THE UNIVERSITY of ADELAIDE

Investigating the role of Toll-like receptor 4 in myocardial ischaemic-preconditioning and ischaemic-reperfusion injury

A thesis submitted in fulfilment for the degree of

DOCTOR OF PHILOSOPHY IN MEDICINE (PHYSIOLOGY)

in

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Adelaide Medical School

The University of Adelaide

by

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I: Abstract

Ischaemic heart disease remains a significant cause of death throughout the developed world. Although technological advancements and improved health care have significantly reduced mortality rates in the last century, additional research is required. Recently myocardial inflammation has drawn increasing interest as a potential therapeutic against cardiovascular disease. Because inflammation is activated by a broad range of pro-inflammatory mediators, the innate immune response can be suppressed at multiple locations. Currently, most anti-inflammatory agents in phase III trials target specific pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6. These mediators have various roles ranging from the recruitment of white blood cells to triggering cell death. In 2017 CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcomes Study), which investigated the therapeutic benefits of Canakinumab (an IL-1β antagonist) in the cardiovascular setting, was completed. Although the study reported that patients had a reduced risk of inheriting cardiovascular diseases, Canakinumab did not reduce mortality rates. Considering the large number of inflammatory mediators involved in myocardial ischaemic-reperfusion injury, blockade of a single cytokine may be insufficient within the clinical setting. To date, no study has investigated whether the suppression of upstream inflammatory receptors is more effective in attenuating myocardial inflammation during ischaemic-reperfusion injury. This thesis investigates the role of toll-like receptor 4 (TLR4), an immunosurveillance receptor, in both myocardial ischaemic-reperfusion injury and ischaemic-preconditioning. Receptor activity is triggered when DAMPs (danger associated molecular pattern molecules) are released from necrotic cells and bind onto the TLR4 receptor complex.

Evidence in the last two decades suggests that TLR4 can either exacerbate ischaemic-reperfusion injury or trigger a preconditioning response under certain conditions. Genetic or pharmacological blockade of TLR4 has been reported limit infarct size, improve survival rates, and suppress myocardial inflammation in in-vivo infarct animal studies. On the other hand, evidence also suggests that low levels of TLR4 ligands can trigger preconditioning. In the 1990s, studies showed that pretreating animals with lipopolysaccharide, a bacterial ligand recognised by TLR4, could protect animals against ischaemic-reperfusion injury. Additionally, evidence also suggests that DAMPs can also elicit a preconditioning response when administered prior ischaemia. Considering these findings TLR4 signalling may be regulated in a biphasic manner which is dependent on the degree of TLR4 stimulation and the timing of TLR4 activation.

Study 1 and 2 investigated whether the direct administration of a TLR4 antagonist during ischaemic-preconditioning can influence contractile recovery after irreversible ischaemic injury. The isolated heart technique was used to determine whether contractile function was directly influenced by the blockade of TLR4. Study 3 and 4 examined if novel TLR4 antagonists could protect against ischaemic-reperfusion injury in the in-vitro and in-vivo setting. To date, no study has investigated whether the rapid administration of a TLR4 antagonist during ischaemia can protect against myocardial ischaemic-reperfusion injury.

In brief, study 1 and 2's results revealed that the suppression of TLR4 signalling in ischaemic-preconditioned hearts depressed contractile recovery after ischaemic insult. Although protein analyses revealed that cardiac fatty acid binding protein (cFABP) and high mobility group box

one (HMGB1) were influenced by the suppression of TLR4 the data collected was conflicting. Study 3 showed that (+)-naloxone and (+)-naltrexone suppressed LPS induced inflammation but did not improve cell viability after simulated ischaemic-reperfusion injury. Finally (+)-naloxone and TAK242 showed that both compounds could reduce myocardial infarct size and inflammation in an in-vivo left anterior descending artery ligation model. The findings from this thesis highlight the bivalent nature of TLR4 in ischaemic-preconditioning and ischaemic-reperfusion injury in both the acute and chronic setting.

II: Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

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III: Statements of authorship (thesis)

Title of Paper	Investigating the role of Toll-like receptor 4 in myocardial ischaemic-preconditioning and ischaemic-reperfusion injury
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Contribution to the thesis	Primary contributor for study design, performing experiments, data analyses, writing, proof-reading and editing of this thesis		
Overall percentage (%)	90%		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Role in this PhD	Co-supervisor		
Contribution to the thesis	Assisted in study design and data analysis / interpretation of this project. Also contributed to the proof-reading and editing of this thesis		
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IV: Revised publications

The literature review in Chapter 2: “The role of Toll-like receptor 4 (TLR4) in myocardial ischaemic-reperfusion injury, cardioprotection and ischaemic-preconditioning”, is a revised version of a previous manuscript published by Mr Samuel Man Lee [1].

1. Lee, S. M., Hutchinson, M. & Saint, D. A. The role of Toll-like receptor 4 (TLR4) in cardiac ischaemic-reperfusion injury, cardioprotection and preconditioning. *Clinical and Experimental Pharmacology and Physiology* **43,** 864–871 (2016).

V: Acknowledgements

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VI: Abbreviations

Adenosine tri-phosphate	ATP
Ca^{2+}	Calcium ions
CANTOS	Canakinumab Anti-inflammatory Thrombosis Outcomes Study
Cardiac troponin	CTnT
CCL12	Chemokine (C-C motif) ligand 12
CK	Creatine kinase
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
cFABP	Cardiac fatty acid binding protein
CVD	Cardiovascular disease
CXC10	C-X-C motif chemokine 10
DAMP(s)	Danger associated molecular patterns
DMEM	Dulbecco's Modified Eagle's Medium
ERK 1/2	Extracellular signal regulated kinases 1/2
FBS	Foetal bovine serum
GPCR	G-coupled protein receptors
H_2O_2	Hydrogen peroxide

HSP(s)	Heat shock proteins
HSC	Heat shock cognate protein
HMGB1	High mobility group box one
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
I-35	Ischaemia for 35 minutes
IHD	Ischaemic heart disease
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-10	Interleukin 10
IFN-γ	Interferon gamma
IRF-3	Interferon regulatory factor 3
iNOS	Inducible nitric oxide synthase
NOS2	Nitric oxide synthase 2
IPC	Ischaemic-precondition(ing)(ed)
IRAK	Interleukin-1 receptor associated kinase
JNK 1/2	C-jun N-terminal kinase 1/2
K^+	Potassium ion
K_{ATP}	Potassium ATP channels

LAD	Left anterior descending
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LVD	Left ventricular diastolic pressure
LVDP	Left ventricular developed pressure
LVS	Left ventricular systolic pressure
LV%	Left ventricular contractile recovery
MAPK	Mitogen activated protein kinase
miRNA	MicroRNA
MCP	Monocyte chemoattractant proteins
MMP-9	Matrix metalloproteinases-9
mPTP	Mitochondrial permeability transitional pores
mitoK$_{ATP}$	Mitochondrial potassium ATP channels
MyD88	Myeloid differentiation primary response gene 88
Na^+	Sodium ions
NaK	Sodium potassium channel
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NOD like receptors	Nucleotide-binding oligomerization domain like receptors
NO	Nitric oxide
NOS	Nitric oxide synthase
NPC	Non-precondition(ing)(ed)
PRR	Pattern recognition receptor
$ONOO^-$	Peroxynitrite
RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SERCA	Sarco/endoplasmic reticulum Ca2+-ATPase
SEM	Standard error of the mean
SD	Standard deviation
STAT	Signal Transducer and Activator of Transcription
TAK-1	Transforming growth factor beta-activated kinase 1
TAK-242	Ethyl (6R)-6-[N-(2-chloro-4-fluorophenyl) sulfamoyl] cyclohex-1-ene-1-carboxylate
TBK-1	TANK-binding kinase-1
TBS	Tris base saline

TCC	2, 3, 5-Triphenyltetrazolium Chloride
TIRAP	Toll-interleukin 1 receptor domain containing adaptor protein
TLR	Toll-like receptor
TLR2	Toll-like receptor 2
TLR3	Toll-like receptor 3
TLR4	Toll-like receptor 4
TNF-α	Tumour-necrosis factor alpha
TRAF-6	TNF-receptor associated factor-6
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
Ubc13	Ubiquitin-conjugating enzyme E2 13
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VIII: Statements of authorships (chapter 1)

Title of Paper	General Introduction + Hypothesis & Aims
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	General outline of the causations of myocardial ischaemic-reperfusion injury (IRI) and ischaemic-preconditioning (IPC). Toll-like receptor 4 is discussed in the context of IRI and IPC. Therapeutics targeting myocardial inflammation and TLR4 antagonists, (+)-naloxone, (+)-naltrexone and TAK242, are also examined. Finally, study design for this thesis is discussed in this chapter as well as the hypotheses and aims for each study.

Principal Author

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Contribution to the Paper	Writing, proof-reading and editing.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Professor David Saint		
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Chapter 1: General introduction + Hypothesis & Aims

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1.1: The socio-economic impact of ischaemic heart disease in Australia

Ischaemic heart disease (IHD) remains a major cause of mortality throughout the developed world. In 2015 IHD was reported to have caused 19,777 deaths in Australia making it the leading cause of mortality in that year [1]. Approximately 7.9 million dollars is reported to be spent annually treating cardiovascular diseases placing a significant strain on the country [1]. Although the incidents of IHD related deaths have declined over the last century the prevalence of heart failure has risen [1,2]. This change is likely attributed to a rising elderly population and increasing rates of people suffering chronic heart conditions. Myocardial ischaemia develops when coronary blood flow is restricted by thrombosis or atherosclerosis. Without sufficient oxygen and nutrients, the ischaemic region is unable to maintain metabolic function leading to tissue damage [3]. Although acute periods of ischaemia can be tolerated, depriving the myocardium of oxygen and nutrients for prolonged periods increases the risk of injury. This damage is exacerbated even further when the ischaemic myocardium becomes reperfused. Attempts to re-establish physiological homeostasis during early reperfusion can exacerbate injury and potentially lead to IHD.

1.2: The molecular mediators of ischaemic-reperfusion injury.

The molecular mechanisms which promote ischaemic-reperfusion injury is due to the combined effects of disruptions within the cellular and extracellular environment [4]. Without early reperfusion, cells begin to experience adenosine tri-phosphate (ATP) deprivation [5,6], accumulation of reactive oxygen (ROS) / nitrogen species (RNS) [6,7] and disruptive changes in the membrane potential [8–10]. As the duration of ischaemia becomes prolonged, cell death signalling cascades [10–13] become active leading to irreversible tissue damage. While

reperfusion is critical for restoring contractile function, additional damage is caused when the ischaemic myocardium attempts to restore environmental and cellular homeostasis. Increased production of reactive species [6,7,14], opening of mitochondrial permeability transitional pores (mPTP) [6,8–10], and intracellular calcium (Ca^{2+}) release [6,9], have all been shown to be enhanced upon reperfusion. The physiological consequences of ischaemic-reperfusion injury include depressed contractile function, increased risks of developing cardiac arrhythmias, and death [4]. How these factors drive ischaemic-reperfusion injury are explored in further detail in chapter 2. Another important regulator of reperfusion injury, as well as facilitating tissue repair, is inflammation.

1.3: Inflammation's role in tissue injury and repair contribute to the development of myocardial infarctions.

It is now clear that inflammation plays a significant role to promoting tissue damage and repair after ischaemic insult [15–18]. Inflammatory signalling is triggered when immunosurveillance receptors recognise endogenous proteins secreted from macrophages [19] (white blood cells) or released from damaged / necrotic cells. Defined as alarmins or DAMPs (danger associated molecular pattern molecules) a large number of endogenous proteins can be recognized by immunosurveillance receptors [20–24]. Receptors such as the toll-like receptor (TLR) family, RAGE (receptor for advanced glycation end products) or nucleotide-binding oligomerization domain (NOD)-like receptors have all been reported to activate and promote inflammatory signalling during pathological or sterile injury [25,26]. The downstream signalling cascades triggered by these receptors leads to the release, activation, or de-novo synthesis of pro-inflammatory mediators. Nuclear factor kappa-light-chain-enhancer of activated B cells

(NF-κB) [27–29], the interleukin family [30–32], tumour necrosis factor alpha (TNF-α) [30,33], and monocyte chemoattractant proteins (MCP) [34,35] are all examples of inflammatory mediators associated with ischaemic-reperfusion injury. During the early stages of reperfusion, the innate immune response which is triggered is rapid, broad and non-specific. Pro-inflammatory cytokines can either trigger cell death pathways or promote the recruitment of white blood cells into the ischaemic region. Neutrophils for example, are recruited into the area of injury and release collagenases such as matrix-metalloproteinase-9 (MMP-9) [36,37] that degrades the extracellular matrix. Eventually the injury phase of inflammation is resolved, and the same mediators involved in triggering tissue damage also initiates the wound healing phase. Aside from phagocytosing cellular debris and apoptotic bodies, macrophages also facilitate in angiogenesis during the repair phase [15]. Although this is just a brief synopsis of the role of myocardial inflammation in ischaemic-reperfusion injury, it highlights potential targets of therapeutic intervention. In recent years, clinicians have explored whether the use of anti-inflammatory agents could be used as a therapeutic to treat cardiovascular diseases.

1.4: A new approach; can anti-inflammatory agents be used to treat IHDs?

Originally, the therapeutic benefits of suppressing inflammation were discovered as a side effect; pharmacological treatments such as aspirin [38] and statins [39] were already reported to be beneficial in the cardiac setting. It was only through later studies that these compounds were reported to exhibit anti-inflammatory properties [40]. However, the efficiency of these drugs has been shown to be limited by factors such as sex, age and pre-existing co-morbidities [40]. For example, while statins are known to supress inflammation in cardiovascular diseases [41–43], several clinical trials suggest that the suppression of inflammation does not improve

cardiac function [42]. However, in recent years specific pro-inflammatory cytokine blockers such as Canakinumab (IL-1β blocker) and Methotrexate (IL-6 and TNF-α blocker) have been tested in large scale clinical trials [40]. Recently, the Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS), which examined the therapeutic benefits of Canakinumab was completed [44]. Ridker *et al* [44] reported that while Canakinumab significantly reduced the risks of inheriting cardiovascular disease, mortality rates were not reduced. Considering that a significant number of pro-inflammatory mediators are released during reperfusion it may be more beneficial to suppress immunosurveillance receptors. This thesis studied toll-like receptor 4 (TLR4), an immunosurveillance receptor, which triggers myocardial inflammation during ischaemic-reperfusion injury [24,45–48].

1.5: Toll-like receptor 4 influences cardiac ischaemic-reperfusion injury

The toll-like receptor (TLR) family is composed of 13 known TLRs which are involved in the regulation of inflammation [49]. Each receptor is involved in recognising and responding to specific immunogens found on bacteria, viruses, and fungi. The ability to recognise specific antigens is attributed to the fact that TLRs are pattern recognition receptors (PRR). This allows TLRs to recognise conserved molecular patterns on endogenous proteins. Nishimura *et al* [50] reported that of the 13 members, toll-like receptor 2 (TLR2) and toll-like receptor 4 (TLR4), are the most highly expressed TLRs in the heart. Although evidence has reported the involvement of TLR2 and TLR4 in ischaemic-reperfusion injury, most of the literature currently available suggests that TLR4 is the predominant TLR [51]. TLR4 is particularly well characterized because of its ability to recognise lipopolysaccharide (LPS), a conserved structural component found on gram negative bacteria [52–54]. However, because of its PRR ability TLR4 has been

reported to recognise DAMPs such as high mobility group box 1 (HMGB1) [22,23], heat shock protein 60 (HSP60) [24] and the S100 [22] family. Unlike its family members, TLR4 possesses two major signalling pathways which allows it to modulate innate and adaptive immunity [49]. The MyD88 (Myeloid differentiation primary response gene 88) pathway [55] is the first cascade to be activated when TLR4 recognises DAMPs in the extracellular environment. Through MyD88, NF-κB, a potent inflammatory transcription factor, is activated in the cytosol and migrates to the nucleus. This triggers the de-novo synthesis of the pro-inflammatory cytokines previously discussed [23,24,45,47–49,55]. TLR4's alternative pathway, the TRIF pathway, is triggered when the TLR4 receptor complex is internalized which prevents its interaction with MyD88. Triggered after MyD88 signalling, the TRIF response modulates adaptive immunity through cytokines such as interferon gamma (IFN-y) and IL-10 [49,56,57]. Genetic and pharmacological knockout studies have shown that attenuation of TLR4 has a significant effect on inflammation limiting tissue damage and infarct size [46–48]. Finally, because of the ubiquitous nature of TLR4; the immunosurveillance receptor is also expressed on macrophages [19], neutrophils [23,58], endothelial cells [45,50] and cardiomyocytes [45,50] within the heart.

1.6: Can the biphasic response of TLR4 signalling be manipulated to promote a preconditioning response? Or is the complete blockade of TLR4 signalling more beneficial?

To date most of the literature which has examined myocardial TLR4 signalling have only focused on the detrimental role of the receptor in ischaemic heart disease. However, in the last 20 years, evidence has suggested that pre-treating animals with TLR4 ligands can

precondition hearts to protect against ischaemic-reperfusion injury. The first studies to report this was studies examining the effects of pre-treating animals with low dosages of LPS [59–61]. Researchers discovered that LPS pre-treated animals had improved contractile function and reduced tissue damage after ischaemic-reperfusion insult. Furthermore, the fact that LPS preconditioning has been reported in other organ models of ischaemic-preconditioning suggests that this mechanism is not cardiac specific [61,62]. Considering TLR4's pattern recognition ability, it is likely that DAMPs recognised by TLR4 also elicit a similar preconditioning response. Multiple studies have reported that the administration of DAMPs prior to ischaemic-reperfusion injury reduces inflammation and limits tissue damage [63,64]. These findings suggest that under conditions of sublethal stress, DAMPs may be released into the myocardium and trigger a preconditioning response. One of the areas in which myocardial TLR4 signalling remains poorly defined is its role in cardiac ischaemic-preconditioning [65]. In brief, by exposing animals to acute bouts of ischaemia and reperfusion, prior irreversible ischaemic insult, triggers a preconditioning response. Since its original discovery [65], the molecular mechanisms which promote myocardial ischaemic-preconditioning has been under significant investigation [66–69]. Examples include the regulation of ion channels [68], suppression of reactive oxygen / nitrogen species [7,9,14], and the attenuation of kinase signalling such as protein kinase C [70,71], mitogen activated protein kinases (MAPK) [66,72] or G-coupled protein receptors (GPCR) [73]. Ischaemic-preconditioning has also been reported to promote the de-novo synthesis of proteins involved either tissue repair or cardioprotection during the later stages of reperfusion [74,75]. How these mechanisms protect against ischaemic-reperfusion injury are discussed in the further detail in chapter 2. Although ischaemic-preconditioning is known to influence inflammation [28,76] it is uncertain whether ischaemic-preconditioning itself

directly influences TLR4 signalling. It may be possible that DAMPs released during ischaemic-preconditioning binds onto TLR4 triggering its desensitization.

The other side of the spectrum is whether complete blockade of TLR4 signalling is more beneficial than the suppression of specific pro-inflammatory cytokines. As mentioned earlier in this chapter, while the CANTOs study [44] was reported to have reduced the risks of inheriting cardiovascular diseases, mortality rates did not improve. Considering that a large range of pro-inflammatory cytokines and inflammatory mediators regulate innate immunity blockade of an upstream regulator may be more beneficial. It remains to be seen whether suppression of TLR4 during early reperfusion would be more effective in reducing tissue damage. While Shimamoto *et al* [47] reported that eritoran, a TLR4 antagonist, limited myocardial inflammation and suppressed infarct size, the antagonist was administered before ischaemia. Considering the limited amount of pharmacological evidence is available it is still uncertain as to whether suppression of TLR4 in the clinical setting is beneficial. Hence several novel TLR4 antagonists which will be tested in this thesis will be briefly discussed.

1.7: TLR4 antagonists - (+)-naltrexone & (+)-naloxone and TAK242

Naloxone and naltrexone are opioid receptor blockers used to treat alcohol and opioid addiction [77]. Both compounds share structural similarities with the only difference being that the tertiary amine methyl component found in naloxone is replaced by methylcyclopropane in naltrexone. Although stereoisomeric derivatives of both compounds exist, the (+) isoform has a very low binding affinity for mu opioid receptors compared to the (-)-stereoisomers [78].

In 2008, Hutchinson *et al* [77] discovered that both compounds, irrelevant of their stereoisomeric configuration, suppressed TLR4 activity within the in-vitro setting. These findings were then supported by in-silico and in-vivo experiments showing that naloxone and naltrexone limits LPS induced TLR4 signalling [79]. On the other hand, more recent evidence by Wang *et al* [80] reported that (+)-naloxone and (+)-naltrexone suppresses the production of TRIF, but not MyD88, dependent inflammatory mediators. Interferon regulator factor 3 (IRF-3) and interferon gamma (IFN-γ) production was suppressed in LPS stimulated BV-2s. In the context of myocardial ischaemic-preconditioning, several studies have used (-)-naloxone to study the involvement of opioid receptors in myocardial ischaemic-preconditioning [81,82]. These findings reported that (-)-naloxone could abolish this preconditioning response [83,84] as shown by the depression in contractile recovery. However, because the authors of these studies were unaware of the TLR4 antagonistic properties in naloxone, it is uncertain as to whether the suppression of contractile function in ischaemic-preconditioned hearts was mediated through TLR4 inhibition. To avoid the possibility of suppressing opioid receptor signalling, this thesis only examined (+)-naloxone and (+)-naltrexone. TAK242 (or Resatorvid), is more recent novel compound specifically synthesised to block TLR4 signalling [85–87]. Significant research into the pharmacokinetics of TAK242 suggests that it causes complete blockade of the TLR4 signalling network. To our knowledge, no study has investigated (+)-naloxone, (+)-naltrexone, or TAK242 in myocardial ischaemic-preconditioning or ischaemic-reperfusion injury.

1.8: Gaps in the field / Hypothesis & Aims

This thesis explores two separate, but interlinking questions.

1) Does the administration of TLR4 antagonists during ischaemic-preconditioning directly influence acute contractile recovery?

2) Would the rapid administration of a TLR4 antagonist, prior reperfusion, limit myocardial inflammation and infarct size?

The studies in this thesis were designed to explore how TLR4 blockade influences cardiac function both at the physiological and molecular level. Using in-vitro, ex-vivo, and in-vivo experiments, the suppression of TLR4 signalling in ischaemic-preconditioning and ischaemic-reperfusion was studied at both acute and chronic timepoints of recovery. To investigate question 1, the isolated heart technique [88–90] was used to study whether (+)-naloxone could directly influence acute contractile function in isolated rat hearts. Perfusate from perfused hearts were collected to measure lactate dehydrogenase (LDH) which is a predictive biomarker of injury. At the end of each experiment the left ventricle from each heart was studied for c-Jun N-terminal (JNK) phosphorylation. JNK is a MAPK known to be activated through the TLR4-MyD88 [52] pathways. Activation of JNK during reperfusion has been reported to trigger TNF-α and is involved in cell death [47,91,92]. Finally, HMGB1, which is known to act as a DAMP during myocardial ischaemic-reperfusion injury [22,23], was also investigated.

1.8a: Study 1 (chapter 3) – Investigating the effects of (+)-naloxone in isolated ischemic-preconditioned rat's hearts.

HYPOTHESIS: Direct administration of (+)-naloxone during ischaemic-preconditioning will influence acute contractile recovery in ex-vivo Langendorff perfused rat hearts.

AIM 1.1: To investigate if direct administration of (+)-naloxone during ischaemic-preconditioning influences contractile recovery in isolated rat hearts.

AIM 1.2: To measure injury biomarker, lactate dehydrogenase, in perfusate collected from the isolated rat hearts from aim 1.1.

AIM 1.3: To determine whether JNK phosphorylation in (+)-naloxone treated hearts are suppressed using western blots.

AIM 1.4: To measure whether HMGB1 levels are influenced in (+)-naloxone treated hearts using western blots.

After study 1's findings, there was a concern as to whether data collected was influenced by the model itself (discussed in further detail in chapter 4 and chapter 10). Hence the constant pressure isolated mouse heart model was used in study 2. Furthermore, (+)-naltrexone was

also examined to see whether a more potent, longer acting, TLR4 antagonist would influence contractile function.

1.8b: Study 2 (chapter 5) – Investigating the effects of (+)-naloxone and (+)-naltrexone in isolated ischemic-preconditioned mouse hearts.

HYPOTHESIS: Administration of (+)-naloxone or (+)-naltrexone alters acute contractile recovery in ischemic-preconditioned ex-vivo Langendorff perfused mouse hearts.

AIM 2.1: To investigate whether direct administration of (+)-naloxone or (+)-naltrexone in ischaemic-preconditioned isolated mouse hearts influences contractile recovery.

AIM 2.2: To quantify pro-inflammatory cytokine, IL-1β, and biomarkers of injury, HMGB1 & cardiac fatty acid binding protein, from aim 2.1 hearts using western blots

While study 2 reported that (+)-naltrexone suppressed acute contractile recovery, the project was revised accordingly to examine a more pressing question. Would the rapid administration of a TLR4 antagonist, prior reperfusion, limit myocardial inflammation and reduce infarct size? Before conducting any animal work, in-vitro cell work experiments were performed to: 1) Confirm whether (+)-naloxone or (+)-naltrexone, suppresses myocardial inflammation in LPS

stimulated cells; and 2) Does either compound improve cell viability when exposed to simulated ischaemic-reperfusion. Hence H9C2s, cardiac cells derived from the left ventricle, were tested under these conditions and examined for inflammatory markers and cell viability.

1.8c: Study 3 (chapter 7): To investigate whether (+)-naloxone and (+)-naltrexone influences inflammation and cell survival in H9C2s exposed to hypoxic / normoxic conditions.

HYPOTHESIS: (+)-Naloxone and (+)-naltrexone limits H9C2 cell death when exposed to simulated ischaemia-reperfusion and suppresses inflammation when stimulated with LPS.

AIM 3.1: To optimize an in-vitro hypoxic / normoxic protocol using the H9C2 cell line

AIM 3.2: To determine whether (+)-naloxone and (+)-naltrexone limits NF-κB nuclear translocation in LPS stimulated H9C2s using immunohistochemistry.

AIM 3.3: To determine whether (+)-naloxone or (+)-naltrexone protect against a loss in cell viability in H9C2s exposed to the hypoxic / normoxic protocol (optimized from aim 3.1) using the neutral red assay.

AIM 3.4: To determine whether LPS stimulated H9C2s, pretreated with (+)-naloxone, suppresses IL-1β, NF-κB, IL-6, TNF-α and TLR4 gene expression.

After (+)-naloxone was reported to suppress acute myocardial inflammation, in-vivo experiments were performed for the final study. TAK242, a more recent TLR4 antagonist, was also tested. Although Shimamoto *et al* [47] reported that eritoran reduced infarct size the compound was administered prior ischaemia. To make study 4 more clinically relevant, (+)-naloxone or TAK242 was administered directly into the heart prior the restoration of blood flow. Animals were then allowed to recover for 48 hours before the hearts were stained for infarct size. Using protein (western blot) and gene expression (RT-PCR) quantification techniques, heart tissue was studied for multiple inflammatory markers. Finally, predictive biomarkers of injury, caspase-3 and matrix metalloproteinase-9 (MMP-9), were examined using western blots.

1.8d: Study 4 (chapter 9): To determine whether (+)-naloxone and TAK-242 limit infarct size and inflammation in a rat infarct model.

HYPOTHESIS: (+)-Naltrexone and TAK-242 limit infarct size and the production of caspase-3, MMP-9, and inflammatory markers in rats given myocardial infarctions in vivo.

AIM 4.1: To perfuse (+)-naloxone or TAK-242, in rats given left anterior descending artery ligation followed by a 48-hour recovery period.

AIM 4.2: To quantify infarct size in from AIM 4.1 hearts using Evan's blue and 2, 3, 5-Triphenyltetrazolium Chloride (TCC) staining.

AIM 4.3: To quantify inflammatory markers, IL-1β and NF-κB, and tissue injury markers, caspase-3, and MMP-9, in tissue homogenates collected in LAD rats (from AIM 4.1) using western blots.

AIM 4.4: To quantify the gene expression of inflammatory markers, IL-1β, NF-KB, TLR4, TNF-α and IL-6 in tissue homogenates collected from rats in AIM 4.1 though RT-PCR.

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IX: Statements of authorships (chapter 2)

Title of Paper	The role of Toll-like receptor 4 (TLR4) in cardiac ischaemic-reperfusion injury, cardioprotection and preconditioning
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Publication Details	Modified literature review published in 2016 examining Toll-like receptor 4's involvement in myocardial ischaemic-preconditioning and ischaemic-reperfusion injury. Furthermore, the theory of DAMP induced preconditioning and examination of novel TLR4 antagonists are also discussed.

Principal Author

Name of Principal Author	Mr Samuel Man lee		
Contribution to the Paper	Writing, proof-reading and editing.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Chapter 2: The role of Toll-like receptor 4 (TLR4) in myocardial ischaemic-reperfusion injury, cardioprotection and ischaemic-preconditioning.

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2.1: Abstract

Ischaemic heart disease remains the largest cause of mortality throughout the developed world. When blood flow is restricted, metabolic function within the ischaemic myocardium is disrupted leading to adenosine triphosphate (ATP) deprivation and accumulation of metabolic waste (i.e. reactive oxygen species). While reperfusion is critical in restoring contractile function, tissue damage can be exacerbated even further as the ischaemic environment attempts to re-establish physiological homeostasis. The damage caused during this phase can have a significant impact on the later stages of reperfusion and contributes to the activation of myocardial inflammation. Inflammation triggered during reperfusion plays an important role in both the promotion and resolution of tissue damage. Innate immunity is triggered when intracellular components are released from damaged / necrotic cells; Also known as DAMPs (danger associated molecular patterns) these proteins / molecules which bind onto immunosurveillance receptors. Evidence suggests that Toll-like receptor 4 (TLR4), an immunosurveillance receptor, is known to enhance ischaemic-reperfusion injury by activating innate immunity. Genetic and pharmacological knockout studies have shown that the removal or suppression of TLR4 reduces infarct size and supresses myocardial inflammation. On the other hand, evidence also suggests hearts can be preconditioned by TLR4 ligands and thus protect against ischaemic-reperfusion injury. However, the exact mechanisms of how this occurs remains poorly defined. Although recent clinical studies suggest that the suppression of inflammation reduces the risk of ischaemic heart disease, TLR4 has not been examined within the clinical setting. This review explores the role of TLR4 in myocardial ischaemic-reperfusion injury, novel TLR4 blockers available, and the theory of TLR4 induced preconditioning.

2.2: Clinical relevance of ischaemic-reperfusion injury.

Ischaemic heart disease (IHD) remains a significant cause of mortality throughout the developed world [1]. IHD develops when blood flow is restricted by either dislodged clots / plaques or narrowing of coronary arteries. Consequently, the ischemic region becomes deprived of oxygen and nutrients causing significant stress on the myocardium. Although reperfusion is critical for survival, the return of blood flow after a chronic period of ischaemia can exacerbate tissue damage [2]. Severe ischaemic-reperfusion injury leads to depressed contractile performance, increased risk of developing arrhythmias and co-morbidities such as stroke. The Australian Bureau of Statistics reported that in 2015 alone, IHD was responsible for 19,777 deaths in Australia (www.abs.gov.au). Although technological and therapeutic advancements have resulted in a decline in cardiac related mortalities, the number of people living with chronic heart problems has increased significantly. Approximately 7.9 billion dollars is spent annually in Australia on cardiovascular diseases, placing a significant strain on the public health sector and the economy [1]. With the aging population expected to double in the next 20 years, and rising obesity levels (www. demographics.treasury.gov), CVD related deaths are likely to rise. By investing in basic science focused on myocardial pathologies such as ischaemic-reperfusion injury, novel treatments can be developed to ease the human and economic costs associated with IHD.

2.3: The translation of cell damage to cell death.

While the aetiology of IHD may vary, the molecular mechanisms which cause ischaemic-reperfusion injury are the same. In most cases the damage caused from acute ischaemic-reperfusion can be reversed, though this is dependent on multiple factors i.e. age, sex or pre-

existing co-morbidities [3–6] . However, if ischaemia persists cell damage can become irreversible and translates into apoptotic or necrotic mediated cell death [5] . The ratio between the two types of cell death is dependent on the duration of ischaemia with cellular necrosis becoming more prominent as ischaemia persists [7,8]. The initial pathway triggered is apoptosis, a regulated breakdown of the cell which is mediated through the activation of pro-apoptotic factors such as the caspase family, Bax, or Cytochrome C [9,10]. Through this process the cell is broken down into contained cellular fragments called apoptotic bodies which are then endocytosed by white blood cells such as macrophages [9,10]. The alternative pathway is cell necrosis where the intracellular components are not packaged within cellular fragments and are thus released into the extracellular environment [11,12]. In these cases, endogenous molecules are defined as DAMPs (danger associated molecular patterns) and can enhance tissue damage by activating immunosurveillance receptors. These pattern recognition receptors (PRRs) recognise foreign molecules or DAMPs through conserved molecular regions and trigger an inflammatory response when stimulated [13]. Heat shock proteins (HSPs) [14–16] and high mobility group box 1 (HMGB1) [11,17,18], are just two examples of proteins reported to act as DAMPs during myocardial ischaemic-reperfusion injury. Activation of these receptors triggers the innate immune response promoting the release or de-novo synthesis of pro-inflammatory cytokines [13,19]. Because the events before the inflammatory cascade are important in defining the severity of reperfusion injury, the non-inflammatory factors which emerge during ischaemic insult will be briefly covered in this review.

2.4: ATP deprivation

Upon the restriction of blood flow ischaemic regions become deprived of oxygen which is critical for ATP production. As the main energy source for most mammals, ATP is used to maintain intracellular metabolic function [20]. It plays an important role in maintaining cardiac (Ca^{2+}) ion transporters, sodium-potassium (Na-K) ATPase, sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), as well as driving muscle contraction [21]. In normal conditions, ATP is produced from the breakdown of glucose derivatives through the krebs cycle [20]. However, the lack of oxygen in an ischaemic region can prevent aerobic respiration leading to a significant reduction in ATP production [22,23]. This causes the depletion of intracellular glycogen reserves until metabolic regulation is no longer maintained inside the cell. As a consequence, the environmental equilibrium is disrupted resulting in a loss of membrane potential, decreased pH levels (leadings to acidosis), and the accumulation of metabolic waste within the ischemic environment [24–27].

2.5: Accumulation of reactive oxygen species (ROS) within ischaemic areas

Another consequence of ischaemia is the accumulation of ROS. A by-product of metabolic reactions, ROS are highly reactive oxygen molecules which play a role in both defence and maintaining the environmental equilibrium [28]. However, when present in high levels, their reactive nature causes DNA damage, lipid peroxidation and protein denaturation [4,29,30]. Examples of ROS include, superoxide, hydrogen peroxide and hydroxyl all of which are associated with ischaemic-reperfusion injury [30]. In normal conditions, ROS is removed via the blood stream or converted into less reactive derivatives with antioxidants. However,

during ischaemia, the restriction of blood flow and a finite number of antioxidants fails to maintain the redox equilibrium [29,30].

2.6: Disruption of membrane potential disrupts myocardial autorhythmicity.

Dramatic shifts in the ionic equilibrium between the intracellular and extracellular environment also contributes to ischaemic-reperfusion injury. The regulation of sodium (Na^+), calcium (Ca^{2+}), and potassium (K^+) ion gradients is critical in maintaining cardiac autorhythmic function [31]. Disrupting the homeostasis of these ions can increase the risk of arrhythmias in the either the atria or ventricles and thus disrupt blood circulation. During ischaemia, the disruption of the ionic equilibrium alters intracellular H^+ [22,32], Na^+ [33–35] and Ca^{2+} gradients [21,25,26,36]. Increased intracellular Ca^{2+} levels can be particularly detrimental due to its potent agonistic effects on signalling kinases and contractile function. Pharmacological studies have demonstrated that elevated cytosolic Ca^{2+} stimulates protease activity, increases diastolic pressure, and promotes the production of ROS [21,25,26,36] during ischaemic-reperfusion injury. Another consequence of enhanced intracellular Ca^{2+} levels is the formation of mitochondrial permeability transitional pores (mPTPs) [36].

2.7: Mitochondrial permeability transition pore formation in ischaemic-reperfusion injury promotes cell death.

The formation of mPTPs on mitochondrial membranes is a common mechanism of intracellular mediated cell death [36]. These non-selective pores allows the influx of proteins less than 1.5 kilodaltons in size into the mitochondria [37]. Unable to regulate the movement

of ions within the mitochondrial environment, the membrane potential is lost resulting in the disruption of the electron transport chain [37]. The influx of proteins can also cause the organelle to rupture due to the excessive osmotic load. This causes the release of proteases, phosphatases and nucleases which can trigger cellular breakdown [36]. If damaged cells lose the ability to promote apoptosis, the cell's structural integrity eventually collapses causing the release of DAMPs. Although mPTP formation occurs during ischaemia, low pH levels in the ischaemic environment prevent the pores from opening. Once blood flow is restored and pH levels return to normal levels the pores open [36].

2.8: Reperfusion injury and the role of inflammation in damage and repair.

As blood flow returns throughout the ischaemic region, attempts to re-establish environmental and metabolic homeostasis causes further damage during the early stages of reperfusion. Enhanced generation of ROS [38,39], increased cytosolic calcium levels [21,36,40] and opening of mPTPs [22,36] causes additional cell death. The release of DAMPs caused by these events bind onto immunosurveillance receptors and triggers inflammatory signalling leading to the de-novo synthesis, or release, of pro-inflammatory cytokines. In-vivo infarct studies have confirmed that pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) [41–43] and multiple interleukin family members [43–47] are secreted or synthesized during the early phases of reperfusion. Working in conjunction with these cytokines, chemokines and adhesion molecules are also released. Chemokines such as, monocyte chemoattractant protein-1 (MCP-1) [48,49] or interferon gamma (IFN-γ) [50,51], trigger the recruitment of immune cells into the ischemic region which then adhere to cells which have been tagged by adhesion molecules [52,53]. The effects of these inflammatory

cells vary, macrophages for example, phagocytose apoptotic bodies and cellular debris as well as triggering angiogenesis [49,52]. This highly regulated process is designed to clear cellular debris, remove damaged cells, and trigger the wound healing cascade which occurs during the later stages of reperfusion. On the other hand, neutrophils can exacerbate myocardial injury by release ROS and degradative proteases which causes additional tissue damage [29,47,54]. It is clear now that the balance between the harmful and protective factors of myocardial inflammation requires careful regulation. Animal and clinical studies have reported that suppressing components of the inflammatory response influences the wound-healing process, and in some cases, can exacerbate injury [52,55,56]. Taking these findings things into consideration, focus should be aimed at regulating the inflammatory response where the injury phase is dampened during early reperfusion without weakening its role in tissue repair and angiogenesis.

2.9: Ischaemic preconditioning

In 1986, Murry *et al* [57] discovered that acute bouts of ischaemia and reperfusion, preceding harmful ischaemic insult, is cardioprotective. Occlusion of the left circumflex artery in 4 x 5-minute bouts was performed in dogs followed by a forty-minute occlusion. Hearts preconditioned with acute bouts of ischaemia were reported to have significantly smaller infarct sizes. Now classified as 'ischaemic-preconditioning', Murry's findings have been successfully replicated in multiple animal models such as pigs, rabbits, and mice [58–60]. Additionally, the protective phenomenon has also been reported to protect against brain [61], liver [62], and kidney [63] models of ischaemic-reperfusion injury. These findings suggest that the protective mechanisms which trigger ischaemic-preconditioning shares common

signalling cascades. Murry's *et al* [57] findings encouraged a new field of cardiac research examining not only the molecular mechanisms which drive ischaemic-preconditioning, but how to reproduce them pharmacologically. Well established mechanisms associated with ischaemic-preconditioning include opening of mitochondrial potassium ATP channels (mitoK_{ATP}) channels [64], adenosine receptor activation [65–67] and protein S-nitrosylation [68,69]. Signalling networks are also influenced by ischaemic-preconditioning by either direct modification of kinase phosphorylation sites or through positive / negative feedback systems [70]. Although ischaemic-preconditioning limits the early stages of reperfusion injury this cardioprotective window only last for several hours; this first window is often referred to as "classical preconditioning". However, ischaemic-preconditioning also confers a second window of cardioprotection, known as delayed preconditioning, which emerges 24 hours later [66,71–73]. Aside from influencing mediators involved in classical preconditioning, delayed preconditioning also triggers cardioprotective pathways [74]. For instance, delayed preconditioning has been hypothesized to promote the synthesis of micro RNA (miRNA) which are small non-coding RNAs that inhibit mRNA activity. These transcriptional regulators have been shown to activate cardioprotective proteins such as cyclooxygenase-2 (COX-2) or nitric oxide synthases (NOS) [75].

2.10: Sub-lethal stress can promote a preconditioning response.

The concept of preconditioning can be defined as stress stimuli which either desensitizes stress response pathways or triggers ones associated with cardioprotection. It is now known that alternative methods of preconditioning exist where acute stress promotes a preconditioning response. For example, induction of ischemic-preconditioning in the limbs of

animals can protect against myocardial ischaemic-reperfusion injury [76,77]. Although multiple clinical trials have shown promising results in the last decade [78–80], a more recent large scale trial by Hausenloy *et al* [81] suggests that remote preconditioning does not improve recovery after cardiac surgery. Another method of stress induced preconditioning is through heat stress [82]. Exposure to high body temperatures prior to a myocardial infarction promotes a delayed preconditioning response. It is believed that heat shock induced preconditioning is attributed to the upregulation of specific HSP isoforms [83].

Perhaps the most interesting form of preconditioning in the context of this review is the literature surrounding lipopolysaccharide (LPS) induced preconditioning. LPS is the main cell wall structural protein found in gram-negative bacteria [19,84] and is recognised by the immunosurveillance receptor, toll-like receptor 4 (TLR4). When LPS is recognized, the innate immune response is rapidly triggered to minimize bacterial infections [19,84,85]. However, during pathological events such as sepsis, the systemic immune response generated by LPS causes severe vasodilation, endothelial dysfunction, and organ failure which can lead to death [86]. Despite this, studies have shown that the low dose administration of LPS can elicit a delayed preconditioning response. In 1989, Brown *et al* [87] examined the effects of administering LPS in Sprague Dawley rats 24 hours before myocardial ischaemic-reperfusion injury. Pre-exposure to LPS was shown to improve contractile function in ischaemic-reperfused hearts and increase catalase activity, a ROS scavenger. Although these findings have been supported by several cardiac studies [71,88,89] it is still unknown as to whether LPS preconditioning is mediated through TLR4 causing its desensitization. If this link exists, then the second consideration is whether TLR4 DAMPs can also elicit a preconditioning

response. The remainder of this review will focus on the literature examining TLR4, its role in cardiac ischaemic-reperfusion injury, as a target of preconditioning, and novel TLR4 pharmacological blockers which will be examined throughout this thesis.

2.11: The toll-like receptor family – TLR4

The Toll-like receptor (TLR) family is a series of immunosurveillance receptors involved in the recognition and response of foreign pathogens within the host. To date, there are 13 known Toll-like receptors (10 of which are known to be functional) which are expressed ubiquitously throughout the human body [90]. Through in-vivo infarct studies, Frantz *et al* [45] was the first to detect the presence of TLR4 in both rat and mouse cardiac tissue. Although a large proportion of TLR4 was detected on microvascular endothelial cells, detectable levels of TLR4 were observed in adult and neonatal cardiomyocytes. However, when isolated rat cardiomyocytes were stimulated with LPS, or when hearts were given myocardial infarctions, TLR4 was significantly upregulated in the myocardium [45]. Of the 10 receptors, TLR4 is the most prevalent TLR in the myocardium followed by TLR2 [90]. Finally, unlike its other family members TLR4 has two independent downstream signalling networks, the myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) dependent pathway, which both regulate innate and adaptive immunity.

2.12: TLR4 extracellular complex

While every TLR recognises specific antigen types they all share similar structural and signalling proteins. Each TLR is comprised of an extracellular domain, a single transmembrane helix and an intracellular signalling domain (figure 1). The extracellular domain of every TLR

exists as a multimeric protein complex comprised of the TLRs themselves and several accessory proteins [19,91,92]. TLR4's extracellular complex is particularly well characterized and will be briefly discussed in terms of LPS stimulation. Circulating LPS monomers bind to LPS binding proteins (LBP), a circulating plasma protein, which collects LPS into aggregates and delivers them to the TLR4 receptor complex. Before LPS binds to TLR4, the LBP-LPS aggregate is presented to CD14 (cluster of differentiation 14) [48,91], an accessory protein, which then transfers the LPS monomers to another accessory protein, MD-2. Containing an LPS binding pocket, MD-2 [93,94] triggers the TLR4 homodimer complex to dimerize [84] resulting in the activation of the MyD88 dependent signalling pathway [93,95].

2.13: MyD88 dependent pathway

Of the two signalling pathways downstream from TLR4, the MyD88-dependent pathway is the first network to be triggered (figure 1). After TLR4 dimerization, TIRAP (TIR-domain-containing adapter protein), migrates to the membrane where it interacts with the extracellular TLR4 complex from the cytosol [96,97]. A signal transduction cascade is then triggered leading to the recruitment of Myeloid differentiation factor 88 (MyD88), interleukin-1 receptor associated kinase-1 (IRAK-1), IRAK-2 [98] and IRAK-4 [99] to form a multimeric protein complex. Once IRAK-1 and IRAK-4 become phosphorylated, both kinases dissociate

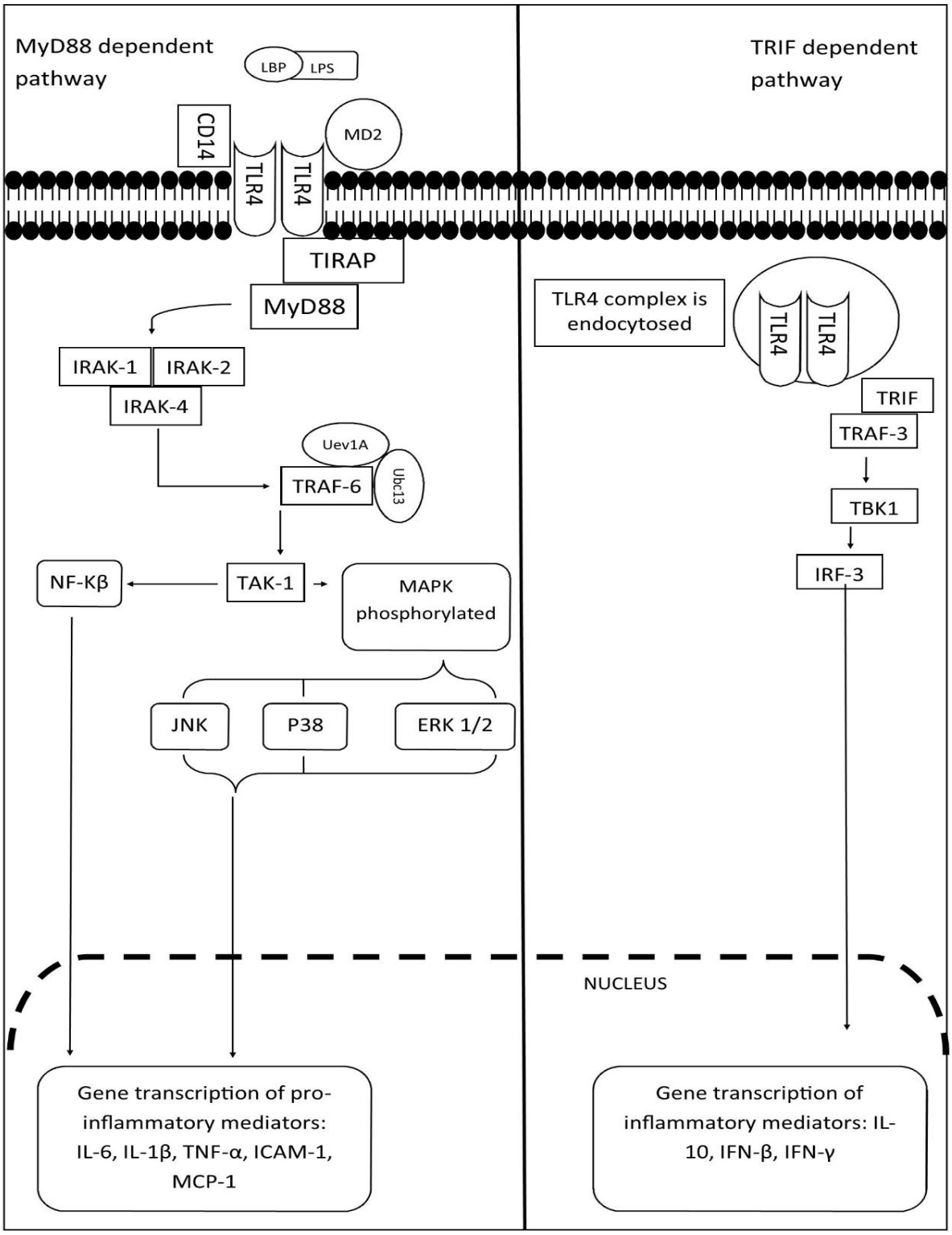

Figure 1: Diagram outlining the TLR4-MyD88 and TRIF pathways. The MyD88 dependent pathway is activated when TLR4 dependent ligands (i.e. LPS, HSPs, HMGB1) bind onto the TLR4 extracellular complex. This triggers the recruitment of TIRAP to the cellular membrane and begin MyD88 signalling.

The TLR4-TRIF pathway is associated with resolving innate immunity and activation of adaptive immunity; the alternative pathway is triggered when the TLR4 receptor complex is endocytosed.

from the protein complex migrating to TNF-receptor associated factor-6 (TRAF-6), a member of the TRAF family. TRAFs modulate inflammation by either interacting with cell surface proteins or, in TRAF-6's case, by enhancing signal transduction [100]. Forming a protein complex with ubiquitin-conjugating enzyme E2 13 (Ubc13) and ubiquitin-conjugating enzyme E2 variant 1A (Uev1A), both ubiquitin-conjugating enzymes, TRAF-6 then binds with Transforming growth factor beta-activated kinase 1 (TAK-1), a member of the MAPK family [92,101,102]. This leads to the activation of p38, c-Jun N-terminal kinases (JNK) and extracellular signal–regulated kinases 1/2 (ERK 1/2) which triggers the release or de-novo synthesis of pro-inflammatory cytokines and cell death regulators [103].

2.14: TRIF pathway

The TRIF (TIR-domain-containing adapter-inducing interferon-β), or the MyD88 independent pathway, is TLR4's secondary main signalling pathway (figure 1) [104]. Activated after MyD88 signalling, the TRIF pathway suppresses innate immunity while triggering the adaptive immune response at the same time. Unlike the MyD88 pathway, the TLR4 extracellular complex is internalized and thus prevents TIRAP interaction. Although several authors have proposed that this endocytosis process is regulated through CD14 [105],[106] this theory is still under debate [91]. The internalization of TLR4 causes the recruitment of TRIF to the endocytosed receptor followed by the recruitment of the adaptor molecule TRAM (TRIF-related adaptor molecule) [92]. This complex signals the recruitment of TRAF-3 followed by

activation of TANK-binding kinase-1 (TBK-1) [107,108]. TBK1 then phosphorylates Interferon regulatory factor-3 (IRF-3) a transcription factor which enters into the nucleus and begins the de-novo synthesis of type 1 interferons [109],[19]. These cytokines have a broad range of responses ranging from the synthesis of interleukin 10 (IL-10) [110],[111], an anti-inflammatory cytokine, to the production of chemokines, such as C-X-C motif chemokine 10 (CXC10) or Chemokine C-C motif ligand 12 (CCL12), which modulates the recruitment of immune cells [19]. Although its role in ischaemic-reperfusion injury remains poorly defined it is likely that the TRIF response resolves innate immunity, while stimulating the repair phase post-ischaemic injury. However, in recent years, researchers have discovered that specific B and T cells contribute either tissue damage or repair in ischaemic-reperfusion injury. While B [112] and Natural Killer T cells [113] have been reported to exacerbate ischaemic-reperfusion injury, regulatory T cells [55,56] may limit infarct size and be cardioprotective .

2.15: TLR4's role in cardiac ischaemic-reperfusion injury

In the last decade, multiple groups have shown that the removal or suppression of TLR4 signalling reduces myocardial ischaemic-reperfusion injury. Chong *et al* [114] and Oyama *et al* [54] were the first two groups to show this using genetic knockout in-vivo models. In Oyama's *et al* [54] study mouse hearts were given LAD ligations for 1 hour followed by 24 hours of reperfusion in wild type (C57-BL/10 ScSn & C3H/OuJ) and TLR4 knockouts strains (C57-BL/10-ScCr & C3H/HeJ). Compared to the vehicle groups, both TLR4 knockouts had reduced infarct sizes however survival rates only improved in the C57-BL/10-ScCr group. Further analysis of the C57-BL/10-ScCrs revealed significant reductions of ROS and complement-3 (small proteins which trigger the complement response during innate

immunity [52]). Chong *et al* [114] also observed similar findings when examining C3H/HeJ mice showing a depression in infarct size and production of inflammatory cytokines / chemokines (i.e. TNF-α, IL-1β, MCP-1, IL-6). Though limited, pharmacological knockout of TLR4 has also been reported to protect against ischaemic-reperfusion injury. Shimamoto *et al* [115] showed that by pre-treating C-57BL/10 mice with Eritoran, a TLR4 antagonist, prior left anterior descending artery (LAD) ligations, reduced myocardial infarct sizes. These findings were supported by molecular analyses of the tissue which revealed decreased JNK phosphorylation, and reduced IL-6 production. These findings, which are supported by multiple cardiac [44,46,116] and non-cardiac [18,117,118] ischaemic-reperfusion studies which have studied TLR4, suggests that pharmacological intervention against TLR4 signalling within the clinical setting may be beneficial. Although TLR4 signalling during ischaemic-reperfusion injury is mediated through DAMPs it is uncertain how many TLR4 ligands trigger MyD88 dependent signalling. Recently, Zhang *et al* [11] observed that the administration of supernatant from necrotic myocardial tissue into the hearts of mice caused myocardial inflammation and fibrosis. Western blot analysis of the tissue supernatant revealed the presence of HMGB1, Galatectin3, and multiple S100 family members all of which are recognised by TLR4. When the necrotic supernatant, was either heat denatured, or administered into TLR4 knockouts, the supernatant failed to cause inflammation or fibrosis in mouse hearts [11].

2.16: HMGB1 and its role in TLR4 associated ischaemic-reperfusion injury.

HMGB1 is a well-known DAMP which triggers inflammation through multiple immunosurveillance receptors [11,119–121]. As a transcriptional regulator and chaperone

protein, HMGB1 is normally located in the nucleus [120]. However, during pathological conditions HMGB1 is either released from necrotic cells, or secreted by macrophages to act as a DAMP [120]. Evidence in the last decade suggests that HMGB1 acetylation in the cytosol is required before it is recognised by pattern recognition receptors [120]. If released through secretion, the protein is packaged into secretory lysosomes as shown by Bonaldi *et al* [122] and released through exocytosis. This post-translational event has been recently hypothesized to be directed through JAK/STAT1 signalling [123]. Andrassy *et al* [124] was the first group to observe that the administration of HMGB1, prior to LAD ligation, increased infarct size and increased TNF-α / IL-6 expression in myocardial tissue. However, myocardial inflammation and infarct size was reported to be suppressed when HMGB1 box-A (a HMGB1 antagonist) was administered 1-hour prior infarction. Although in-vitro [11],[48,119] and in-vivo [11],[119],[17,18] experiments have confirmed that TLR4 recognizes exogenous HMGB1, its role in myocardial ischaemic-reperfusion injury requires additional investigation. For instance, it is uncertain whether HMGB1 induced signalling during ischaemic-reperfusion is predominantly mediated through TLR4.

2.17: DAMP induced preconditioning and the role of TLR4.

Although this review has explored the role of TLR4 as a mediator of innate immune injury evidence behind LPS induced preconditioning suggests that transient TLR4 signalling can be cardioprotective. Considering these findings and TLR4's PRR ability, it is still uncertain as to whether TLR4 dependent DAMPs can trigger a similar TLR4 signalling response. While limited, evidence suggests that the desensitization of TLR4 through low dosages of TLR4 ligands protects against ischaemic-reperfusion injury. To our knowledge, the only evidence to suggest

DAMP induced preconditioning in cardiac ischaemic-reperfusion injury is by Hu *et al* [125]. Pretreatment with HMGB1 24 hours prior to LAD occlusion significantly reduced infarct size and attenuated the production of cardiac biomarkers, LDH and creatine kinase (CK). This delayed preconditioning response also supressed inflammation as reported by the reduction of TNF-α and IL-6 levels. While Hu *et al's* [125] study did not examine the role of TLR4, hepatic [126] and renal [127] ischaemic-reperfusion studies have documented a link between HMGB1-TLR4 induced preconditioning. Considering the ubiquitous nature of TLR4, and the large range of DAMPs that TLR4 recognises, it is uncertain as to whether these preconditioning responses are specifically mediated through HMGB1. Hence additional research is required to determine whether low levels of DAMPs can trigger TLR4 desensitization and thus promote myocardial preconditioning. Additionally, it is also important to know whether ischaemic-preconditioning itself is directly responsible for triggering TLR4 desensitization. To date, it is still unknown how TLR4 signalling is influenced during ischaemic-preconditioning. It may be possible that during the preconditioning phase, minor levels of DAMPs are released from the ischaemic myocardium which triggers TLR4 desensitization. While this is entirely theoretical, the literature examined throughout this review supports this theory and warrants additional investigation.

2.18: Anti-inflammatory agents currently under clinical review

In the past decade clinicians have come to understand the role of innate immunity in cardiovascular disease. The suppression of cardiac biomarkers such as c-reactive proteins (CRP) or pro-inflammatory cytokines such as IL-6, TNF-a, and IL-1β can significantly reduce the risks of cardiovascular disease [128]. Aspirin [129] and statins [74,130] were the first drugs to

be discovered which showed that the suppression inflammation which could reduce the risk of cardiovascular diseases. These initial findings encouraged clinicians to examine other treatments such as methotrexate [131] or salsalate [128] which, aside from their original properties, also display anti-inflammatory properties which are correlated to reducing the risk of inheriting cardiovascular disease. This has encouraged the development of specific cytokine blockers such as Canakinumab (IL-1β) [132,133] and Infliximab (TNF-α) [134,135] which have both shown encouraging results in the clinical setting [128]. Recently the CANTOs study, a phase III clinical trial examining Canakinumab within the cardiovascular setting, was completed [136]. Although the trial revealed that Canakinumab reduced the risk of cardiovascular diseases by 15%, mortality rates were not reduced. These findings may be attributed to the fact that innate immunity is regulated by a large range of pro-inflammatory mediators which enhance ischaemic-reperfusion injury. Hence the suppression of IL-1β may be insufficient in triggering a beneficial response. It is interesting to point out that of all the anti-inflammatory agents currently under clinical trials, no study has examined suppression of TLR4. As discussed throughout this review, TLR4 is an important regulator of both innate and adaptive immune signalling. Since both phases of inflammation have been reported to contribute to ischaemic-reperfusion injury targeting TLR4 may be more beneficial. Like methotrexate [131], which has been proven to be effective at low doses, transient suppression of TLR4 may suppress innate immunity. This would require regulated time and dose dependent studies to determine how to suppress TLR4 signalling without disrupting the wound healing process.

2.19: Examination of novel TLR4 antagonists in the treatment of myocardial ischaemic eperfusion injury: (+)-naloxone and (+)-naltrexone

As mu opioid receptor antagonists, naloxone and naltrexone are used to treat sufferers of opioid or alcohol addiction [137]. Although steroisomeric variants exist for both compounds the (+) isomers have poor binding affinity towards mu opioid receptors [138]. Although they both share similar pharmacological structures the tertiary amine methyl structure found in naloxone is replaced by methylcyclopropane in naltrexone. This difference means that naltrexone has a longer half-life and greater potency compared to naloxone [139,140]. In 2008, Hutchinson *et al* [141] discovered through in-vitro studies that naloxone and naltrexone, independent of steroselectivity, suppressed TLR4 signalling and cytokine production. These finding were supported by additional experiments in 2010 through in-vitro, in-silico and in-vivo experiments [94]. Although this TLR4 blockade response was originally hypothesized to be directed through inhibition of the MyD88 pathway more recent evidence suggests that the TRIF pathway is supressed [142]. Wang *et al* [142] reported that both (+)-naloxone and (+)-naltrexone suppressed TRIF, but not MyD88, mediators IRF-3 and IFN-γ in LPS stimulated BV-2s. While limited, researchers have examined (-)-naloxone in the myocardial setting. After the discovery of opioid induced myocardial preconditioning [143],[72], investigators in the 1990s examined whether (-)-naloxone could block ischaemic-preconditioning [143,144]. While these findings were hypothesized to be mediated through the blockade of opioid receptor signalling, researchers were unaware of these compounds TLR4 antagonistic properties. Although opioid receptors are still believed to trigger myocardial preconditioning, the suppression of TLR4, in conjunction with mu-opioid receptor signalling, may explain why the preconditioning responses reported in these studies were

abolished. To our knowledge, no studies have studied whether this blockade effect is partially mediated through TLR4 or maintained in the positive stereoisoforms.

2.20: Examination of novel TLR4 antagonists in the treatment of myocardial ischaemic eperfusion injury: TAK242

TAK242 (Ethyl (6R)-6-[N-(2-chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate) is a TLR4 antagonist synthesized in the mid-2000s [145]. Significant research into the pharmacological properties of TAK242 [146–148] has revealed it to be potent TLR4 blocker suppressing inflammation through the TIR intracellular domain of the TLR4 receptor complex [147]. Matsunaga *et al* [148] explored this even further observing that TAK242 pretreatment can block both TIRAP and TRAM dependent NF-κB activity in HEK293 transfection models. These findings suggest that TAK242 is able to completely suppress TLR4's signalling network. Although TAK242 has been shown to protect against cerebral [149] ischemic-reperfusion injury it is uncertain as to whether TAK242 can protect against myocardial infarction. Additionally, whether the complete blockade of TLR4's signalling network influences tissue repair after ischaemic insult is unknown.

2.21: Conclusions

Although it is now clear that inflammation plays an important role in ischaemic-reperfusion injury the ability to regulate it remains poorly defined. TLR4 is an attractive target because of its broad influence on both the innate and adaptive immunity. Furthermore, the evidence behind LPS and DAMP induced preconditioning suggests that transient TLR4 signalling can

promote a delayed preconditioning response. Whether ischaemic-preconditioning can trigger TLR4 desensitization through the release of DAMPs during the preconditioning phase is unknown. It is also important consider how TLR4 is desensitized; whether this is through the interference of the TLR4 receptor complex or by enhanced negative feedback signalling requires additional investigation. On the other hand, considering the findings from the CANTOs study [136], suppression of TLR4 signalling is more clinically relevant. While blocking TLR4 in patients suffering from cardiovascular disease may be beneficial, additional pharmacological studies are required. Timing of administration, pre-existing co-morbidities, and the possible side effects of suppressing adaptive immunity, are all questions that need to be addressed in future studies.

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X: Statements of authorships (chapter 3)

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Publication Details	This study examined whether (+)-naloxone, administered during ischaemic-preconditioning, could influence contractile recovery in ischaemic-reperfused isolated rat hearts. Perfusate collected during early reperfusion was tested for LDH. Finally, left ventricles were test for JNK phosphorylation or HMGB1 protein levels using western blots.

Principal Author

Name of Principal Author	Mr Samuel Man lee		
Contribution to the Paper	Writing, proof-reading, editing, performed experiments and data analyses.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	11-01-2018

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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Chapter 3: Examining the effects of (+)-naloxone on contractile recovery in isolated ischaemic-preconditioned rat hearts.

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3.1: Abstract

Introduction: Ischaemic heart disease remains a significant cause of mortality throughout the developed world. In recent years clinical studies have shown that the suppression of innate immunity reduces the risk of cardiovascular disease. A major component which enhances myocardial inflammation is toll-like receptor 4, an immunosurveillance receptor, which triggers the innate immune response. However, evidence in the last 20 years suggests that transient TLR4 activity can trigger a preconditioning response. What remains unclear is whether ischaemic-preconditioning influences TLR4 signalling by triggering its desensitization. This study examined whether (+)-naloxone, a TLR4 blocker, could influence ischaemic-reperfusion injury and ischaemic-preconditioning.

Methods: Sprague Dawley (300-400g) rat hearts were isolated and perfused using a constant flow apparatus. All hearts were exposed to 30 minutes of no flow ischaemia (LTI) followed by 40 minutes reperfusion. Ischaemic-preconditioning (IPC) was induced by 3 × 5 minutes of ischaemia and 5 minutes of reperfusion. (+)-Naloxone was infused at 10 μM for 15 minutes prior LTI in non-preconditioned hearts (NPC). For IPC hearts, (+)-naloxone was infused during each 5-minute bout of reperfusion during IPC. Effluent collected 3 minutes after LTI was tested for lactate dehydrogenase (LDH). After each experiment, the left ventricle was isolated, and flash frozen in liquid nitrogen. Western blot analysis was used to detect total HMGB1 protein levels and JNK phosphorylation from left ventricular tissue.

Results: Ischaemic-preconditioning protected against LTI as observed by the significant improvement in left ventricular contractile function (LV%) (Ischaemic-preconditioned: 95.9 ± 7.56% vs non-preconditioned: 26.1 ± 13.6%) ($P < 0.05$). Although (+)-naloxone caused a depression in LV% in both non-preconditioned (10.9 ± 3.09%) and ischaemic-preconditioned hearts (76.9 ± 15.9%), no statistical significance was observed ($P>0.05$). Quantification of LDH revealed that IPC suppressed the release of LDH after ischaemic insult whereas NPC exacerbated its release (IPC-veh: 0.11 ± 0.05 vs NPC-veh: 0.47 ± 0.09) ($P < 0.05$). While LDH levels did not change in (+)-naloxone + NPCs (0.52 ± 0.13) hearts, LDH release was significantly higher when (+)-naloxone was administered in IPCs (0.60 ± 0.09) ($P < 0.05$). Ischaemic-preconditioning or (+)-naloxone treatment did not influence HMGB1 and JNK-phosphorylation levels ($P>0.05$).

Conclusion: (+)-Naloxone did not influence contractile recovery in ischaemic-preconditioned and non-preconditioned hearts. However, (+)-naloxone was shown to enhance the release of LDH which was conferred through ischaemic-preconditioning.

3.2: Introduction

Ischaemic heart disease remains a significant cause of mortality throughout the developed world. The loss or restriction of blood flow within the coronary vasculature initiates cellular stress mechanisms as the ischaemic myocardium becomes deprived of oxygen. While reperfusion is critical for survival, tissue injury can be exacerbated even further as the myocardium attempts to re-establish the environmental equilibrium [1–4]. Depending on the duration of ischaemia, reperfusion injury can be enhanced through myocardial inflammation [5]. Endogenous proteins released from necrotic resident cells or secreted from macrophages can bind onto immunosurveillance receptors within the ischaemic myocardium [6–9]. One of these receptors is Toll-like receptor 4 [10–13] (TLR4) which recognises lipopolysaccharide (LPS) a structural component found on gram negative bacteria [14]. However, during myocardial ischaemic-reperfusion injury, TLR4 recognises proteins such as high mobility group box 1 (HMGB1) [9,15] or heat shock proteins 60 (HSP60) [16,17], which are released during tissue damage and cell death. During this pathological event, the endogenous proteins which are released into the extracellular environment are termed as danger associated molecular patterns (DAMPs) [8]. Once activated, TLR4 triggers MyD88 [18] signalling resulting in the activation of signalling kinases such as p38 or c-Jun N-terminal kinases (JNK) [19]. Aside from triggering the production of pro-inflammatory cytokines [20], these kinases also promote cell death signalling during ischaemic-reperfusion injury [4,21–23].

3.21: Ischaemic-preconditioning and the possible involvement of TLR4

In 1986, Murry *et al* [24] discovered the cardioprotective phenomenon now known as ischaemic-preconditioning. In brief, Murry's *et al* [24] findings showed that acute bouts of

ischaemia and reperfusion, preceding 30 minutes of ischaemia, reduced infarct size. Research into the causations of this phenomenon have found that mitochondrial potassium ATP channels [25,26], protein nitrosylation [27,28], or activation of opioid receptors [29–31], promotes this preconditioning response. However, significant research gaps within the field still exist, especially in the field of myocardial inflammation. Although ischaemic-preconditioning is known to attenuate the production of inflammatory mediators [32–34], its impact on upstream signalling pathways, such as TLR4, remains poorly defined. Another reason why TLR4 is of interest is because of the literature surrounding LPS preconditioning. Studies have shown that pre-administration of low dose LPS, prior to ischaemic insult, elicits a myocardial preconditioning response [35–37]. Additionally, LPS preconditioning has been shown to be effective in other organ models of ischaemic-reperfusion injury suggesting this mechanism is not cardiac specific [38–40]. These findings suggest that TLR4 possesses bivalent signalling mechanisms which are dependent on the timing and degree of TLR4 activation. Although LPS preconditioning suggests that the TLR4 signalling cascade is somehow desensitized, it is unknown as to whether this also occurs during ischaemic-preconditioning. This study examined whether (+)-naloxone, a TLR4 antagonist, could influence contractile recovery in ischaemic-preconditioned hearts. Unlike the positive isoform, (-)-naloxone is used to treat alcohol and opioid addiction due to its ability to block mu-opioid receptors [41,42]. Research in the 1990s reported that (-)-naloxone could abolish the protective properties of myocardial ischaemic-preconditioning [32,33]. Although these findings were hypothesized to be mediated through the blockade of opioid receptors, the authors at the time were unaware of the compound's TLR4 inhibitory properties. To avoid the suppression of opioid induced preconditioning, (+)-naloxone was selected for this study.

Hence this study hypothesized that (+)-naloxone would influence contractile recovery in ischaemic-preconditioned hearts through the inhibition of TLR4 signalling.

3.3: Method

3.31: Animals ethics

Male Sprague Dawley rats (300 – 400 grams) were used for this study. Ethics for this project was approved by The University of Adelaide's Animal Ethics Committee (M-2014-048).

3:32: Isolated Langendorff rat technique

Hearts were rapidly excised from rats, placed into ice cold Krebs Henseleit buffer solution, and cannulated onto the constant flow Langendorff apparatus in less than 3 minutes. Krebs Henseleit buffer (Recipe: 130 mM NaCl, 4 mM KCl, 0.6 mM MgCl, 24 mM $NaHCO_3$, 24 mM NaH_2PO_4, 12 mM D-Glucose and 1.5 mM of $CaCl_2$), was heated at 37˚C and gassed with carbogen. The left auricle was then removed, and a latex balloon connected to a pressure transducer was inserted into the left ventricle. The balloon was then filled with milliQ water to allow for left ventricular diastolic (LVD) and systolic pressure (LVS) recordings with Labchart 5 (Adinstruments, Sydney, Australia). Left ventricular developed pressure (LVDP) was calculated as LVS – LVD. Flow rate was adjusted between 5 – 10 mls / min and LVD was adjusted to 10 mmHg. Schematic timelines of groups are shown in figure 1. Before drug infusion, (+)-naloxone was diluted at 10 µM into a separate bottle of Krebs buffer and the infusion line was switched to the drug solution when required. Long term ischaemia was induced by turning off the peristaltic pump for 30 minutes during which the heart was

submerged into warm, nitrogenated Krebs buffer. Left ventricular contractile recovery was determined by comparing post-ischaemic LVDP recordings to pre-ischaemic baseline measurements. The following equation was used: Left ventricular contractile recovery (LV%) = (post-ischaemic left ventricular developed pressure / pre-ischaemic left ventricular developed pressure baseline recordings) × 100%.

3.33: Tissue homogenization

After each Langendorff experiment the left ventricle was dissected, and flash frozen in liquid nitrogen. Cardiac tissue was homogenized in lysis buffer [50 mM Tris base saline pH 8.0, 0.5 ml Triton-X100, 0.5 ml protease inhibitor cocktail, 1,4-Dithiothreitol (DTT) and 2×SDS buffer] using a GentleMAC Octo dissociator (Macquarie Park, NSW, Australia). A Thermofisher Pierce BCA kit was used according to the manufacturer's instructions for protein quantification.

3.34: Western blot

35 µg of protein was loaded into 12 % agarose gels and separated at 100 volts for 1 hour using a Mini-protean electrophoresis tank (Biorad, NSW, Australia). The gels were then transferred onto nitrocellulose membranes using a trans-blot semi-dry electronic transfer system (Biorad, NSW, Australia) for 1 hour at 25 volts. Membranes were then blocked with 3% skim milk, diluted in PBS-tween 0.05% (PBS-T), for 1 hour at room temperature (RT) followed by an overnight incubation with one of the following primary antibodies: SAPK/JNK phos (Thr183/Tyr185) (9251), SAPK/JNK (9252) (Cell Signalling Technology, Danvers, USA), HMGB1 (GTX101277) all at 1:1000, and house keeper, GAPDH rabbit (GTX10018), 1:10,000 (Genetex, Irvine, USA) at 4˚C. Primary antibodies were diluted in PBS-T. After 3 PBS-T washes,

membranes were incubated with either Donkeynrabbit-700 or Streptavidin-800 for 1 hour, RT in the dark. The membrane was washed again 3 times with PBS-T followed by 1 PBS wash before scanning. A Li-cor Odyssey scanner (Li-cor, Lincoln, USA) was used to scan the membranes at 700 and 800 λ. Image analyses was performed using ImageJ software (National Institute of Health, Maryland, USA). Data is presented as the relative expression of the protein interest after controlled with the GAPDH. The same method was also performed for total JNK and JNK-phos before JNK-phos / GAPDH was divided by total JNK / GAPDH.

3.35: LDH quantification

Perfusate was collected immediately after the 30 minutes of ischaemia and stored in a -20 freezer. Samples were collected 3 minutes after ischaemia. A Lactate Dehydrogenase Assay kit (KA0878) (Abnova, Taipei, Taiwan) was used according to the manufactures instructions. The assay was read using a Millenium Science Biotek plate reader (Mulgrave, Victoria, Australia)

3.36: Data analyses (isolated heart and western blot data)

To minimize the effects of intergroup variability, Langendorff data timepoints, 30 – 65 mins, were averaged for each recording for both LVDP% and LVD measurements. All data was formatted in Microsoft Excel (Redmond, Washington, USA) before being analysed with GraphPad Prism (GraphPad Software Inc, California, USA). Multiway ANOVAs with post-hoc TukeyHSDs were constructed to analyse both the Langendorff and western blot data. All data is presented as mean ± standard error of the mean (μ ± SEM), statistical significance was defined as ($P < 0.05$).

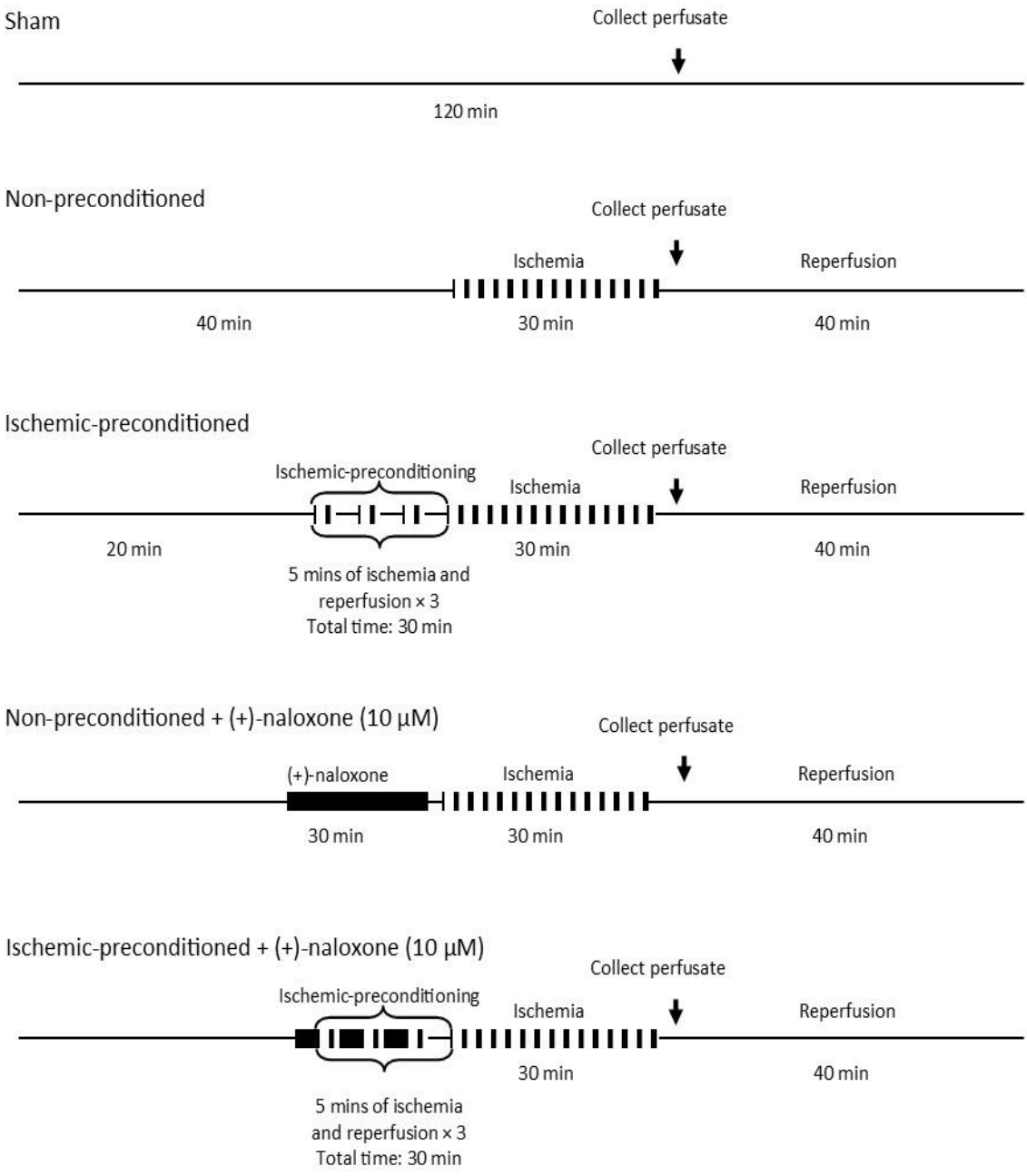


Figure 1: Schematic timeline of the groups examined in isolated rat heart experiments. Bars with the vertical lines represent no flow ischemia. Full bars represent drug infusions of (+)-naloxone at 10 µM at noted times. Perfusate was collected 2 minutes after reperfusion for analysis of LDH levels.

3.37: Contingency tests (isolated heart data)

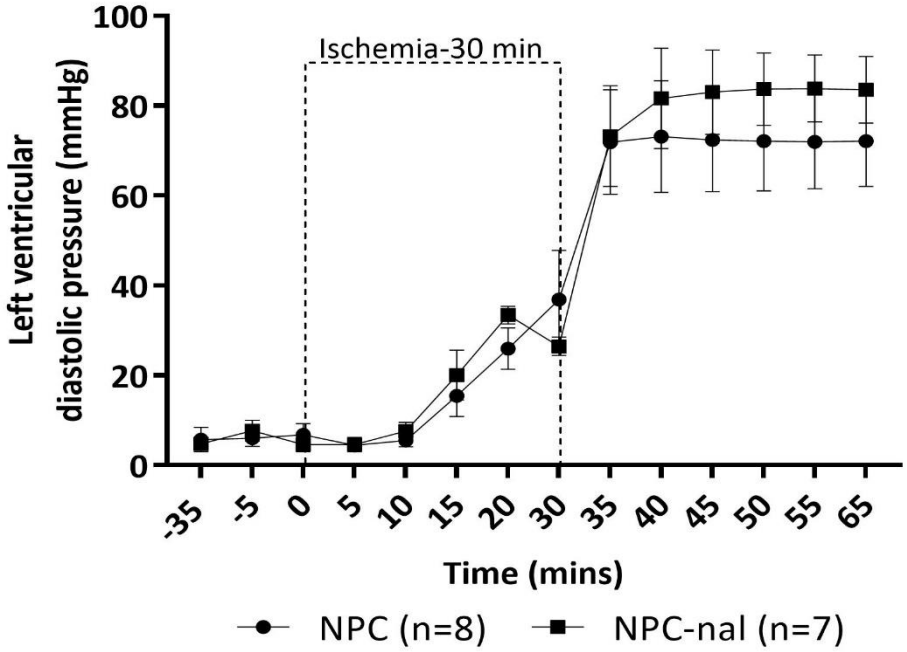
A significant amount of intergroup variability was observed in ischaemic-preconditioned hearts treated with (+)-naloxone. To determine whether this variability could be explained by (+)-naloxone, two-sided Chi-square tests with Fisher's exact corrections, were constructed. Recovery from ischaemic insult was defined as hearts with LV% greater than or equal to 70%.

3.4: Results

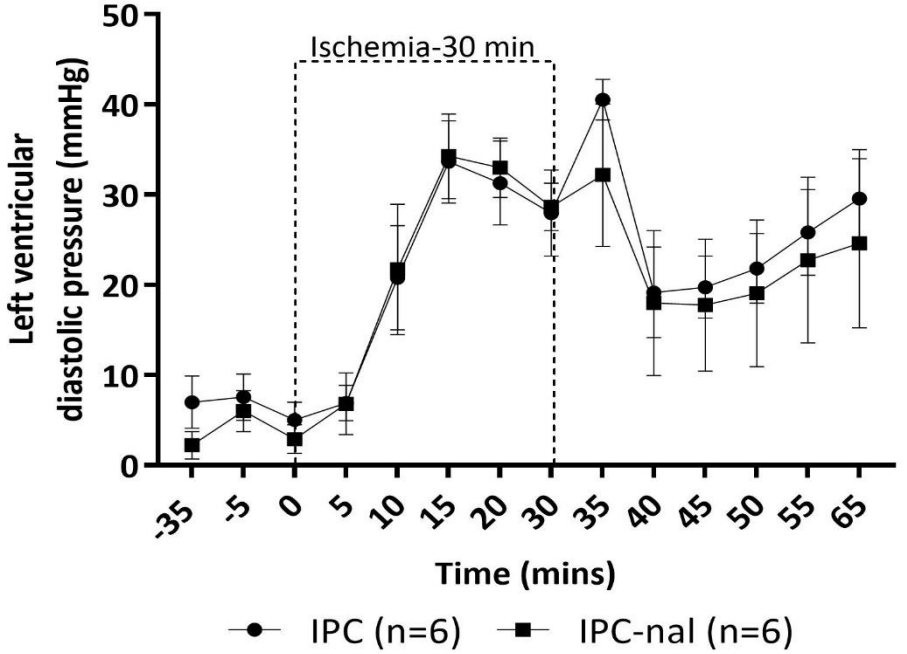
3.41: (+)-Naloxone does not influence contractile recovery in non-preconditioned and ischaemic-preconditioned hearts.

Left ventricular contractile recovery was used to determine whether (+)-naloxone could influence non-preconditioned and ischaemic-preconditioned hearts (figure 2). While two-way ANOVA revealed that ischaemic-preconditioning protected against ischaemic-reperfusion injury ($P < 0.05$) (+)-naloxone did not influence LV% or LVD function ($P > 0.05$) (figure 3). Non-preconditioned hearts to lost approximately 74% of their original left ventricular function recorded prior ischaemic insult (26.1 ± 13.6%) (figure 3b). Additionally, LVD in non-preconditoned hearts rose to 72 mmHg (72.2 ± 10.8 mmHg) suggesting reduced relaxation of the heart during diastole (figure 3a). When exposed to ischaemic preconditioning, hearts were protected against ischaemic insult as shown by the significant improvement in LVD recovery and LV% (LVD: 29.0 ± 6.0 mmHg and LV% 95.9 ± 7.6%) ($P < 0.05$) (figure 3). Although (+)-naloxone depressed LV% for both non-preconditioned (10.9 ± 3.1%) and ischaemic-preconditioned hearts (76.9 ± 16.0%), no statistical significance was observed ($P > 0.05$) (figure 3).

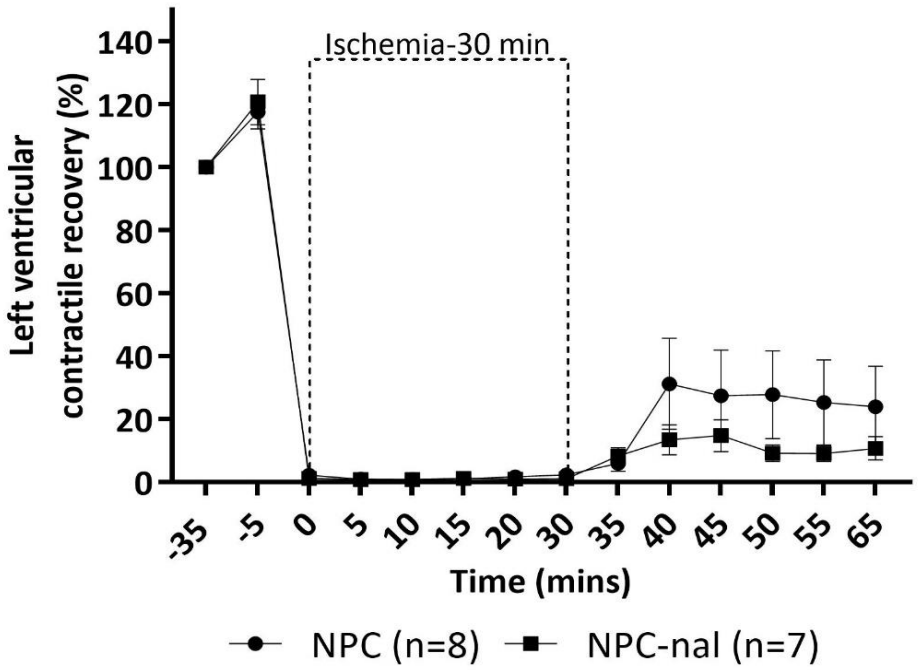
2a


Ischemia-30 min
Left ventricular diastolic pressure (mmHg)
100
80
60
40
20
0
-35
-5
0
5
10
15
20
30
35
40
45
50
55
65
Time (mins)
NPC (n=8)
NPC-nal (n=7)

2b


Ischemia-30 min
Left ventricular diastolic pressure (mmHg)
50
40
30
20
10
0
-35
-5
0
5
10
15
20
30
35
40
45
50
55
65
Time (mins)
IPC (n=6)
IPC-nal (n=6)

2c


Ischemia-30 min
Left ventricular contractile recovery (%)
140
120
100
80
60
40
20
0
-35
-5
0
5
10
15
20
30
35
40
45
50
55
65
Time (mins)
NPC (n=8)
NPC-nal (n=7)

2d

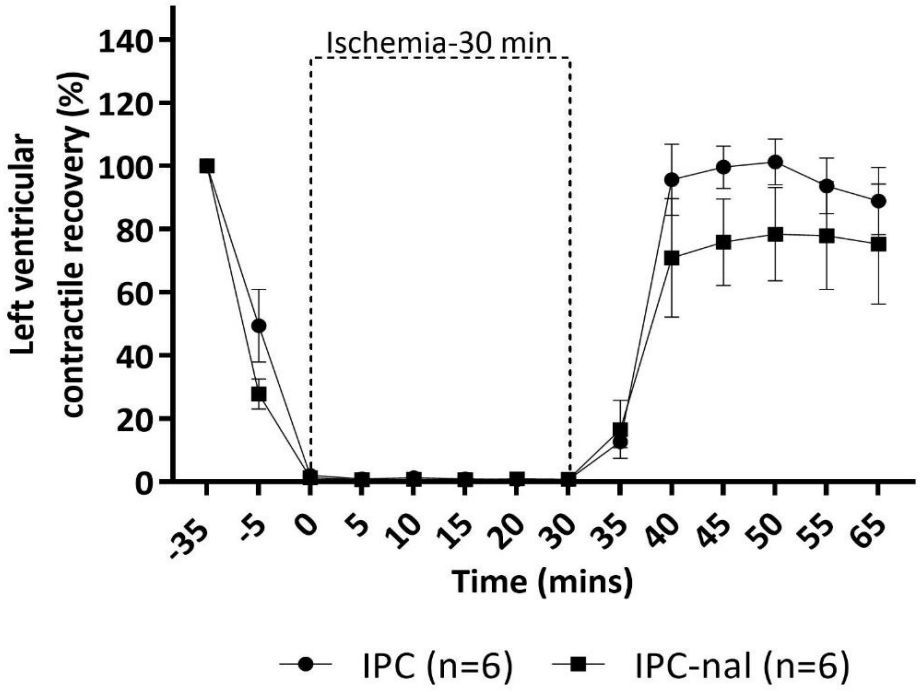

Ischemia-30 min
Left ventricular contractile recovery (%)
140
120
100
80
60
40
20
0
-35
-5
0
5
10
15
20
30
35
40
45
50
55
65
Time (mins)
IPC (n=6)
IPC-nal (n=6)

Figure 2: Complete timeline recordings of LV% and LVD of ischaemic-preconditioned rat hearts treated with or without ischaemic-preconditioning. Left ventricular diastolic pressure of non-preconditioned (2a) or ischaemic-preconditioned (2b) hearts treated with or without (+)-naloxone. 2c-2d) Left ventricular contractile recovery (LV%) of non-preconditioned (2c) or ischaemic-preconditioned (2d) hearts treated with or without (+)-naloxone. Data presented as mean ± SEM, n = 6 - 8.

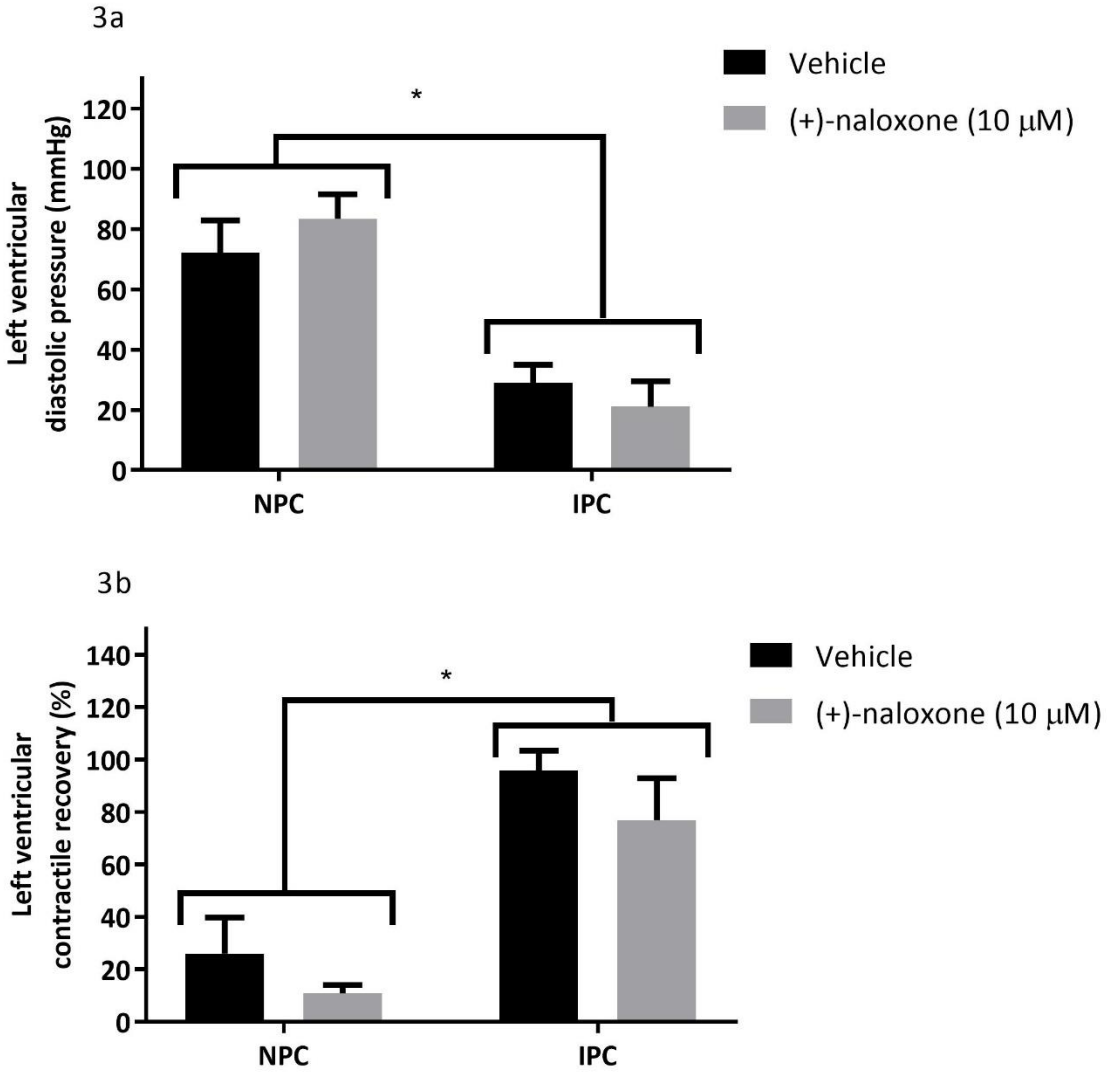


Figure 3: Late reperfusion summaries (averages between 30-65 mins) recording of the isolated rat heart experiments. 3a) Left ventricular diastolic pressure, 3b) left ventricular contractile recovery. *

Represents statistical significance observed between groups ($P < 0.05$). Data presented as mean ± SEM, n = 6 - 8.

Similar observations were also made when LVD was examined (figure 3a). By the end of the experiment LVD rose to 72 mmHg (72.2 ± 10.8 mmHg) in non-preconditioned hearts (figure 3a) whereas ischaemic-preconditioning limited its rise (29.0 ± 6.0 mmHg) ($P < 0.05$). (+)-Naloxone did not influence LVD in either non-preconditioned (83.5 ± 8.1 mmHg) or ischaemic-preconditioned hearts (21.0 ± 8.5 mmHg) ($P > 0.05$) (figure 3a).

3.42: (+)-Naloxone does not explain the variability observed in ischaemic-preconditioned hearts treated with (+)-naloxone.

Because of the significant level of variability observed in (+)-naloxone treated ischaemic-preconditioned hearts, two-sided Chi-square tests (with Fisher's exact P-value corrections) were constructed (table 1). Functional recovery after ischaemic insult was defined as a LV% value of 70% or greater. Under these parameters, all ischaemic-preconditioned hearts recovered from ischaemic insult. Without ischaemic-preconditioning, 78% of non-preconditioned hearts did not recover from ischaemic insult (table 1). Chi-square analyses revealed statistical significances between non-preconditioned vehicle vs ischaemic-preconditioned vehicle hearts ($P < 0.05$) (table 1). The proportion of hearts which recovered from 30 minutes of ischaemia in the ischaemic-preconditioned group treated with (+)-naloxone was only 42.9%. Although this value is smaller compared to ischaemic-preconditioned vehicle hearts, chi-square analyses reported no statistical significance

between the two groups (ischaemic-preconditioned vehicle vs ischaemic-preconditioned + (+)-naloxone, P = 0.07) (table 1).

Recovery (≥70%)	**Non-preconditioned (n=8) ***	**Ischaemic-preconditioned (n=6) ***	**Non-preconditioned (+)-naloxone (n=7)**	**Ischaemic-preconditioned (+)-naloxone (n=6)**
Yes	22.2%	100 %	0 %	42.9%
No	77.78%	0%	100 %	57.1%

Table 1: Two side Chi-square test with Fisher's exact P-value corrections examining the percentage of hearts which survived ischaemic insult. * = P < 0.05

3.43: (+)-Naloxone caused an increase release of LDH in ischaemic-preconditioned hearts.

LDH is a ubiquitous enzyme located in the cytosol. As a predicative biomarker of injury, its release into the extracellular environment often signals tissue injury. Two-way ANOVA showed an interactive effect between ischaemic-preconditioning and (+)-naloxone treatment (figure 4) (P < 0.05). Effluent collected at the beginning of reperfusion revealed that there was a significantly higher level of LDH in non-preconditioned vehicle hearts (0.47 ± 0.09 mU/ml) compared to those which were given ischaemic-preconditioning (0.11 ± 0.05 mU/ml) (P < 0.05). However, when (+)-naloxone was administered into ischaemic-preconditioned hearts, LDH levels were significantly higher (0.60 ± 0.09 mU/ml) compared to ischaemic-preconditioned vehicle hearts (figure 4). Infusion of (+)-naloxone prior to ischaemia did not influence LDH levels in non-preconditioned hearts (0.52 ± 0.13 mU/ml).

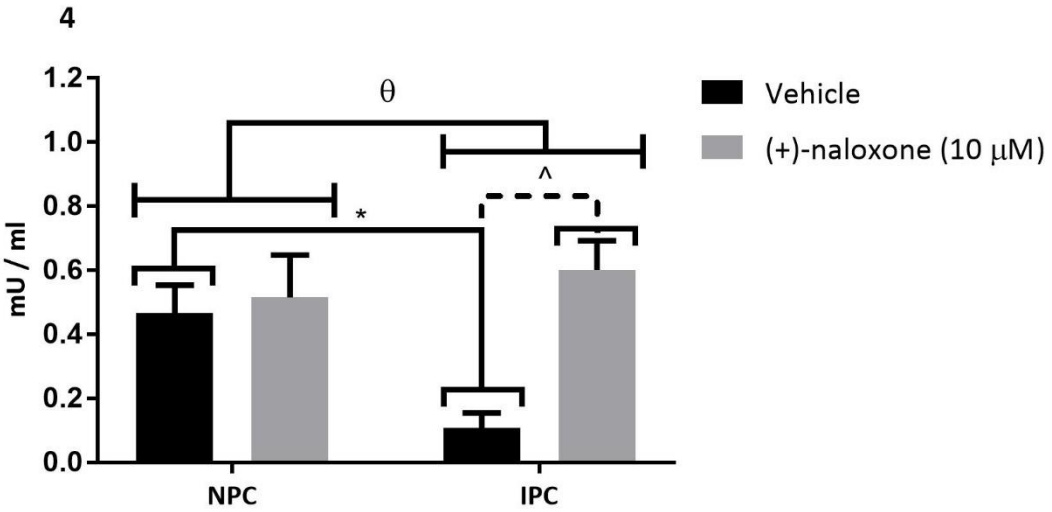


Figure 4: LDH release from isolated rat hearts after ischaemic insult. NPC: Non-preconditioned, IPC: Ischaemic-preconditioned. * represents the statistical difference observed between NPC-veh vs IPC-veh ($P < 0.05$). ^ represents the statistical difference observed between IPC-veh vs IPC-naloxone ($P < 0.05$). θ represent the statistical interaction reported between ischaemic-preconditioning and (+)-naloxone treated hearts. Data presented as mean ± SEM, n = 6-8.

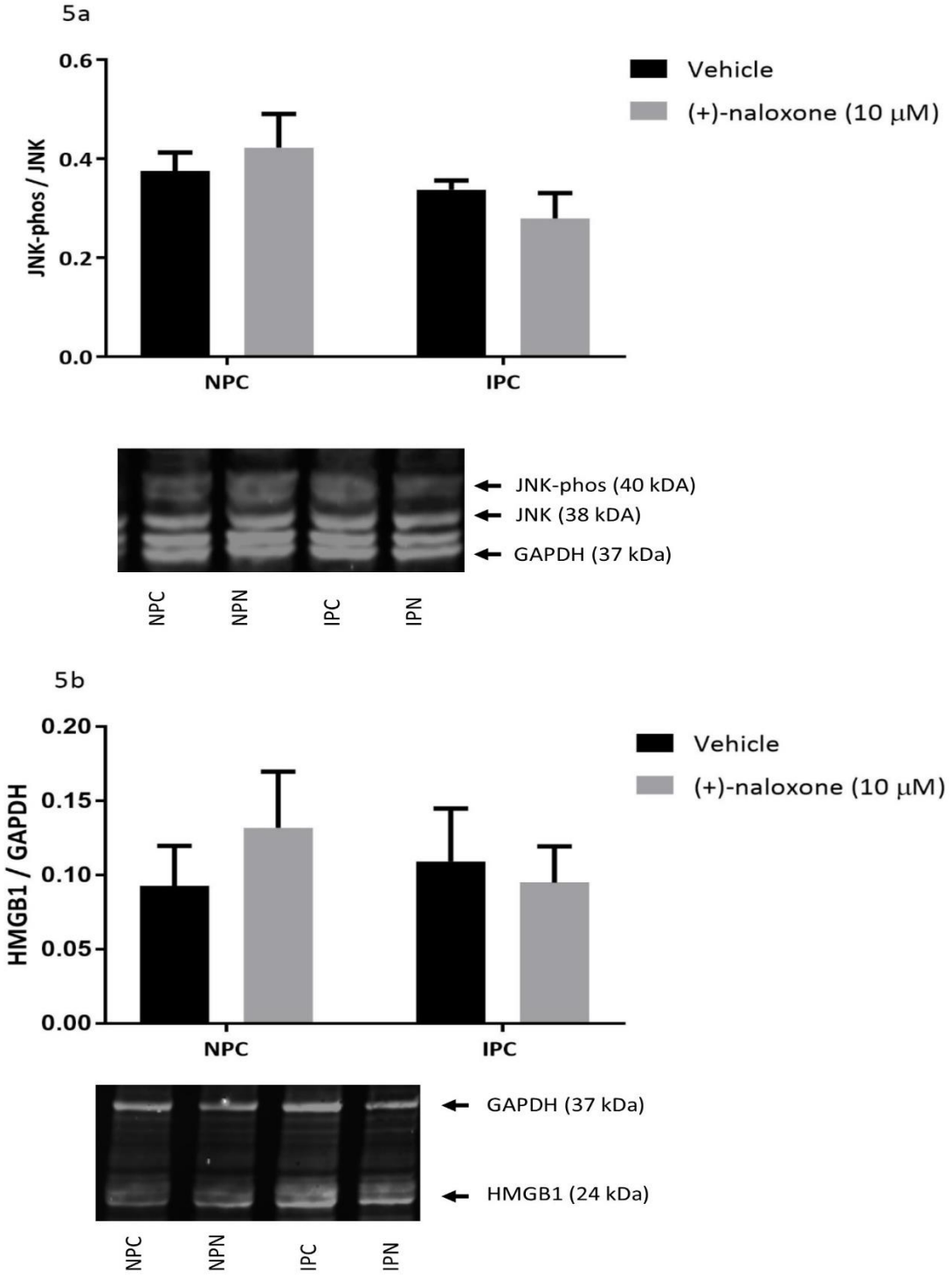


Figure 5: Western blot analyses of JNK phosphorylation (5a) and HMGB1 (5b) levels from isolated rat hearts. NPC: non-preconditioned hearts, NPN: non-preconditioned + (+)-

naloxone, IPC: ischaemic-preconditioned, IPN: ischaemic-preconditioned + (+)-naloxone. Data presented as mean ± SEM, n = 6-7.

3.44: Ischaemic-preconditioning and (+)-naloxone did not influence JNK phosphorylation or HMGB1 expression.

JNK activation is associated with the activation of cell death signalling cascades and promoting the de-novo synthesis of the pro-inflammatory cytokine, TNF-α. Two-way ANOVA revealed that ischaemic-preconditioning and (+)-naloxone did not influence either factors ($P > 0.05$) (figure 5a). Ischaemic-preconditioning did not influence JNK phosphorylation when compared to non-preconditioned hearts (0.38 ± 0.04) ($P > 0.05$). Furthermore, (+)-naloxone did not influence phosphorylation activity in either non-preconditioned (0.42 ± 0.07) or ischaemic-preconditioned hearts (0.28 ± 0.05) ($P > 0.05$) (figure 5a). Furthermore, quantification of HMGB1 revealed that neither ischaemic-preconditioning or (+)-naloxone influenced protein levels (figure 5b) ($P > 0.05$). No differences were observed between ischaemic-preconditioned hearts treated with or without (+)-naloxone. Finally, (+)-naloxone did not affect HMGB1 expression in non-preconditioned hearts (figure 5b).

3.5: Discussion

It is now known that myocardial inflammation is influential in the initiation and resolution of late reperfusion injury [5]. Through the recognition of DAMPs released [15–17] during ischaemia and reperfusion, TLR4 triggers the MyD88 signalling pathway resulting in the de-novo synthesis or activation of inflammatory mediators [14]. While ischaemic-

preconditioning is known to suppress inflammatory mediators [26,33,34], its impact on TLR4 signalling during early reperfusion remains poorly defined. This study examined whether (+)-naloxone, a TLR4 antagonist, could influence left ventricular recovery when administered directly during ischaemic-preconditioning. The negative stereoisomer of naloxone is commonly used to treat opioid or alcohol addiction due to its ability to block mu opioid receptors, a property which (+)-naloxone lacks. During the 1990s, (-)-naloxone was tested to see whether it could block opioid receptor signalling triggered during myocardial ischaemic-preconditioning. Chien *et al* [29] and Tomai *et al* [44] both reported that (-)-naloxone could block contractile recovery in ischaemic-preconditioning hearts through the suppression of opioid receptors. However, because (-)-naloxone [45,46] is also known to suppress TLR4 it is uncertain whether the blockade of ischemic-preconditioning is partially mediated through TLR4. To avoid the possibility of suppressing opioid receptor signalling, this study selected (+)-naloxone which lacks the ability to inhibit mu opioid receptors [47]. Because no study has examined (+)-naloxone using isolated hearts the concentration tested was determined from studies conducted by Hutchinson *et al* [45,46]. Furthermore, a recent study conducted by Wang *et al* [48] reported that (+)-naloxone is able to bind onto MD2, an adaptor protein involved in activating the TLR4 receptor complex, at low concentrations.

3.51: (+)-Naloxone does not influence contractile recovery in ischaemic-preconditioned hearts but does enhance the release of LDH.

The findings from this study suggest that (+)-naloxone does not influence contractile recovery in ischaemic-preconditioned hearts. While contractile function was depressed in (+)-naloxone treated ischaemic-preconditioned hearts no statistical significance was observed. Because of

the significant intergroup variability reported in this group, chi-square analyses were then performed to determine if this variability was associated with (+)-naloxone. While a lower ratio of hearts was defined as "recovered" (which was defined as greater than, or equal to, 70% LV% in this study) in the (+)-naloxone-ischaemic-precondition group, no differences were seen. These findings support Chien *et al's* [29] original study which showed that (-)-naloxone, but not (+)-naloxone, blocked the infarct limiting effects of ischaemic-preconditioned hearts. On the other hand, it should be noted that Chien *et al* [43] study used 1) the in-vivo infarct technique on rabbit hearts, and 2) administered naloxone before ischaemic-preconditioning. Additionally, since dose-dependent experiments have not been conducted on (+)-naloxone, the lack of change reported in this study and Chein *et al* [43] results may be attributed to the concentration used.

This study also reported an interactive effect between (+)-naloxone and ischaemic-preconditioned hearts when LDH was examined. Residing within the cytosol, the release of LDH into the bloodstream or extracellular environment is indicative of tissue injury and used as a biomarker of tissue injury [49,50]. Since LDH release is a common predictive biomarker of tissue injury [51,52], LDH was selected for this study. Analysis of perfusate from isolated hearts revealed that while ischaemic-preconditioning attenuated the release of LDH, higher levels of LDH were released in (+)-naloxone + ischaemic-preconditioned hearts. These findings suggest that (+)-naloxone may have partially blocked the protective properties of myocardial ischaemic-preconditioning. The differences between the contractile function and LDH data are conflicting. While it was originally believed that the lack of significance in the isolated heart recordings was attributed to intergroup variability, the chi-square tests conducted do

not support this theory. Additionally, because LDH is not considered a cardiac specific biomarker it is difficult to directly correlate changes in contractile function to LDH release. Analysis of a more cardiac specific biomarkers such as cardiac troponin I (cTnT) [51],[53], creatine kinase (CK) [53] or cardiac fatty acid binding protein (cFABP) [54] may be more reflective of the changes in contractile function. On the other hand, because the isolated heart was used, any LDH collected would have been released directly from the heart. Considering these findings, and the duration of reperfusion for each heart, it would be interesting to see whether perfusing hearts for a longer period would show a more prominent difference in contractile function after ischaemic insult. Furthermore, since myocardial inflammation emerges during the later stages of reperfusion [5], it may be possible that a more prominent effect would be observed 24 hours after the original ischaemic insult.

3.52: (+)-Naloxone and ischaemic-preconditioning did not influence JNK phosphorylation and HMGB1 levels.

This study also showed that neither (+)-naloxone or ischaemic-preconditioning effected HMGB1 levels. HMGB1 is known to act as a DAMP during ischaemic-reperfusion injury [55,56] when released into the extracellular environment. However, prior to its release, HMGB1 translocates into the cytosol where it undergoes hyperacetylation [57,58]. Like LDH, this study was interested in seeing as to whether HMGB1 could be used as an acute biomarker of injury. While HMGB1 levels did not change between groups it does suggest that its release from the myocardium does not occur during early reperfusion. However, since the protein needs to translocate into the cytosol before its release, future experiments could measure the levels of nucleus and cytosolic HMGB1 during early reperfusion.

Finally, this study showed that JNK phosphorylation was not influenced by ischaemic-preconditioning or (+)-naloxone. The JNK group is derived from the mitogen protein kinase (MAPK) family. All three JNKs isomers (all termed as JNK1, JNK2 and JNK3) exist within the heart and play specific roles in the myocardium [59]. Ma *et al* [23] was the first to show that, in the context of ischaemic-reperfusion injury, the phosphorylation of JNK develops as quickly as 10 minutes after reperfusion before returning to baseline after 90 mins. Early studies reported that JNK phosphorylation triggers the activation of apoptotic signalling pathways resulting in cardiac injury [4,22,60,61]. These findings are also supported by Kaiser *et al* [62] who showed in in-vivo mouse infarct studies that the genetic knockout of JNK1 and JNK2 reduced infarct size. However, the same study [62] also reported that the transgenic overexpression of MKK7, a upstream activator of JNK, also reduced infarct size. The role of JNK is complicated even further when examining its role in ischaemic-preconditioning. Sato *et al* [63] showed that ischaemic-preconditioning itself promoted the upregulation of JNK phosphorylation. However, when curcumin, a JNK antagonist, was administered prior ischaemic-preconditioning, cardioprotection was abolished. These studies suggest that JNK has a biphasic role in ischaemic-reperfusion injury and ischaemic-preconditioning. Timing and duration of JNK signalling clearly determines where the MAPK attenuates or enhance ischaemic-reperfusion injury. It is uncertain as to why no differences were observed in our study. Whether this is related to technical issues or due to our experimental design requires additional investigation.

3.6: Conclusion

This study suggests (+)-naloxone may have blocked the cardioprotective effects of ischaemic-preconditioning as shown by increased release of LDH from the myocardium. Although LDH release is a predictor of tissue damage, the lack of changes observed in left ventricular contractile recovery, JNK signalling, and HMGB1 levels is conflicting. Additionally, significant intergroup variability was reported in ischaemic-preconditioned hearts treated with (+)-naloxone. Whether this is attributed to the experimental design of this study requires additional investigation.

3.7: Acknowledgements

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XI: Statements of authorships (chapter 4)

Title of Paper	Design flaws of the constant flow isolated rat model from study 1 and the preliminary experiments conducted for study 2
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Linking chapter between study 1 and study 2. Preliminary studies for study 2 are also in this chapter. Duration of ischaemia was troubleshooted in isolated mouse hearts. Ischaemic-preconditioning was also optimized in this chapter.

Principal Author

Name of Principal Author	Mr Samuel Man lee		
Contribution to the Paper	Writing, proof-reading, editing, performed experiments and data analyses.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Proof-reading, provided funding to this study, and allowed me to me use his lab during my stay at the University of Queensland.		
Signature		Date	8/1/18

Chapter 4: Design flaws of the constant flow isolated rat model from study 1 and the preliminary experiments conducted for study 2

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4.1: Limitations of study 1

The original aim of chapter 3 was to determine whether (+)-naloxone could directly influence the contractile function of non-preconditioned and ischaemic-preconditioned hearts. Although these findings were initially promising, the significant amount of intergroup variability observed in (+)-naloxone treated hearts influenced the data analyses. Furthermore, while the constant flow isolated heart technique is simple to perform, it is not the optimal ex-vivo model to use when studying ischaemic-reperfusion injury [1]. Because the flow rate is maintained at a constant state the myocardium cannot adjust vascular flow when required. During events where the myocardium requires increased oxygen demand, vascular flow is increased [2]. This is can be problematic in ischaemic-reperfusion studies when the heart requires a higher flow rate after ischaemic insult. This can lead to sub-optimal coronary perfusion and cause additional damage throughout the reperfusion stage.

4.2: Experimental design of study 2 and optimisation of the ischaemic-reperfusion protocol and ischaemic-preconditioning.

Because of these issues, the 2nd study of this thesis re-examined the hypothesis of study 1 using a different model, the constant pressure isolated heart technique [1],[3]. Furthermore, because there was a concern that the variability observed was due to (+)-naloxone itself a more potent TLR4 antagonist was also examined. Hence (+)-naltrexone was also tested in study 2. Similar to naloxone, in terms of its molecular structure and pharmacological properties, naltrexone has an longer half-life and greater drug potency regarding its ability to block mu-opioid receptors [4,5]. Finally, because of the lactate dehydrogenase (LDH) data

collected in study 1, I decided to look at cardiac fatty acid binding protein (cFABP) levels within the cytosolic fractions of the left ventricles [6]. Like LDH, cFABP is an acute biomarker of injury which is released during early reperfusion. CFABP was selected over LDH for study 2 because it is only located in the heart and thus would be a more accurate marker of myocardial injury compared to LDH.

While no difference in HMGB1 release was observed in study 1 this may be attributed to the fact that total HMGB1 (both cytosol and nuclear) content was examined. Because HMGB1 requires cytosolic translocation from the nucleus before its release [7,8], study 2 investigated whether ischaemic-preconditioning or (+)-naloxone / (+)-naltrexone influenced HMGB1 translocate. Finally, study 2 also investigated whether interleukin-1 beta (IL-1β) activity could be detected in the myocardium. Because acute reperfusion was studied, it would be unlikely that the pro-inflammatory cytokine would be upregulated within 1 hour. IL-1β was selected because unlike other pro-inflammatory cytokines, pre-cursor forms of IL-1β exist in the cytosol [9]. Also known as pro-IL-1β it's activation by inflammasomes, inflammatory complexes which are present in the cytosol, cleave pro-IL-1β releasing the active form [9]. Hence study 2 examined whether the cleaved form of cytosolic IL-1β could be detected during early reperfusion.

As reported by Reichelt *et al* [3], differences in ischaemic resistance have been reported between mouse strains. BALB/c mice (which were selected for study 2) were discovered to exhibit increased resistance to ischemic insult when compared to other strains such as C57BL/6s or 129/sv [3]. BALB/cs were selected for study 2 because I was originally interested

in looking at TLR4 knockout BALB/c mice. Because of these findings, preliminary studies were conducted to determine the appropriate parameters. Hence this study also examined what is the appropriate duration of ischaemia for BALB/c mice and 2), triggering ischaemic preconditioning (IPC) in isolated mouse hearts.

4.3: Methods

4.31: Constant pressure isolated heart mouse model

The protocol used to conduct these preliminary studies is as described in Reichelt et al [3]. A detailed description of constant pressure isolated mouse heart technique is described in study 2, chapter 5. Left ventricular diastolic pressure (LVD), left ventricular developed pressure (left ventricular systolic – left ventricular diastolic pressure) (LVDP) was measured. Left ventricular contractile recovery (LV%) from ischaemic insult was determined by the following equation: (Left ventricular developed pressure post-ischaemic measurements / left ventricular developed pressure pre-ischaemic baseline measurements) × 100%. LVDP was used as a measure of contractile recovery. Ischaemic insult was induced for either 30, 32.5 or 35 mins followed by either 40 or 45 mins of reperfusion. After 35 mins of ischaemia was determined to cause a significant reduction in contractile recovery, ischaemic-preconditioning was then optimized. Two protocols were tested for ischaemic-preconditioning. Protocol 1: 3 bouts of 2.5 mins of ischaemia and 2.5 mins of reperfusion. Protocol 2: 3 bouts of 2.5 mins of ischaemia and 5 mins of reperfusion. All data is presented as mean ± SEM. Averages of the last 30 minutes of reperfusion were analyzed with one-way ANOVAs with post-hoc TukeyHSD. Data presented as mean ± SEM, $P < 0.05$ represents statistical significance.

4.4: Results

4.41: 32.5 and 35 mins of no-flow ischaemia causes a significant depression in left ventricular contractile recovery.

Complete recordings of isolated hearts exposed to different periods of ischaemia are represented in figure 1. The preliminary experiments conducted in this study support Reichelt *et al* [3] findings. BALB/c hearts exposed to 30 mins of ischaemia recovered approximately 70% of their original contractile recovery and reduced LVD. NPC – 30 mins: LVD – 3.4 ± 0.2 mmHg, LV%: 73.7 ± 6.3 % (figure 4a, 4c). However, when BALB/c hearts were exposed to either 32.5 mins or 35 mins of ischaemia, contractile function was significantly reduced ($P < 0.05$). LVD increased to 22 mmHg (22.0 ± 0.5 mmHg) when exposed for 32.5 mins ischaemia and was exacerbated even further when exposed for 35 mins (44.3 ± 4.7 mmHg) (figure 4a). One-way ANOVA reported are difference between each of these groups ($P < 0.05$). Although LV% was depressed after 32.5 or 35 mins of ischaemia (NPC – 32.5 mins: 44.5 ± 7.3% & NPC – 35 mins: 52.1 ± 7.5%) statistical significance was only reported between the NPC – 30 mins and NPC – 32.5 mins ($P < 0.05$) (figure 4c). The lack of statistical significance between LVDP%: NPC - 30 mins & NPC – 35 mins may be attributed to intergroup variability. 35 minutes of ischaemia was selected for study 2 because of the significant increase in LVD (figure 4a).

4.42: 2.5 minutes of ischaemia and 2.5 minutes reperfusion for 3 times triggers myocardial ischaemic-preconditioning.

Complete recordings of non-preconditioned isolated hearts compared to ones given ischaemic-preconditioning are presented in figure 2 (IPC – 2.5I / 2.5 R) and figure 3 (IPC - 2.5I / 5R). Of the two protocols tested, one-way ANOVA revealed that repeated bouts of 2.5 mins

of ischaemia and 2.5 mins of reperfusion protected against 35 mins of ischaemia (figure 5a, 5c). Compared to non-preconditioned hearts, ischaemic-preconditioning (IPC – 2.5I / 2.5 R) reduced the rise in LVD (NPC: 45.6 ± 6.6 vs IPC – 2.5I / 2.5 R: 20.5 ± 1.3 mmHg) (figure 5a) and improved contractile recovery (NPC: 37.4 ± 3.1 vs IPC – 2.5I / 2.5 R: 72.3 ± 6.4 mmHg) (figure 5c). The second ischaemic-preconditioning protocol which was examined did not protect against 35 mins of ischaemic insult (figure 5).

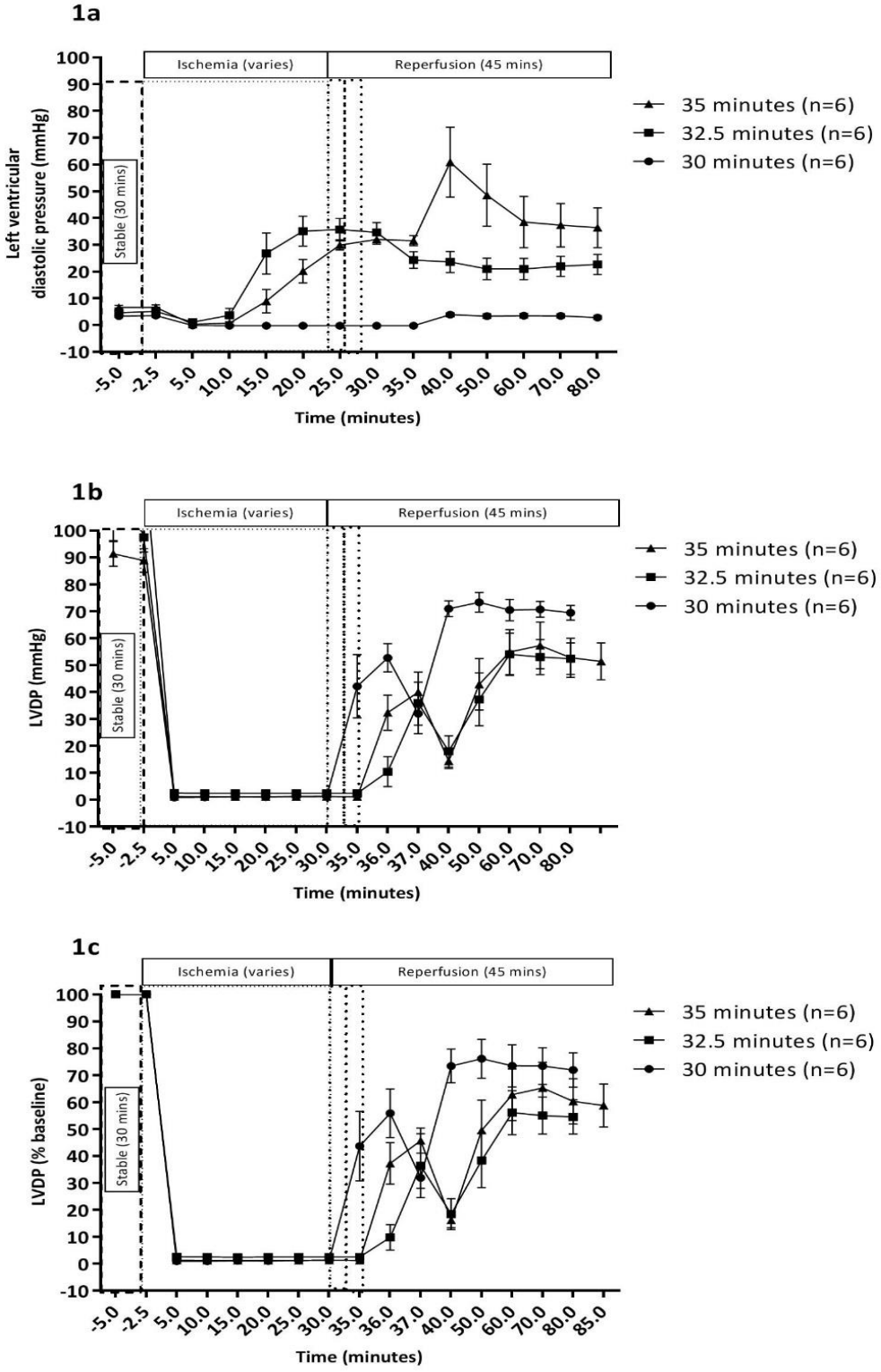

Figure 1: Isolated mouse hearts exposed to 30, 32.5 and 35 mins of ischaemia. Complete timeline recordings, 1a) LVD, 1b) LVDP and 1c) LVDP%. Data presented as mean ± SEM.

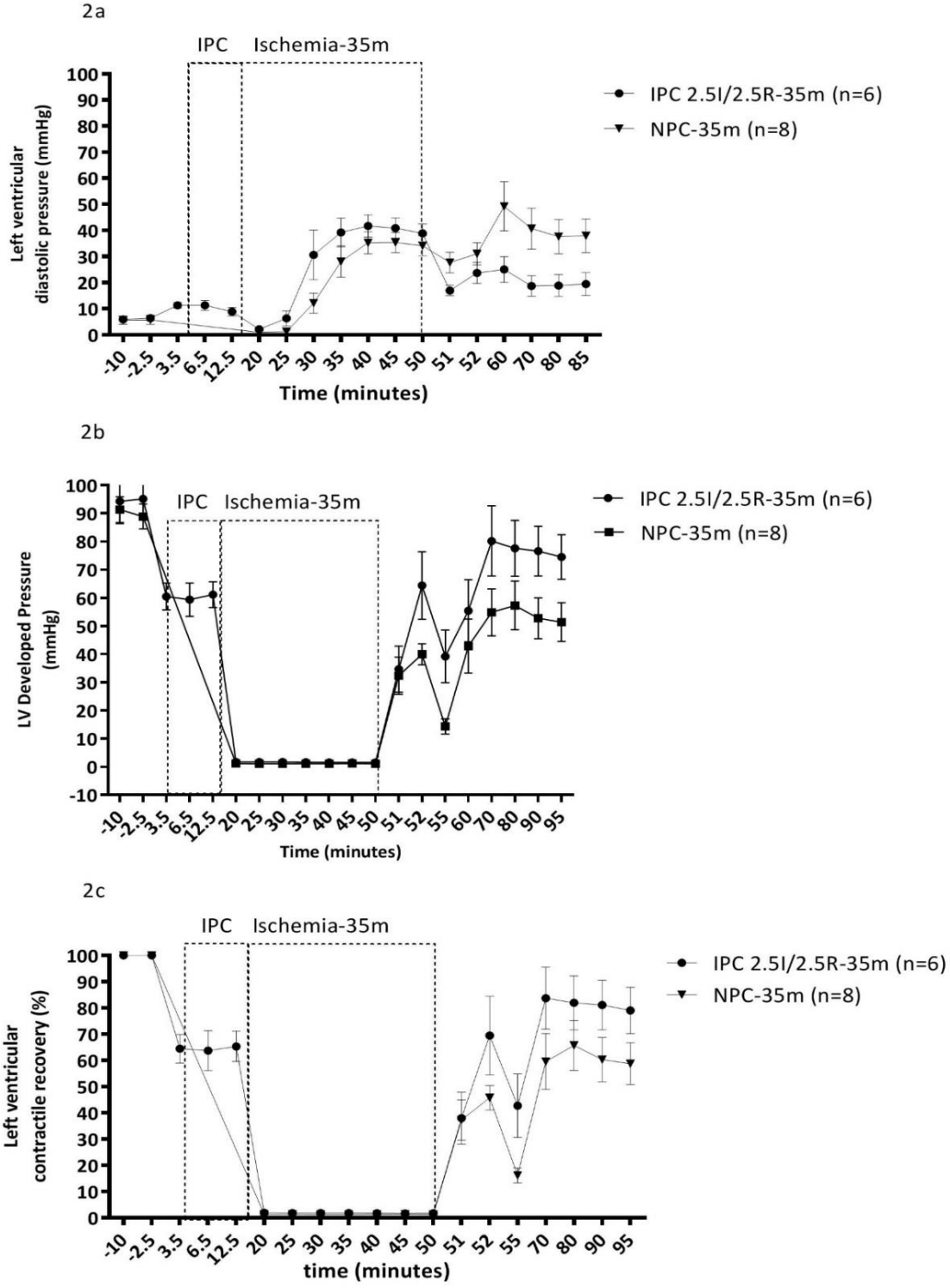

Figure 2: Isolated mouse hearts examining, ischaemic preconditioning (3 × 2.5 mins of ischaemia and 2.5 mins of reperfusion). 2a) LVD, 2b) LVDP, and 2c) LVDP%. NPC: Non-preconditioned hearts, IPC: Ischaemic-preconditioned hearts. Data presented mean ± SEM. Samples sizes are shown in the graphs.

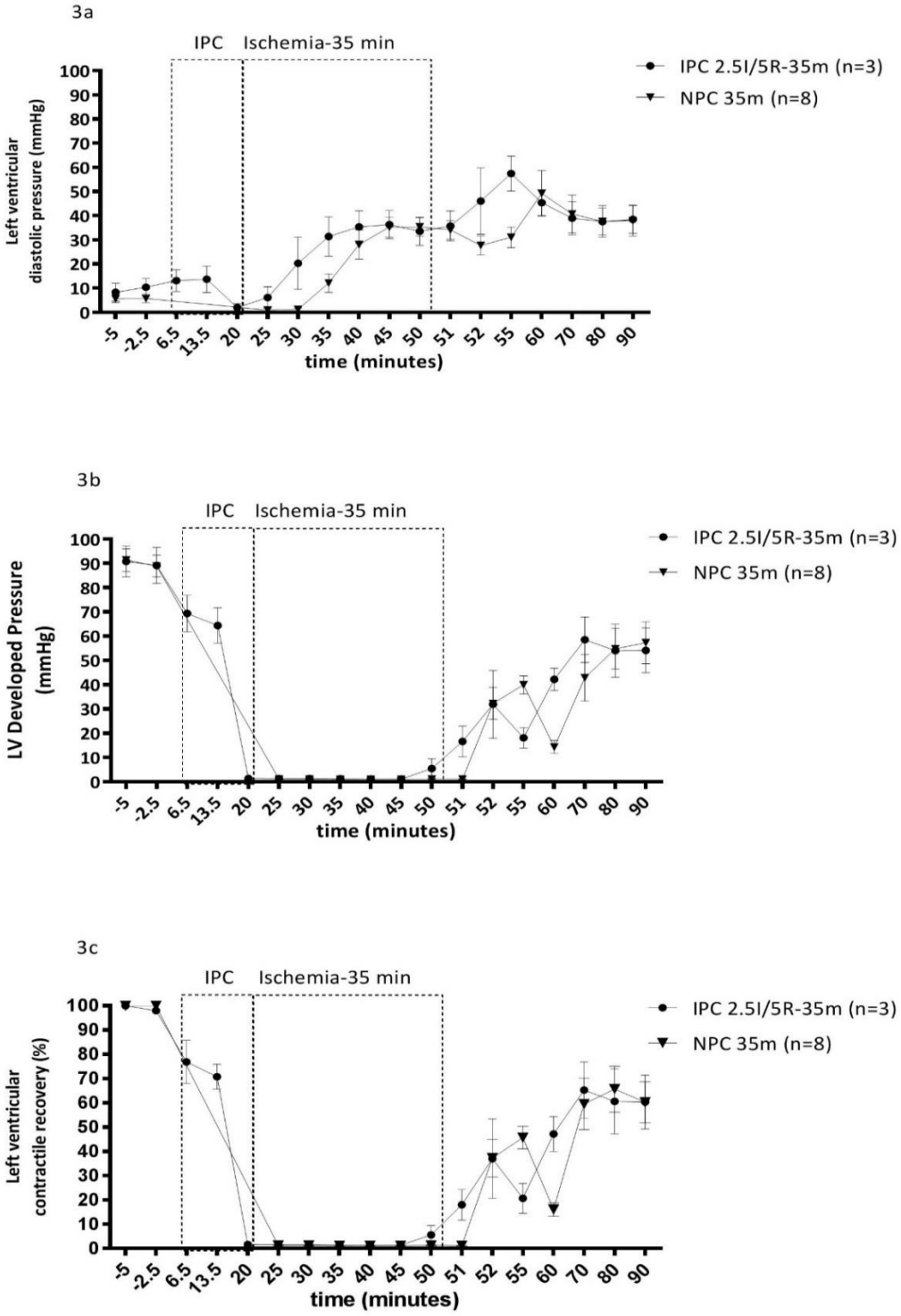

Figure 3: Isolated mouse hearts examining, ischaemic preconditioning (3 × 2.5 mins of ischaemia and 5 mins of reperfusion). 3a) LVD, 3b) LVDP, and 3c) LV%. NPC: Non-preconditioned hearts, IPC: Ischaemic-preconditioned hearts. Data presented mean ± SEM. Samples sizes are shown in the graphs.

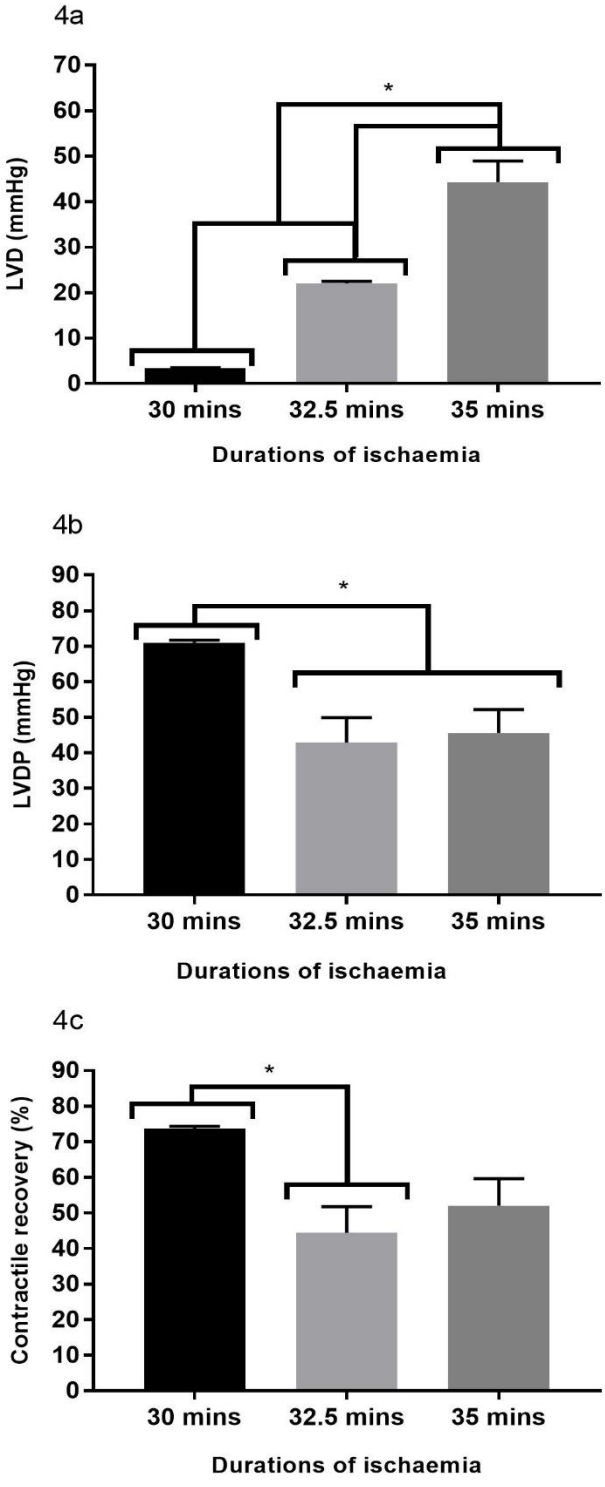

Figure 4: Reperfusion summaries (last 30 minutes of reperfusion) of hearts exposed to different periods of ischaemia. 4a) LVD, 4b) LVDP, and 4c) LV%. Data presented mean ± SEM, * = $P < 0.05$ between groups, n = 6-8 per group.

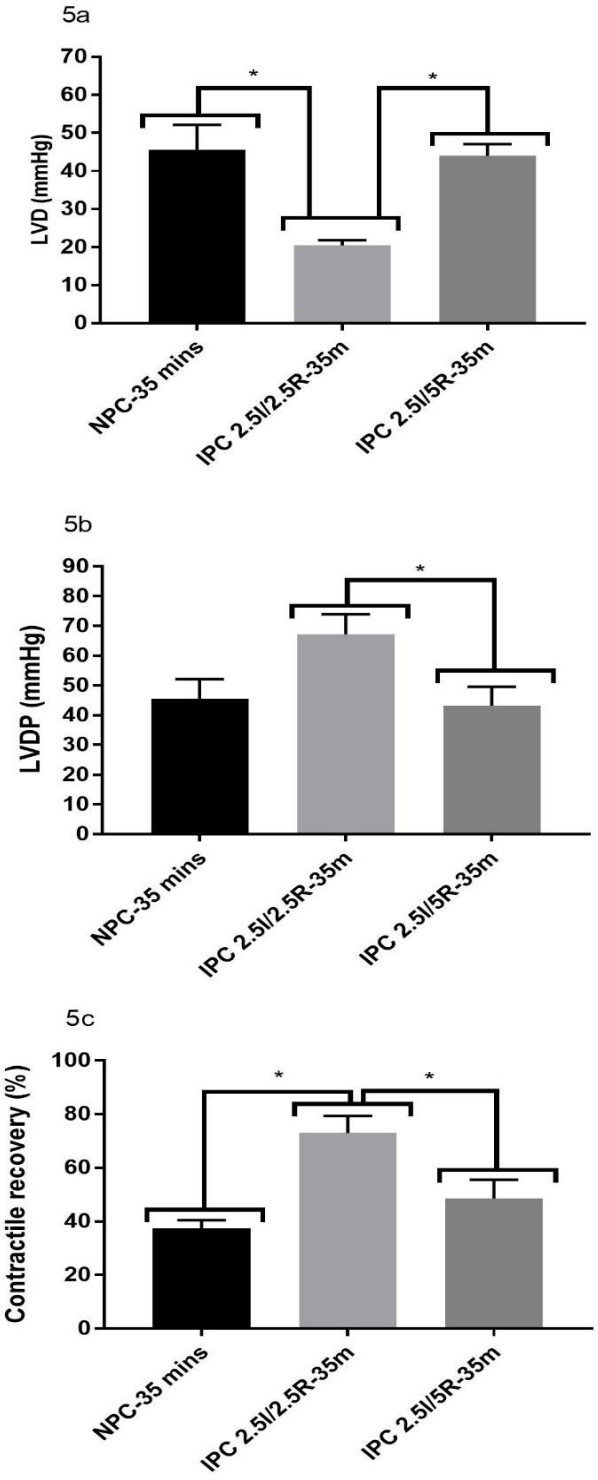

Figure 4: Reperfusion summaries (last 30 minutes of reperfusion) of hearts exposed to different periods of ischaemia. NPC – non-preconditioned hearts, IPC – Ischaemic-preconditioned. 5a) LVD, 5b) LVDP, and 5c) LV%. Data presented mean ± SEM, * = $P < 0.05$ between groups, n = 6-8 per group.

4.5: Conclusion

These findings show that 35 mins of no flow ischaemia is required to see a significant reduction in contractile function. Myocardial ischaemic-preconditioning was stimulated through 3 bouts of 2.5 mins of ischemia and 2.5 mins of reperfusion. From these findings study 2 was tested under these parameters.

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XII: Statements of authorships (chapter 5)

Title of Paper	(+)-Naltrexone reduces contractile recovery in ischaemic-preconditioned mouse hearts exposed to ischaemic-reperfusion injury.
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Study 2 tested whether (+)-naloxone or (+)-naltrexone could influence contractile recovery in ischaemic-preconditioned isolated mouse hearts. To resolve issues addressed in chapter 4, the Constant Pressure Langendorff Heart model. Protein expression of cytosolic cFABP, IL-1β, and HMGB1 in the left ventricle was quantified.

Principal Author

Name of Principal Author	Mr Samuel Man lee		
Contribution to the Paper	Writing, proof-reading, editing, performed experiments and data analyses.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	11-01-2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Professor David Saint		
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Name of Co-Author	Professor Wally-Thomas
Contribution to the Paper	Proof-reading, provided funding to this study, and allowed me to me use his lab during my stay at the University of Queensland.
Signature	Date 8/1/18

Chapter 5: (+)-Naltrexone reduces contractile recovery in ischaemic-preconditioned mouse hearts exposed to ischaemic-reperfusion injury.

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5.1: Abstract

Introduction: Ischaemic-preconditioning remains an effective method of protection against ischaemic-reperfusion injury. Although this phenomenon has been heavily investigated, its direct influence on immunosurveillance receptors remains poorly defined. This study investigated whether anti-inflammatory agents**,** (+)-naloxone and (+)-naltrexone, could directly influence the contractile recovery of isolated mouse hearts exposed to ischaemic-preconditioning.

Methods: Balb/c (25g) mice were studied using the isolated heart technique. All hearts were exposed to 35 mins of ischaemia (I-35) and 50 mins of reperfusion. Ischaemic-preconditioned was (IPC) induced by 3×2.5 mins of ischaemia and 2.5 mins of reperfusion before I-35. (+)-Naloxone or (+)-naltrexone was directly infused into hearts, at 20 μM, 15 mins prior to I-35 in non-preconditioned (NPC) hearts. For IPC, either compound was infused for 10 mins before IPC and during the 1st and 2nd acute bouts of reperfusion during preconditioning itself. To measure contractile recovery, left ventricular measurements taken after I-35 were compared to pre-ischaemic baseline recordings to obtain percentage recovery (LV%). The cytosolic fractions of the heart homogenates were quantified for cFABP, IL-1β, and HMGB1 levels using western blots. Multiway ANOVAs with post-hoc TukeyHSD analyses were constructed, data presented as mean±SEM.

Results: IPC significantly improved contractile recovery after 35 mins of no flow ischaemia (IPC:86.7 ± 4.35%, vs NPC:58.4 ± 3.97%). Although (+)-naltrexone limited contractile recovery

in IPC hearts (69.8 ± 3.6%) ($P<0.05$) no statistical difference was observed in IPCs treated with (+)-naloxone (72.4 ± 3.6%) ($P>0.05$). Neither compound influenced LV% in NPC hearts ($P > 0.05$). Western blot analyses revealed that ischaemic-preconditioning reduced cytosolic HMGB1 levels (NPC: 1.2 ± 0.2 vs IPC: 0.7 ± 0.1) and minimized the loss of cytosolic cFABP (NPC: 0.7 ±0 .1 vs IPC: 1.2 ± 0.04) ($P<0.05$). (+)-Naloxone reduced HMGB1 content in NPC hearts (0.51 ± 0.10) and caused an increased loss in cytosolic cFABP content within IPC hearts (0.9 ± 0.1) ($P < 0.05$). While (+)-naltrexone also protected against HMGB1 cytosolic translocation in NPC hearts (0.6 ± 0.1) ($P < 0.05$), cytosolic cFABP levels was not influenced by the compound when infused into IPCs (1.1 ± 0.12) ($P>0.05$). However (+)-naloxone + ischaemic-preconditioned hearts had lower levels of cFABP compared to ischaemic-preconditioned vehicle hearts ($P < 0.05$). Ischaemic-preconditioning and neither treatments did not influence IL-1β levels ($P>0.05$).

Conclusions: (+)-Naltrexone limits acute contractile recovery in ischaemic-preconditioned hearts. (+)-Naltrexone but not (+)-naloxone reduced the nuclear to cytosolic translocation of HMGB1 in non-preconditioned hearts. Finally, (+)-naloxone blocked ischaemic-preconditioning's ability to preserve cytosolic cFABP within the myocardium.

5.2: Introduction

Ischaemic heart disease remains a significant cause of mortality throughout the developed world. Chronic periods of ischaemia within coronary arteries disrupts physiological homeostasis as the myocardium becomes deprived of oxygen and nutrients. Myocardial damage is exacerbated even further when the ischaemic myocardium is reperfused. Reactive oxygen species (ROS) formation, release of intracellular calcium into the cytosol, and opening of mitochondrial permeability transitional pores (mPTPs), occurs during early reperfusion [1–3]. These changes lead to a loss in contractility, an increase risk of arrhythmogenesis, and in some cases death. The tissue damage and cell death which occurs during reperfusion can be exacerbated through myocardial inflammation. Endogenous proteins released into the extracellular environment, also known as DAMPS (danger associated molecular pattern molecules), binds onto immunosurveillance receptors within the myocardium triggering inflammation [4–7]. The initial immune response (often defined as innate immunity) which is triggered, is rapid and broad, resulting in the release of pro-inflammatory cytokines [8–11] and recruitment of white blood cells (i.e. macrophages, neutrophils and monocytes) throughout the ischaemic region [7,12,13]. One of the most well-known immunosurveillance receptors involved in myocardial ischaemic-reperfusion injury is Toll-like receptor 4 (TLR4) [5,11,14–16]. Unlike its other family members, TLR4 possesses two signalling networks, the MyD88 dependent and TRIF dependent pathway [17]. During reperfusion, DAMPs bind onto TLR4 triggering the MyD88 signalling pathway. This results in the de-novo synthesis of pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin 6 (IL-6), and interleukin 1 beta (IL-1β) [14,17,18]. Substantial evidence from TLR4 genetic and pharmacological knockout studies suggest that the removal of TLR4 improves contractile

recovery, reduces infarct size, and suppresses inflammation in animals exposed to ischaemic-reperfusion injury [5,14–16,19–22].

5.21: Ischaemic-preconditioning and TLR4 antagonists, (+)-naloxone and (+)-naltrexone.

Since its original discovery [23], the molecular mechanisms behind ischaemic-preconditioning have remained under investigation to this day. In brief, exposure to acute bouts of ischaemia and reperfusion, preceding chronic ischaemic insult, limits infarct size and improves contractile recovery. Protein S-Nitrosylation [24], activation of opioid receptors [25,26], and opening of mitochondrial potassium ATP (mitoK_{ATP}) channels [27], are all examples of molecular mechanisms associated with myocardial ischaemic-preconditioning. Although it is now known that ischaemic-preconditioning suppresses inflammation [28,29], its effects on TLR4 activity remains poorly defined. In the last 20 years evidence suggests that transient stimulation of TLR4 with TLR4 dependent ligands can trigger a preconditioning response. While administering LPS [30–32] and HMGB1 [33,34] prior ischaemic insult has been reported to trigger a preconditioning response, it is still unknown whether ischaemic-preconditioning itself triggers TLR4 desensitization. If ischaemic-preconditioning releases low levels of DAMPs during the preconditioning phase it may be possible that the transient stimulation of TLR4 may trigger its desensitization. This study investigated whether (+)-naloxone and (+)-naltrexone, two opioid receptor inactive compounds which possess TLR4 inhibitory actions [35,36], could influence ischaemic-preconditioning. Unlike the (-)-isomers, the (+)-isomeric derivatives lack the ability to block mu opioid receptors. Given that opioid receptors trigger cardioprotective pathways during ischaemic-preconditioning [25]-[26,37], the (+)-isomers were selected for this study to minimise conflicting variables.

We hypothesize that (+)-naloxone and (+)-naltrexone directly influences acute contractile recovery in ischaemic preconditioned hearts. To avoid conflicting systematic variables such as blood or humoral / neuronal factors, the isolated heart technique was used. Because this study examined acute reperfusion recovery, the cytosolic levels of High mobility group box 1 (HMGB1), and cardiac-fatty acid binding protein (cFABP) were investigated. Located in the cytosol, the release of cFABP into the extracellular environment can be used as a predictive biomarker of acute myocardial injury [38]. HMGB1, is a nuclear transcriptional regulator which becomes a DAMP when released from the cells [6,7]. However, prior to its release, HMGB1 translocates into the cytosol and undergoes hyperacetylation [39]. This modification allows HMGB1 to be recognized by immunosurveillance receptors such as TLR4 [4,40,41]. Finally, IL-1β activity was also measured to determine whether the pro-inflammatory cytokine was activated during early reperfusion.

5.3: Methods:

5.31: Animal model

12-week-old male Balb/c mice (20 - 27 grams) were selected for this study. Ethics was approved by The University of Queensland's Animal Ethics committee, (SBMS/253/12/NHMRC).

5.32: Isolated heart technique: Constant Pressure Mouse Langendorff model

The Langendorff setup used follows the similar design as described by Reichelt *et al* [42] Constant pressure was fixed at 80 mmHg while flow rate was monitored using an in-line flow probe 2N In-line. Mice were anaesthetized with ketamine (50 mg/ml) and xylazil (20 mg/ml) via intraperitoneal injection. Anaesthesia was confirmed by paw and reflex test. Hearts were excised, submerged in ice cold Krebs Henseleit buffer cannulated onto the Langendorff system, and perfused with a modified Krebs buffer recipe in under 3 minutes. Buffer recipe: NaCl-119 mM, Glucose-11 mM, $NaHCO_3$-22 mM, KCl-4.7 mM, $MgCl_2 \bullet 6H_2O$-1.2 mM, KH_2PO_4-1.2 mM, EDTA-0.5 mM, CaCl-1.85 mM, Pyruvate-2 mM, heated to 37^oC, and gassed with carbogen. The left arterial appendage was removed and polyethylene tubing (OD: 0.80 mm; ID: 0.50 mm) was inserted through the apex for thebesian drainage. A 9-mm balloon attached to a pressure transducer (ADinstruments, NSW, Australia) was inserted into the left ventricle before filled with milliQ water. Hearts were then submerged into an organ bath, filled with heated Kreb's buffer, and paced between 1-8 volts at 7 hertz during perfusion. Labchart 7 Pro (ADinstruments, NSW, Australia) in conjunction with Powerlab 4125 (ADinstruments, NSW, Australia), was used to measure left ventricular systolic and diastolic pressure. Left ventricular developed pressure (LVDP) was calculated as the average left ventricular systolic (LVS) pressure minus average left ventricular diastolic pressure (LVD). To determine the percentage of contractile recovery after ischaemic insult (LV%) the following equation was used: (LVDP post-ischaemic measurements / LVDP pre-ischaemic baseline measurements) ×100%.

5.33: Schematic timeline / drug infusion

Duration of ischaemia, ischaemic-preconditioning, and drug infusion timelines are described in the schematic timeline (Fig 1). 20 µM of (+)-naloxone or (+)-naltrexone was directly administered into the heart via an infusion port (connected to the langendorff system) using a syringe pump (Harvard Apparatus, MA, USA). Because of the location of the infusion port, and the fact that a constant pressure model was used, the working stock concentration and flow rate had to be taken into consideration. The drug infusion rate was set as 1% of the flow rate which was monitored through Labchart; the infusion flow rate was changed accordingly when the cardiac flow rate changed. Because of the low infusion rate the working drug solution was x 100 more concentrated then the desired concentration. By considering these two factors the concentrated drug solution would be diluted to 20 µM when infused in the running perfusion solution.

5.34: Tissue homogenization / fractionation

After each experiment, the left ventricle was dissected, cut into 1 mm sections, and flash frozen in liquid nitrogen before stored in -80°C freezers. Frozen samples were then weighed and placed into test tubes with 0.1 ml/g of lysis buffer [Tris-HCl pH 7.0 (100 mM), EGTA (5 mM), protease inhibitor cocktail and PhoSTOP Easypack (Roche Diagnostics, Mannheim, Germany)]. An IKA T10 basic ULTRA-TURRAX homogenizer system (John Morris Scientific, NSW, Australia) was used to homogenize the tissue for 15s. 400 µl of tissue homogenate was added to 400 µl of 2 x sample buffer [Tris-HCl pH-6.8 (100mM), SDS (4%), Bromophenol Blue (0.2%), Glycerol (20%) and 2-beta-mercaptoethanol (5%)]. 250 µl of total homogenate was

then centrifuged at 21,500 × g for 10 mins. Cytosolic fraction: 200 µl of supernatant was extracted and added into 200 µl 2 x sample buffer.

5.35 : Total protein quantification (BCA assay)

A Pierce BCA Protein Assay Kit (Thermo-Fisher Scientific, SA, Australia) was used according to the manufacturer's instructions.

5.36: Western blot

25 µg protein was loaded into NuPAGE® Bis-Tris gels in conjunction with Life Technologies mini gel electrophoresis tanks (Thermofisher, Victoria, Australia). Electrophoresis was initially run at 180 V for 15 mins and then 200 V at 30 mins. Gels were transferred onto nitrocellulose membranes using a Life Technologies mini blot module (Thermofisher, Victoria, Australia). Membranes were blocked with 1:4 Odyssey blocking buffer in tris-base saline (TBS) for 1 hr at room temperature (RT) followed by primary antibody incubation overnight at 4 ^{o}C. Antibodies used: 1:1000 GAPDH rabbit (ab9485), 1:20000 GAPDH goat (ab9483), 1:1000 rabbit cardiac-FABP (ab45966), 1:5000 rabbit HMGB1 (ab18256), and rabbit 1:1000 IL-1β (ab9722), all from Abcam (Abcam, Victoria, Australia). This was followed by another 3x5 mins washes with TBS-Tween 0.05% followed by a 1 hr incubation with either 1:10000 Donkeynrabbit-700 or 1:20000 DonkeynGoat-800 in the dark. After another 3x5 min TBS-Tween washes membranes were rinsed with TBS for 5 mins. A Licor Odyssey scanner (Li-Cor, Lincoln, USA) was used to scan membranes. ImageJ (NIHC, Maryland, USA) was used to analyse protein bands. Relative expression of the protein of interest was determined after the protein was normalized by the housekeeper, GAPDH.

5.37: Statistical analysis

All data was formatted in Microsoft Excel (Microsoft, Washington, USA) prior to data analyses. Because of the large level of variability observed during the early stages of reperfusion these measurements were grouped between early reperfusion (timepoints: 11-14) and late reperfusion (15-20) and analysed separately. Multiway ANOVAs with post-hoc TukeyHSDs were constructed using Graphpad Prism (GraphPad Software Inc, California, USA). All data presented in this study is shown as mean ± SEM. Variables for the multiway ANOVAs were defined as the following, preconditioning: non-preconditioning vs ischaemic-preconditioning, and treatment: vehicle, (+)-naloxone and (+)-naltrexone.

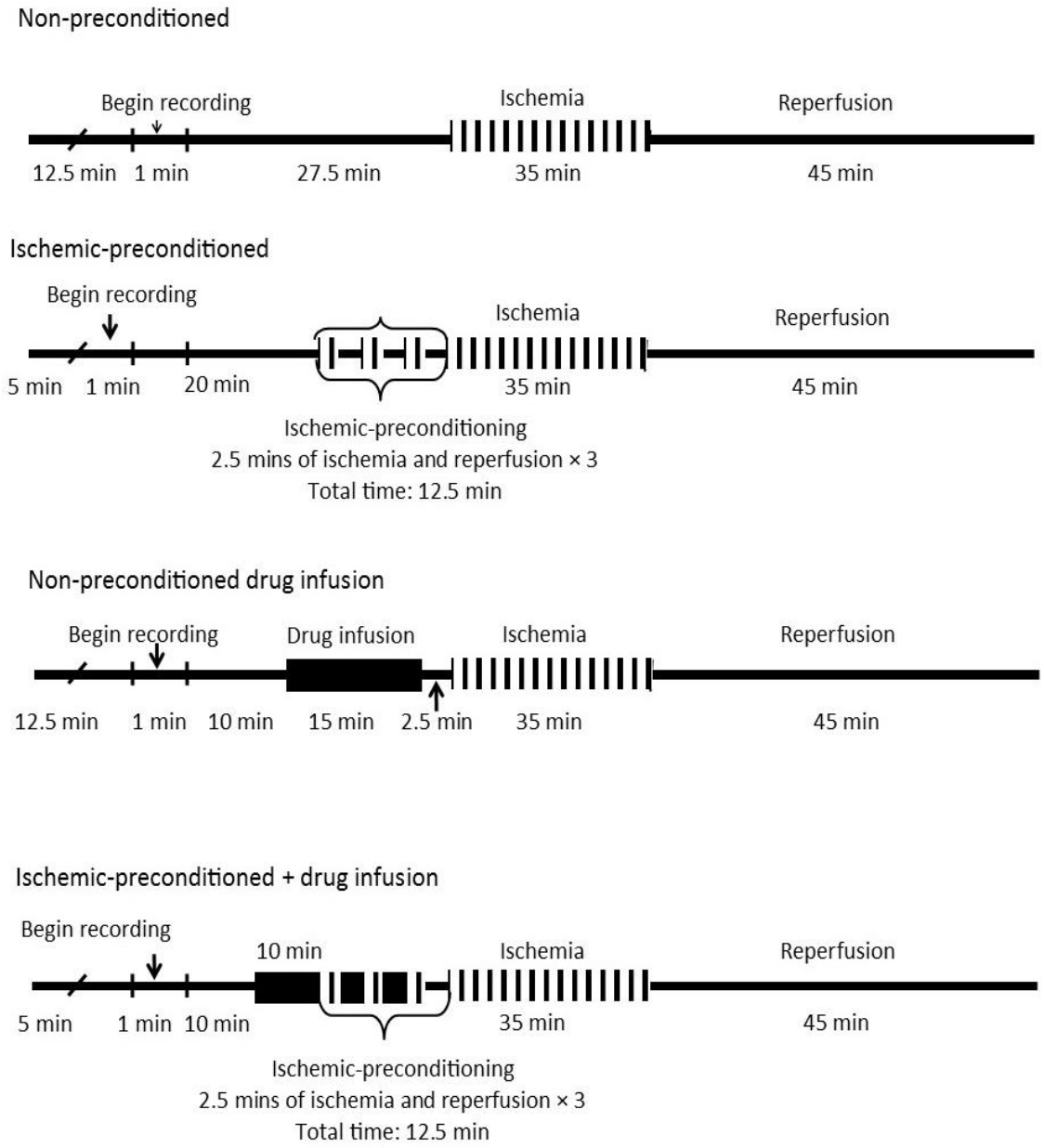


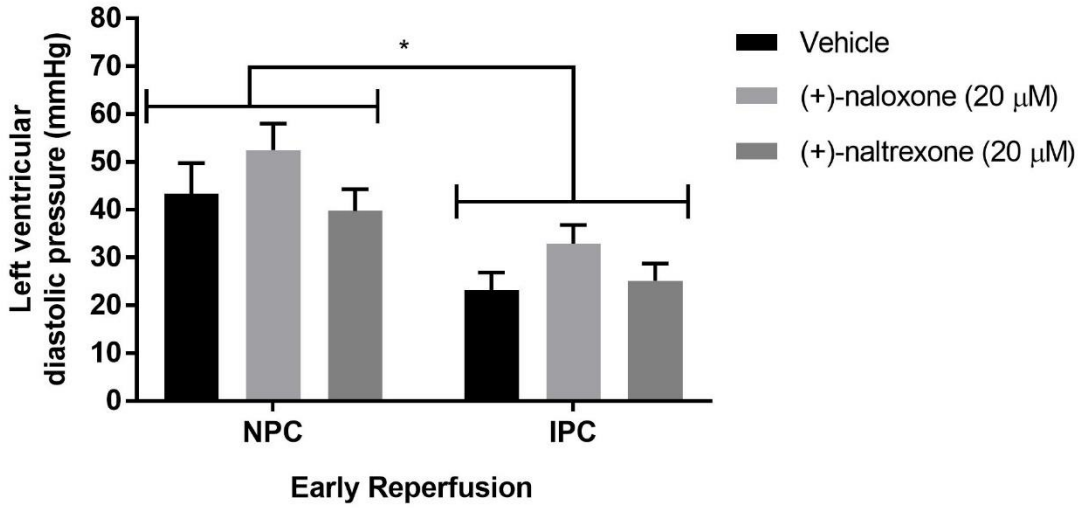
Figure 1: Schematic timeline representing groups studied using the langendorff heart technique. Symbols: — = perfusion, ■ = drug infusion (20 µM), ⁄ = ischemia. Prior recordings, hearts were perfused for either 12.5 or 5 minutes to stabilize LVDP. At the end of each experiment the left ventricle was rapidly dissected and snap frozen in liquid nitrogen.

5.4: Results

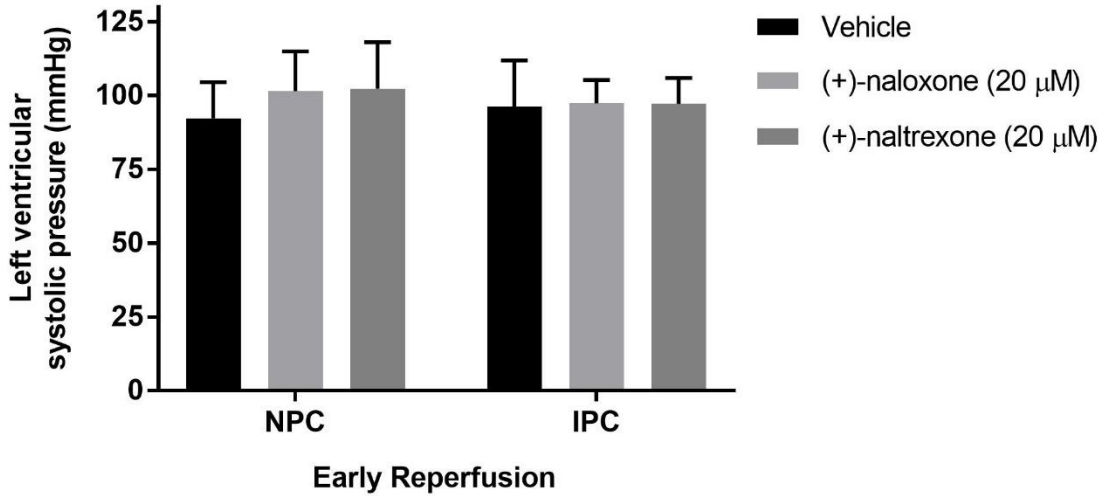
5.41: (+)-Naltrexone depresses left ventricular contractile recovery in ischaemic-preconditioned hearts.

Two-way ANOVAs with post-hoc TukeyHSDs were constructed to determine whether (+)-naloxone or (+)-naltrexone influenced contractile recovery in ischaemic-preconditioned hearts. Ischaemic-preconditioning was shown to preserve LVD and LV% function both during the early and late phases of reperfusion after ischaemic insult (conditioning: $P < 0.05$) (figure 2a, 2c, 3a, 3c). By the end of reperfusion, LVD pressure increased to 37 mmHg in non-preconditioned (37.7 ± 5.3 mmHg) hearts whereas those given ischaemic-preconditioning increased to only 21 mmHg (21.2 ± 3.8 mmHg) (figure 3a) ($P < 0.05$). Ischaemic-preconditioning also preserved contractile function with hearts recovering approximately 85% of their original LVDP after exposure to ischaemic insult (Late reperfusion LV%: non-preconditioned: 55.4 ± 4.0 % vs ischaemic-preconditioning: 86.7 ± 4.4%) (figure 3c). LVS was not influenced by either ischaemic-preconditioning or any treatments throughout reperfusion ($P > 0.05$) (figure 2b and 3b). A strong interactive effect was observed between treatment and conditioning when late, but not early (figure 2), reperfusion LV% was examined (interaction: $P < 0.05$) (figure 3c). Post-hoc TukeyHSD revealed that (+)-naltrexone depressed LV% recovery in ischaemic preconditioned hearts with contractile function dropping to 70% (Late reperfusion: ischaemic-preconditioning-(+)-naltrexone: 69.8 ± 3.6%). Although (+)-naloxone also reduced contractile function (ischaemic-preconditioning-(+)-naloxone: 72.4 ± 3.6%) statistical significance was not observed ($P = 0.06$). Analysis of LVD function suggests that neither compound influenced LVD recovery after ischaemic insult ($P > 0.05$).

2a


Left ventricular diastolic pressure (mmHg)
80
70
60
50
40
30
20
10
0
*
NPC
IPC
Early Reperfusion
Vehicle
(+)-naloxone (20 μM)
(+)-naltrexone (20 μM)

2b


Left ventricular systolic pressure (mmHg)
125
100
75
50
25
0
NPC
IPC
Early Reperfusion
Vehicle
(+)-naloxone (20 μM)
(+)-naltrexone (20 μM)

2c

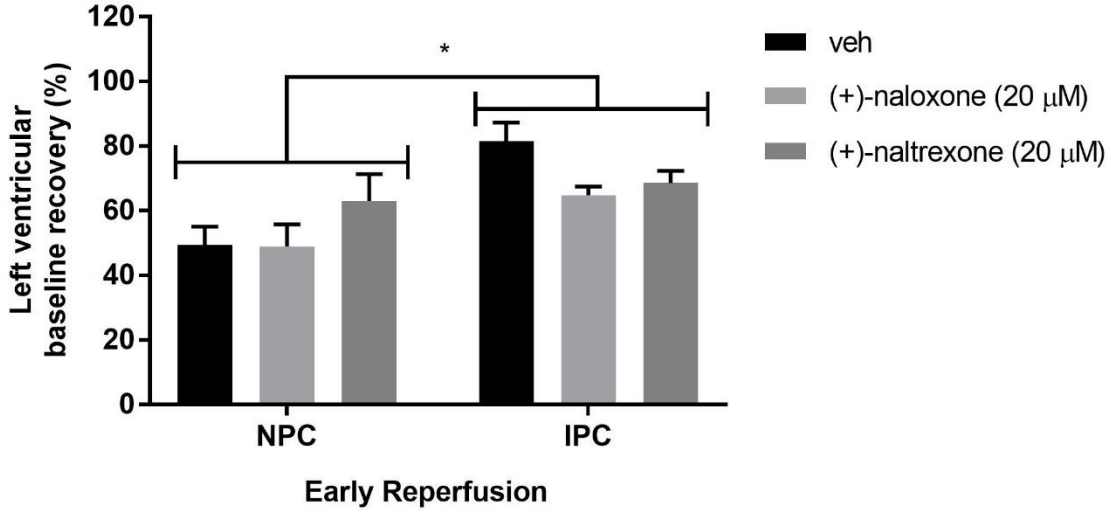
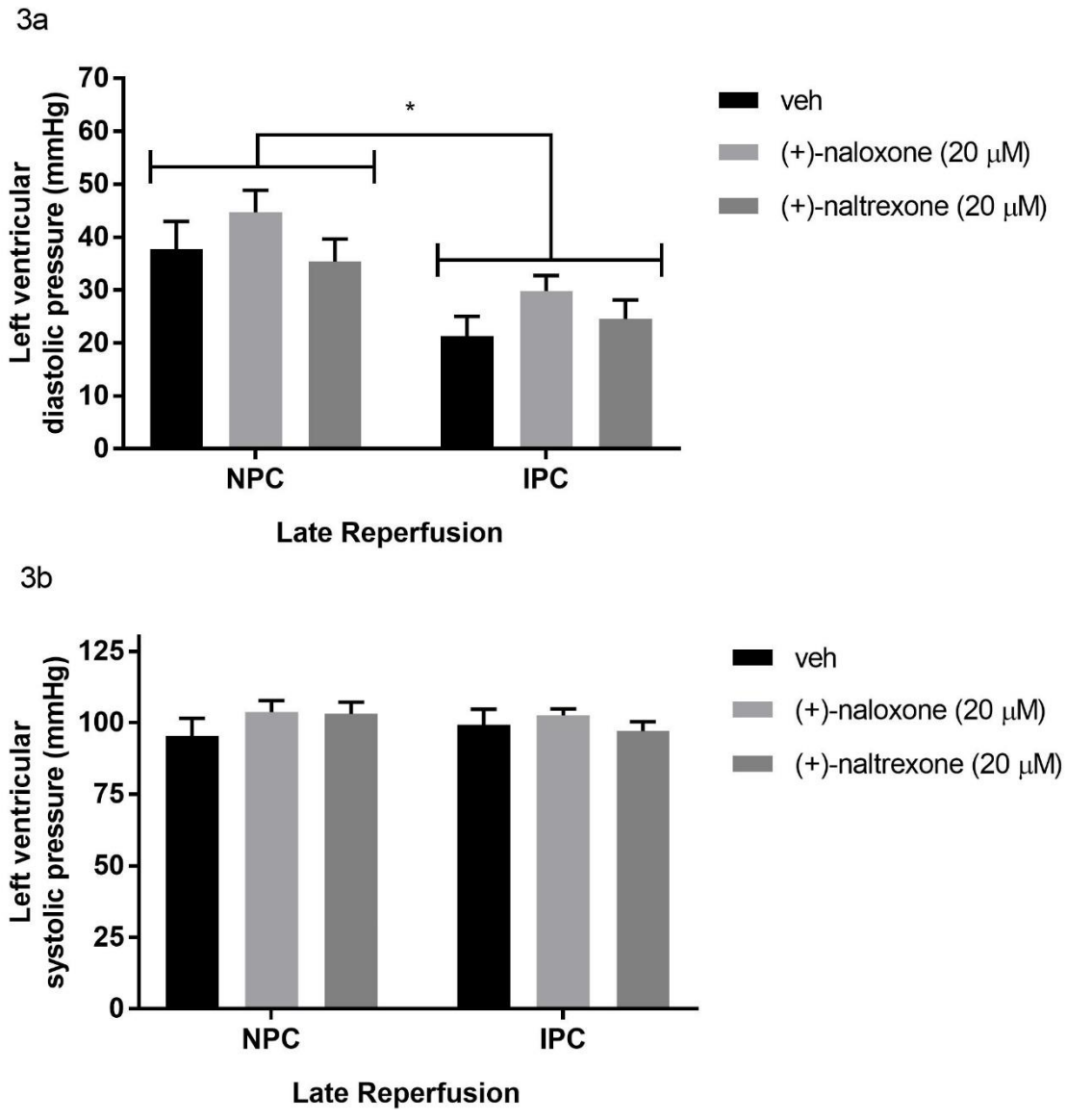

Left ventricular baseline recovery (%)
120
100
80
60
40
20
0
*
NPC
IPC
Early Reperfusion
veh
(+)-naloxone (20 μM)
(+)-naltrexone (20 μM)

Figure 2: Ischaemic-preconditioned isolated mouse hearts exposed with, or without, (+)-naloxone or (+)-naltrexone (20 μM) during the preconditioning phase. Early phase of reperfusion (11-14) studied; NPC-non-preconditioned, IPC-ischaemic-preconditioned. Early reperfusion measurements were taken during the first 15 minutes of reperfusion. 2a) Left ventricular diastolic pressure, 2b) Left ventricular systolic pressure and 2c) Left ventricular baseline contractile recovery after ischaemic insult. Data presented as mean ± SEM, * represents statistical significance ($P < 0.05$) between non-preconditioned and ischaemic-preconditioned hearts, n=7-8.



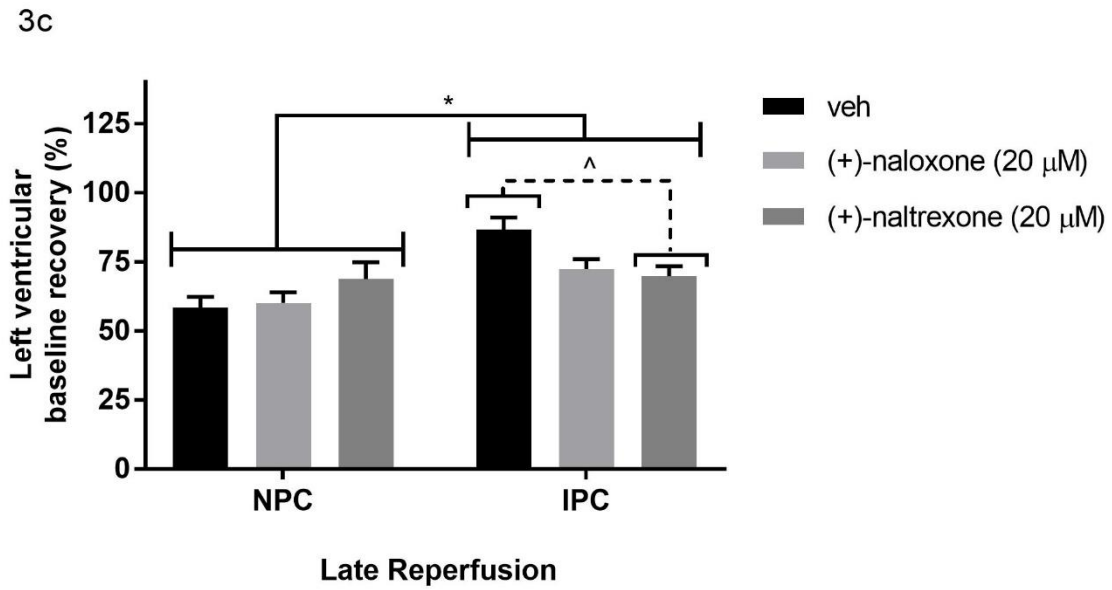


Figure 3: Ischaemic-preconditioned isolated mouse hearts measurements exposed, with or without, (+)-naloxone or (+)-naltrexone (20 μM) during the later stages of reperfusion (15-20 timepoints). NPC-non-preconditioned, IPC-ischaemic-preconditioned. Late reperfusion timepoints taken during the remaining 35 minutes of reperfusion. 3a) Left ventricular diastolic pressure, 3b) Left ventricular systolic pressure and 3c) Left ventricular baseline contractile recovery after ischaemic insult. Data presented as mean ± SEM, * represents statistical significance ($P < 0.05$) between non-preconditioned and ischaemic-preconditioned hearts. ^ represents the statistical significance observed between ischaemic-preconditioned and ischaemic-preconditioned + (+)-naltrexone treated hearts, n = 7-8.

5.42: (+)-Naloxone and (+)-naltrexone reduces HMGB1 cytosolic content in non-preconditioned hearts.

HMGB1 is a nuclear transcriptional regulator which becomes a DAMP when released into the extracellular environment. Because the chaperone requires post-translational modification

prior to its release HMGB1 translocates into the cytosol. Two-way ANOVA revealed that independently, ischaemic-preconditioning and treatments were statistically significant ($P < 0.05$) (figure 4a). Compared to non-preconditioned hearts (1.2 ± 0.2), ischaemic-preconditioning had lower levels of cytosolic HMGB1 detected in the left ventricle (0.7 ± 0.1) ($P < 0.05$). Pre-administration of (+)-naloxone (0.5 ± 0.1) or (+)-naltrexone (0.6 ± 0.1) prior to ischaemia also reduced HMGB1 levels in non-preconditioned hearts ($P < 0.05$). Neither compound influenced HMGB1 levels in ischaemic-preconditioned hearts ($P > 0.05$).

5.43: (+)-Naloxone limited Ischaemic-preconditioning's ability to limit the release of cytosolic cFABP levels

CFABP is a small cytoplasmic protein which is used as a cardiac biomarker of injury during events such as ischaemia. Two-way ANOVA revealed that while ischaemic-preconditioning was able to limit the loss of cytosolic cFABP content (+)-naloxone blocked this effect (Interaction: $P < 0.05$) (figure 4b). Post-hoc analyses revealed that ischaemic-preconditioning preserved cytosolic cFABP content (1.3 ± 0.04) which was significantly reduced in non-preconditioned hearts (0.7 ± 0.1). While administration of (+)-naloxone during ischaemic-preconditioning reduced cytosolic cFABP content (0.8 ± 0.1) ($P < 0.05$), no differences were observed when (+)-naltrexone was examined [ischaemic-preconditioning + (+)-naltrexone: 1.1 ± 0.1] ($P > 0.05$). Finally, neither compound influenced cFABP levels in non-preconditioned hearts [(+)-naloxone + non-preconditioned: 0.9 ± 0.1 and (+)-naltrexone + non-preconditioned: 0.7 ± 0.1] ($P > 0.05$).

5.44: Ischaemic-preconditioning or (+)-naloxone / (+)-naltrexone does not influence IL-1β levels.

Two-way ANOVAs with post-hoc TukeyHSD revealed that neither ischaemic-preconditioning nor treatments influenced IL-1β levels ($P > 0.05$) (figure 4c)

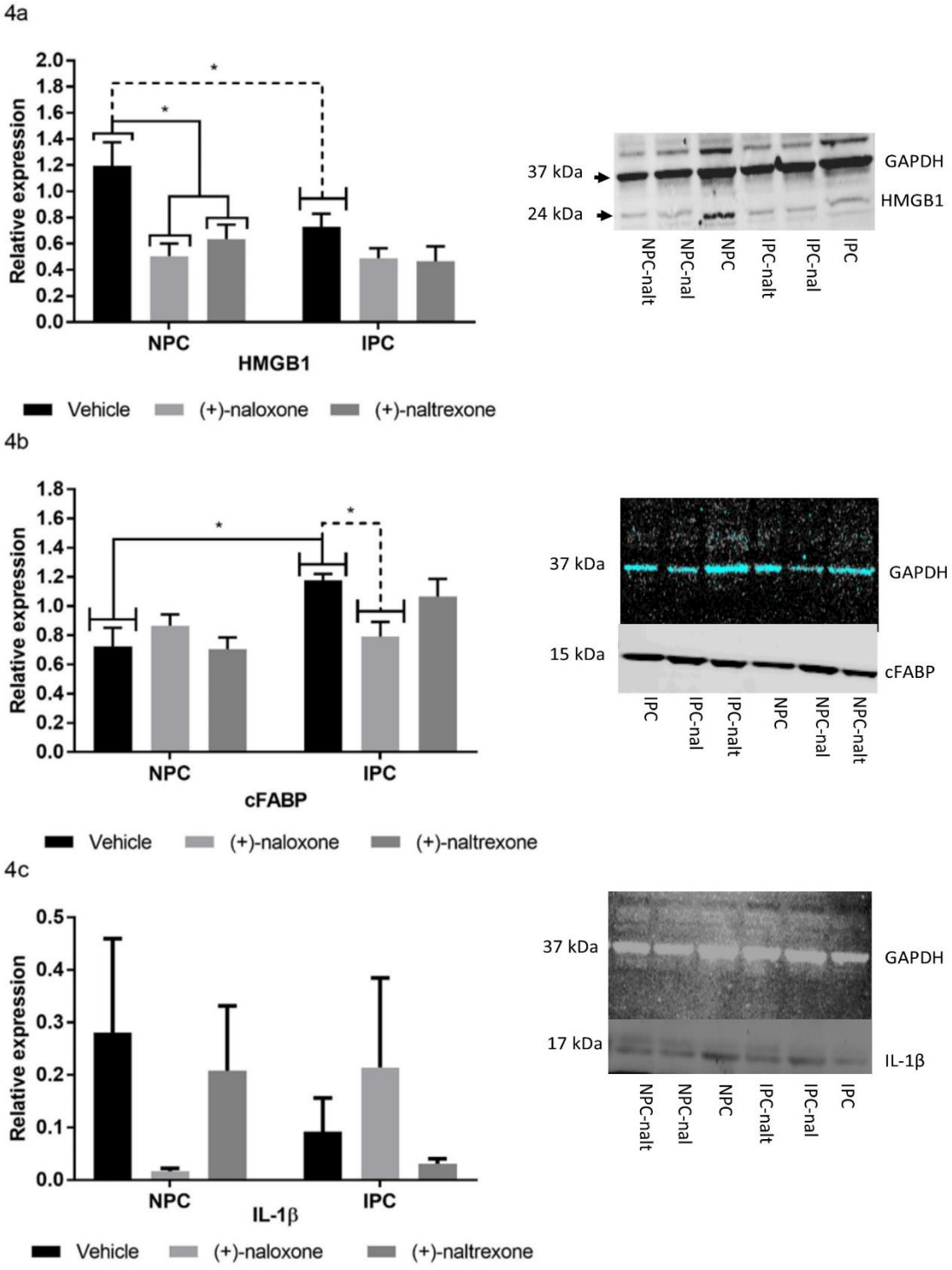


Figure 4: Western blot analyses of left ventricular tissue isolated from langendorff mouse heart experiments. Relative expression of 4a) HMGB1, 4b) cFABP and 4c) IL-1β normalized to GAPDH. * represents the statistical difference observed between noted groups ($P < 0.05$). Data presented as mean ± SEM, n = 6 - 7.

5.5: Discussion

Inflammation is now known to play an important role in promoting tissue injury during the later stages of reperfusion [8]. Innate immunity is triggered when immunosurveillance receptors, such as the toll-like receptor family or receptor for advanced glycation end products (RAGE), recognise DAMPs which are released during ischaemic-reperfusion injury. As a consequence, pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 are released into the ischaemic myocardium [5,14,19,20,22,43]. Although ischaemic-preconditioning can suppress innate immunity [10],[29], its effects on immunosurveillance receptor activity remains poorly defined. This study examined whether direct administration of TLR4 antagonists during ischaemic-preconditioning would alter acute contractile recovery in isolated mouse hearts. Involved in the recognition of LPS from gram negative bacteria [18,30,31,44], or DAMPs [4,6,41,43,45] released from necrotic cells, TLR4 signalling triggers the innate immune response which exacerbates myocardial injury. However, evidence also suggests that transient TLR4 stimulation can promote a delayed preconditioning response. Administration of nonlethal dosages of LPS or TLR4 ligands prior to ischaemic insult has been reported to trigger a preconditioning response [30,31,40,44,46]. These findings suggest that TLR4 has a bivalent signalling response which is dependent on the timing and degree of receptor activation. To test whether TLR4 blockade influences the protective properties of

ischaemic-preconditioning (+)-naloxone and (+)-naltrexone were tested. Aside from their ability to block TLR4, (-)-naloxone and (-)-naltrexone also inhibit mu-opioid receptors [35,36,47]. Evidence in the 1990s reported that (-)-naloxone blocked the protective properties of ischaemic-preconditioning [26,36]. While these finding were hypothesized to be mediated through the suppression of opioid receptor signalling, the authors were unaware naloxone / naltrexone's TLR4 antagonistic properties. Hence the findings from these studies may be partially attributed to the suppression of TLR4 signalling during ischaemic-preconditioning. To minimise conflicting variables, (+)-naloxone and (+)-naltrexone were selected for this study to avoid mu opioid receptor inhibition.

5.51: (+)-Naltrexone depresses left ventricular contractile recovery in ischaemic-preconditioned hearts.

This study showed that (+)-naltrexone, but not (+)-naloxone, reduced contractile recovery in ischaemic-preconditioned hearts. To our knowledge, no study has examined either positive stereoisomeric compounds within the cardiac disease setting. Although (+)-naloxone also reduced LV% recovery in ischaemic-preconditioned hearts, no statistical significance was observed. While both share similar structures, the tertiary amine methyl component of (+)-naloxone is replaced with methylcyclopropane in (+)-naltrexone. This difference means that the minus isomer of naltrexone has an increased half-life and potency compared to naloxone in terms of mu-opioid receptor inhibition [47,48]. The impact on (+)-isomer function remains to be determined. Recently, Wang's *et al* [35] pharmacological work on (+)-naloxone / (+)-naltrexone suggested that both compounds supressed the production of inflammatory mediators associated with the TRIF, but not the Myd88, pathway. As TLR4's secondary

signalling network, the TRIF response is involved in the resolution of innate immunity while activating the adaptive immune response at the same time [17,49]. Considering that the TLR4-TRIF response is delayed before MyD88 signalling [49], and that the depression in contractile recovery was observed within the acute setting, we do not believe that our findings are mediated through the inhibition of the TLR4-TRIF pathway.

Wang's *et al* [35] paper also reported that both compounds suppressed the production of reactive oxygen species (ROS) and nitric oxide (NO) in BV-2 cells. These molecules play important roles in how cells respond to stress and are both involved in ischaemic-preconditioning and ischaemic-reperfusion injury. Produced from the mitochondria, ROS is involved in maintaining metabolic reactions and activating signalling pathways [1]. However, during ischaemia, high levels of ROS can accumulate within the myocardium causing structural damage and activation of cell death pathways [2,50–52]. Because early reperfusion triggers significant ROS production, treating hearts with a ROS scavenger prior to reperfusion can protect against ischaemic-reperfusion injury [1,53]. On the other hand, evidence also suggests that ischaemic-preconditioning triggers low levels of ROS production which promotes the opening of mitoK_{ATP} channels [54],[55]. Since mitoK_{ATP} plays an important role in triggering classical preconditioning its suppression can influence cardioprotection.

Similar to ROS, NO can also protect, or exacerbate, myocardial ischaemic-reperfusion injury. When the redox equilibrium is disrupted during ischaemia and reperfusion, pre-existing NO can react with superoxide to produce peroxynitrite ($ONOO^-$), a highly reactive ROS [56,57]. However, during ischaemic-preconditioning, NO is produced by inducible nitric oxide

synthase (iNOS) and triggers the opening of mitoK_{ATP} channels [53] and promotes protein-S-Nitroslyation [24]. Lebuffe *et al* [53] demonstrated that ROS and NO scavengers could abolish contractile recovery in the classical phase of ischaemic-preconditioning. Furthermore, real time measurements of NO levels revealed that ischaemic-preconditioning promoted NO production. In regards with its relationship with TLR4, Zhu *et al* [15] showed that the genetic knockout of TLR4, MyD88, or iNOS, reduced cell survival in LPS preconditioned cardiomyocytes exposed to serum starvation. Considering these studies, (+)-naltrexone may have partially blocked ischaemic-preconditioning by preventing the regulated production of ROS or NO. However, since the pharmacological evidence to support this is limited [35], additional evidence is required to support the theory of TLR4 induced inhibition of ROS / NO production during myocardial ischaemic-preconditioning.

5.52: (+)-Naltrexone and (+)-naloxone reduced cytosolic HMGB1 levels in non-preconditioned hearts.

Aside from its role as a nuclear transcriptional regulator, HMGB1 acts as a DAMP during ischaemic-reperfusion injury by binding onto TLR4 [7,41] or RAGE [6,40]. However, prior to its release, HMGB1 translocates into the cytosol and undergoes post-translational acetylation via Janus kinase / Signal Transducer and Activator of Transcription (JAK/STAT) signalling [39,58]. This period of cytosolic translocation was used to determine if cytosolic HMGB1 could be used as a predictive biomarker of injury during acute reperfusion. Initial findings showed that ischaemic-preconditioning, regardless of treatments, had lower levels of cytosolic HMGB1 compared to non-preconditioned hearts. To our knowledge, no study has examined the rate of HMGB1 cytosolic translocation from the nucleus into the cytosol within the cardiac

disease setting. Our findings also showed that (+)-naloxone and (+)-naltrexone both limited HMGB1 cytosolic translocation in non-preconditioned hearts. While no improvements in LV% was observed in these hearts, this may be attributed to the short reperfusion protocol. While it is unlikely that TLR4 inhibition influenced the JAK/STAT pathway, the network is known to be influenced by ROS levels. Literature in the early 2000s suggests that hydrogen peroxide (H_2O_2) stimulates JAK/STAT signalling [52,59]. Considering that (+)-naltrexone and (+)-naloxone was reported to suppress superoxide production, it may be possible that the compounds also limits H_2O_2 and thus inhibit JAK/STAT signalling. While the literature supports this theory, it is unknown if (+)-naloxone or (+)-naltrexone can influence H_2O_2 production during ischaemic-reperfusion injury.

5.53: (+)-Naloxone blocked the preservation of cytosolic cFABP content in ischaemic-preconditioned hearts.

Because of the experimental design for this study, the cytosolic levels of cFABP was quantified [38]. Released during acute reperfusion, low levels of cytosolic cFABP suggest significant tissue injury. As expected, cytosolic cFABP content was significantly lower in non-preconditioned hearts compared to ones exposed to ischaemic-preconditioning. These findings correlate with the changes observed in the isolated heart experiments as shown by the depressed LV% in non-preconditioned hearts. However, when hearts were treated with either TLR4 antagonist the data was more conflicting. While (+)-naltrexone triggered increased loss of cFABP in ischaemic-preconditioned hearts, no changes in cFABP content was reported for (+)-naloxone. These findings are interesting considering that (+)-naloxone did not block LV% in ischaemic-preconditioned hearts. CFABP was selected for this study on the

belief that the reduction in cFABP would correlate to changes in contractile function. Hence the lack of change in cFABP levels in (+)-naltrexone treated ischaemic-preconditioned hearts does not match findings observed in the isolated heart data. Furthermore, while (+)-naloxone was not reported to suppress contractile function, it would have been interesting to see whether perfusing hearts for longer periods would have resulted in a more noticeable change. Examining alternative cardiac biomarkers (i.e. myoglobin or creatine kinase) may provide further insight as too whether the molecular changes observed in this study are reflective of the physiological data collected.

5.54: IL-1β levels were not influenced during acute contractile recovery

IL-1β is a pro-inflammatory cytokine triggered during innate immune responses [11,13,22]. Existing in its pre-cursor form, pro-IL-1β, is cleaved into its active state by protein complexes called inflammasomes [8]. Because this study was interested in studying acute contractile recovery, it was unlikely that we would see any changes in the expression pro-inflammatory markers. However, since IL-1β exists in a pre-cursor state, this study was interested in seeing whether the activation of IL-1β could be detected during early reperfusion. Our findings showed that 50 minutes of reperfusion was not enough time to see changes in IL-1β activity within the cytosol. Because of the findings of this study, the next logical step is to use a longer recovery in-vivo infarct model. Considering that inflammation emerges several hours after reperfusion, any changes caused by either compound on innate immunity would be observed during this timeframe.

5.6: Conclusions

Our findings show that (+)-naltrexone was able to limit the protective effects of ischaemic-preconditioning. These findings are of considerable interest since we have shown that (+)-naltrexone directly influenced acute contractile recovery irrelevant of systemic variables (i.e. blood, humoral and neuronal factors). From the literature examined, the blockade of ischaemic-preconditioning may be attributed through the suppression of low dose ROS production and inhibition of iNOS. The changes in cytosolic HMGB1 and cFABP caused by (+)-naloxone and (+)-naltrexone also poses new questions into how these compounds influence acute contractile recovery at the molecular level. The next logical step is to determine whether these compounds can replicate these findings within an in-vivo infarct model.

5.7: Acknowledgements

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5.8: Bibliography

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XIII: Statements of authorships (chapter 6)

Title of Paper	Summary of study 1 & 2 and preliminary experiments for study 3.
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Publication Details	Summary of study 2's finding and why study 3 was conducted. This chapter also contains preliminary experiments conducted for study 3

Principal Author

Name of Principal Author	Mr Samuel Man lee		
Contribution to the Paper	Writing, proof-reading, editing, performed experiments and data analyses.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

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Chapter 6: Summary of study 1 & 2 and preliminary experiments for study 3.

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6.1: Introduction

6.11: Suppression of toll-like receptor 4 (TLR4), by (+)-naltrexone depresses acute contractile recovery in ischaemic-preconditioned hearts.

Study 2 re-examined the hypothesis from study 1: does (+)-naloxone suppress contractile recovery in ischaemic-preconditioned hearts? As mentioned in chapter 4, there were concerns of whether the constant flow isolated heart model [1,2] contributed to the intergroup variability observed in chapter 4. To rectify this, the constant pressure isolated mouse heart model was used for study 2 [3]. (+)-Naltrexone, a more potent and longer acting TLR4 antagonist, was also tested. The findings from study 2 support study 1 results. That is, while left ventricular contractile function is depressed, (+)-naloxone does not influence the cardioprotective properties of ischaemic-preconditioning in the acute setting. On the other hand, when (+)-naltrexone, which has a similar structure to (+)-naloxone, was perfused into ischaemic-preconditioned hearts, acute contractile recovery was impaired. Although (-)-naltrexone has been reported to have an increased half-life / potency compared to (-)-naloxone, these studies have only examined these compounds in the context of mu-opioid receptor inhibition [4,5]. Extensive research conducted by Wang *et al* [6] suggests that in regards to TLR4 inhibition, no differences were reported between the two positive stereoisomers. Additionally, the study [6] also reported that aside from suppressing the TLR4-TRIF dependent signalling, (+)-naloxone and (+)-naltrexone also limited the production of nitric oxide (NO) and reactive oxygen species (ROS) . When upregulated in a controlled manner, these molecules trigger classical preconditioning by opening mitochondrial potassium ATP channels [7–9]. While it is unknown as to whether TLR4 directly influences ROS production, TLR4 has been suggested to regulate inducible nitric oxide synthase (iNOS) activity [10]. INOS has been reported to trigger delayed preconditioning by promoting protein

S-nitrosylation [11–13]. While these findings suggest that (+)-naltrexone can block ischaemic-preconditioning in a TLR4 dependent non-inflammatory manner additional research is required. On the other hand, while Wang *et al* [6] findings are interesting, the study only examined the TLR4 antagonists in the in-vitro setting and thus requires additional validation using animal models. Considering the findings from study 1 and 2, a more prominent response is likely to be observed in an in-vivo infarct model since the heart would be exposed to both innate and adaptive immunity. Finally, since study 2 only perfused the hearts for 50 minutes it is unlikely that changes in contractile function by (+)-naltrexone is mediated through TRIF-dependent signalling. Studying TLR4-MyD88 signalling such as the MAPK family during early reperfusion may determine whether the positive stereroisomers suppresses TLR4.

Study 2 also showed that (+)-naloxone and (+)-naltrexone could influence the release of cardiac fatty acid binding protein (cFABP) and the translocation of high mobility group box one (HMGB1) into the cytosol. These proteins were used to predict myocardial tissue injury during acute reperfusion [14–16]. Compared to the physiological data, the protein analytical results were conflicting. For example, the reduction in cytosolic cFABP levels of (+)-naloxone, but not (+)-naltrexone, in ischaemic-preconditioned hearts do not match the changes in left ventricular contractile recovery (see study 2). Whether this is due to the specific biomarkers selected is uncertain and requires further study. The cytosolic HMGB1 data collected was perhaps the most interesting. Normally residing in the nucleus, its release requires the protein to translocate into the cytosol where it undergoes hyperacetylation [17,18]. Although HMGB1 levels were reduced in (+)-naloxone / (+)-naltrexone ischaemic-preconditioned hearts, no statistical significance was observed. Again, it would be interesting to see whether replicating

this study in an in-vivo infarct model (with a longer recovery period) would show a more prominent difference in HMGB1 cytosolic levels. Additionally, the cytosolic levels of HMGB1 findings were also reported to be significantly reduced in (+)-naloxone and (+)-naltrexone non-preconditioned hearts. Although HMGB1 can be released through macrophages [18,19] or released from necrotic cells [15,20], the progression timeline from translocation to the cytosol to its release remains poorly defined. However, if (+)-naloxone and (+)-naltrexone does limit HMGB1 translocation its release, and thus influence on myocardial inflammation, is likely to occur in the later stages of reperfusion.

6.12: Do (+)-naloxone and (+)-naltrexone suppress myocardial inflammation and protect against ischaemic-reperfusion injury?

The original interest of this thesis was to ask the question, “does ischaemic-preconditioning directly influence TLR4 signalling and if so, is this mediated by DAMP induced desensitization of TLR4?”. While the findings from study 1 and 2 are interesting I was decided that the more important question(s), in terms of clinical relevance, was 1) does (+)-naloxone and (+)-naltrexone suppress myocardial inflammation? 2) Can (+)-naloxone or (+)-naltrexone protect against myocardial infarctions in an in-vivo infarct long-term recovery model? This resulted in the designs of study 3 and 4 which both examined whether each compound suppressed myocardial inflammation and limited cell / tissue death. As mentioned in the beginning of this thesis, it is now clear that myocardial inflammation is a significant risk factor in the development of cardiovascular disease. Although specific anti-inflammatory blockers such as Canakinumab and Infliximab [21] have shown promising results, no study has examined whether blockade of TLR4 is beneficial within the cardiovascular clinical setting. While (+)-

naloxone and (+)-naltrexone did not improve contractile function during acute reperfusion, we considered whether TLR4 blockade is more beneficial in the long term. While it is clear that the suppression of TLR4 signalling protects against ischaemic-reperfusion injury, these studies do not replicate the clinical setting [22–24]. Hence the final study of this project examined whether administrating TLR4 antagonists just before reperfusion would be cardioprotective.

6.2: Study 3 design, do (+)-naloxone and (+)-naltrexone suppress myocardial inflammation in lipopolysaccharide (LPS) stimulated H9C2s cells? And do they protect against simulated ischaemic-reperfusion injury?

Before conducting any animal work, study 3 was designed to explore whether (+)-naloxone and (+)-naltrexone can limit myocardial inflammation and prevent the loss of cell-viability using the H9C2 cell-line [25,26]. Myocardial inflammation was quantified by testing whether; 1) does either compound limit the translocation of the transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) into the nucleus? and 2) Is the gene expression of interleukin-6 (IL-6), interleukin-1 beta (IL-1β), tumour necrosis factor alpha (TNF-α), and TLR4 influenced? Study 3 also investigated whether (+)-naloxone or (+)-naltrexone influences cell viability in H9C2 cells exposed to simulated "ischaemic-reperfusion" injury. A hypoxia incubation chamber was used in conjunction with buffers made to stimulate ischemic and reperfusion environments. When optimized properly this would simulate "ischaemic-reperfusion injury" in the in-vitro setting.

6.21: Limitations of the H9C2 cell line and technical issues.

Although the original aim was to quantify cytokine protein expression, it was not possible to collect sufficient protein yields for western blot analyses. Communication with a lab who works with this cell-line revealed that H9C2s have very low protein yields. Attempts were also made to analyse the supernatant from lipopolysaccharide (LPS) treated H9C2s for TNF-α release, however, the cytokine could not be detected. Finally, it should be noted that towards the end of this study (+)-naltrexone was no longer available for RT-PCR work. Due to the difficulty of obtaining this drug, and because of my thesis timeline, I decided to proceed with the study without looking at (+)-naltrexone's influence on gene expression. These issues are discussed in further detail in the Conclusion chapter.

6.22: Preliminary studies - (+)-Naloxone and (+)-naltrexone limit NF-κB nuclear translocation in H9C2s at 10 and 100 ng of LPS.

To ensure that the appropriate LPS concentration was used, dose dependence experiments were conducted. While numerous studies have examined LPS stimulation in H9C2s, the dosages used are inconsistent [27–29]. Because the quantification of NF-κB nuclear translocation through immunohistochemistry is a relatively quick technique, the protocol was used to test whether 10 or 100 ng of LPS, for 30 minutes, could promote an inflammatory response. Furthermore, (+)-naloxone and (+)-naltrexone were also studied to see whether pre or co-treating cells with either compound, in conjunction with LPS, could limit NF-κB nuclear translocation.

6.3: Methods

6.31: Immunocytochemistry - Investigating dose dependent studies for LPS and the independent effects of (+)-naloxone / (+)-naltrexone on NF-κB nuclear translocation.

Cells were seeded onto poly-d-lysine coated coverslips (which were placed into 24 well plates) at 2×10^4 cells / ml and left to incubate overnight. For LPS dose dependent experiments (figure 1) H9C2s were stimulated with LPS at 10 or 100 ng [(diluted in Dulbecco's Modified Eagle Medium (DMEM)] for 30 minutes at 37^{o}C. (+)-Naloxone and (+)-naltrexone was also tested independently to see whether these TLR4 antagonists influenced NF-κB translocation.

After the final incubation, cells were fixed with 4% formaldehyde with 5% sucrose for 10 mins at room temperature (RT). The slides were washed once with ice cold PBS followed by a 10 mins blocking / permeabilization step with blocking buffer: 3% skim milk + 0.15% triton-X. Each well was then incubated with 1:1000 DonkeynRabbit NF-κB-p65 (diluted in blocking buffer) (Ab7970) (Abcam, Victoria, Australia), for 2 hours at RT. After incubation, cells were washed 3 times with ice cold PBS with 1 min wash periods. Cells were then treated with Alexa Fluor 488 (1:1000) in conjunction with DAPI (1:10,000) (both diluted with blocking buffer) for 1 hour, RT, in the dark. This was followed by another 3 washes with PBS before fixed onto mounting slides. These slides were then viewed using a confocal microscope at wavelengths 488 and 358 λ. For each sample 30 cells were counted per slide (n = 5 per group) and analysed using the macro described in appendices. In total approximately 150 cells were counted per group.

Images from slides collected were quantified using ImageJ (NIH, Maryland, USA). Using the line tool, measurements were drawn across each cell. A custom macro script, used in conjunction with ImageJ, was used to generate data files from the measurements taken (discussed in the appendices). These files were then loaded into Rstudio (Rstudio, Massachusetts, USA) to generate histogram plots of NF-κB and DAPI staining intensity. By overlapping the NF-κB and DAPI histogram plots, NF-κB translocation from the cytosol to the nucleus could be determined. 30 cells were counted for every sample (n = 5 per group), total number of cells counted per group was 150 cells.

6.32: Immunocytochemistry - Examining effects of LPS stimulated H9C2s with either (+)-naloxone / (+)-naltrexone on NF-κB translocation.

Preliminary studies were also conducted to test whether applying either TLR4 antagonists prior, or during, LPS treatment influenced NF-κB translocation. Experiments were performed to test co-treating LPS stimulated H9C2s with either (+)-naloxone or (+)-naltrexone at 100 μM (diluted in DMEM). H9C2s were stimulated with either 10 or 100 ng of LPS for 30 minutes in conjunction with either compound. For pretreatment studies, cells were pretreated with (+)-naloxone or (+)-naltrexone (at 100 μM) for 1 hour; the pre-treatment media was then aspirated and replaced with fresh media containing LPS (100 ng) + (+)-naloxone or (+)-naltrexone (100 μM) for 30 minutes.

6.4: Results

6.41: LPS stimulated H9C2s trigger NF-κB nuclear translocation in a dose dependent manner and (+)-naloxone / (+)-naltrexone does not independently trigger NF-κB activity.

A dose dependent effect was observed in LPS treated cells as shown by the increased number of H9C2s which had NF-κB nuclear translocation (veh: 19 cells; LPS-10 ng: 90 cells; LPS-100 ng: 123 cells out of 150 cells per group) (figure 1a) ($P < 0.05$). (+)-Naloxone and (+)-naltrexone were also tested independently to determine whether the compounds themselves could trigger NF-κB activation. Compared to untreated H9C2s, neither compound trigged significant NF-κB nuclear translocation (figure 1b) ($P > 0.05$).

6.42: (+)-Naloxone and (+)-naltrexone pre-treatment is required to suppress NF-κB nuclear translocation.

H9C2s were co-treated with either (+)-naloxone or (+)-naltrexone (at 100 μM) with LPS (10 or 100 ng) for 30 minutes before being stained for NF-κB activity. While (+)-naloxone and (+)-naltrexone significantly reduced NF-κB translocation when cells were exposed to 10 ng of LPS [LPS-10g: 90 cells, LPS + (+)-nal: 62, LPS + (+)-nalt: 66/150 cells] (figure 2a) no differences were observed when the LPS concentration was increased to 100 ng [LPS-100g: 120 cells, LPS + (+)-nal: 111, LPS + (+)-nalt: 107/150 cells] ($P > 0.05$) (figure 2b). H9C2s were then tested to see whether pre-treating H9C2s with (+)-naloxone or (+)-naltrexone would improve the suppressive effects of NF-κB activation (figure 2c). By using this protocol, the number of LPS stimulated cells which had NF-κB nuclear translocation was significantly reduced [drug pretreatment: 123 cells, LPS + (+)-nal: 94, LPS + (+)-nalt: 114/150 cells].

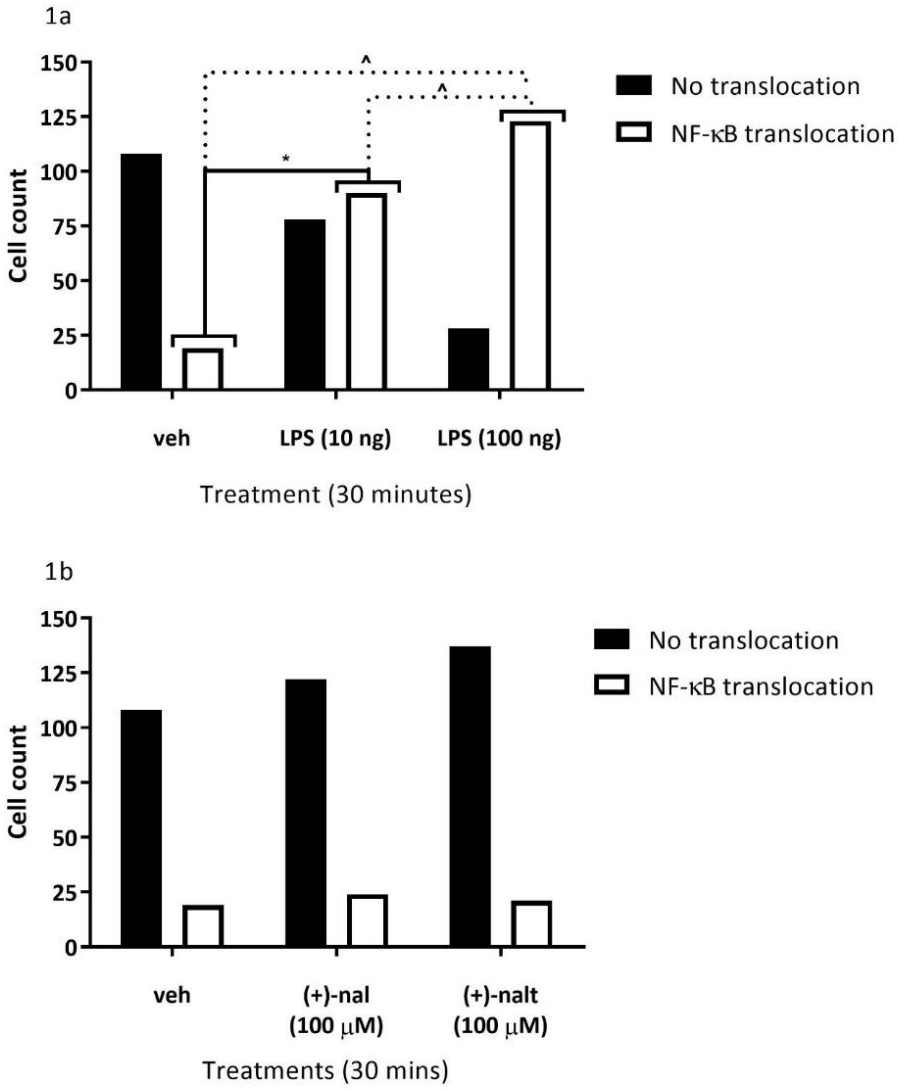
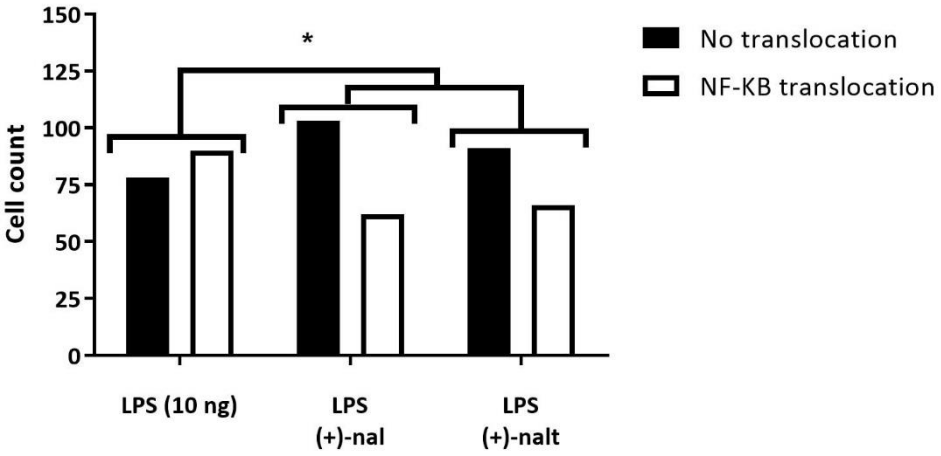


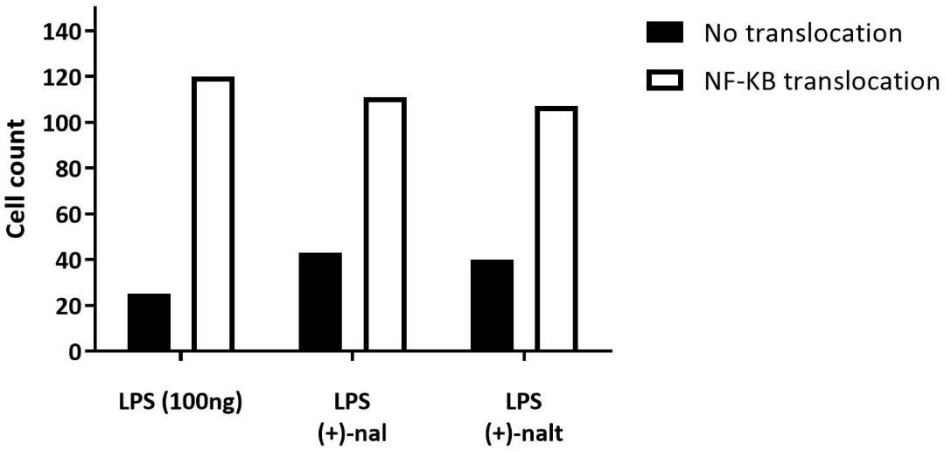
Figure 1: Cell counts examining NF-κB nuclear translocation. 1a) H9C2s exposed to LPS for 30 minutes at 10 and 100 ng. 1b) H9C2s treated with either (+)-naloxone or (+)-naltrexone at 100 μM for 30 minutes. Veh – vehicle, (+)-nal – (+)-naloxone, (+)-nalt - (+)-naltrexone. * = statistical significance of LPS (10 ng) observed when compared to vehicle control groups, ^ = statistical significance of LPS (100 ng) observed when compared to either LPS 10 ng or vehicle control groups ($P < 0.05$) (n =150-160 cells per group).

2a



LPS (10ng) with drug treatment (100 μM) (30 minutes)

2b



LPS (100ng) with drug treatment (100 μM) (30 minutes)

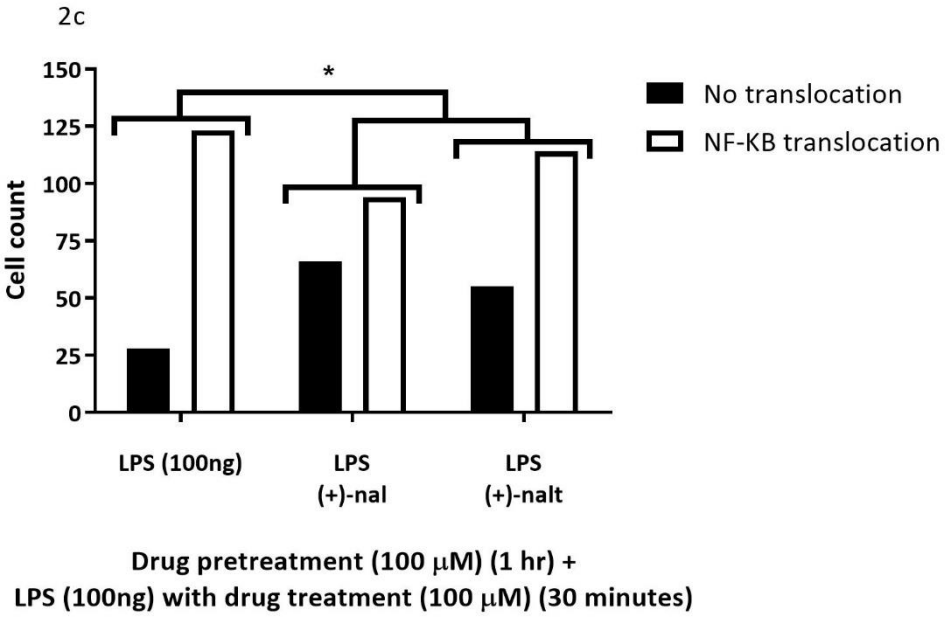


Figure 2: Cell counts examining NF-κB nuclear translocation. 2a) H9C2s co-treated with LPS (10 ng) and either (+)-naloxone or (+)-naltrexone (both at 100 μM) for 30 minutes. 2b) H9C2s co-treated with LPS (100 ng) and either drug (both at 100 μM). 2c) Examining whether pre-treating H9C2s, prior LPS + drug co-treatments, with either compound improved the suppression of NF-κB translocation. (+)-nal – (+)-naloxone, (+)-nalt - (+)-naltrexone. * = statistical significance observed between groups ($P < 0.05$) (n = 150-160 cells per group).

6.5: Conclusion

From these preliminary findings, the same protocol treatments were used for study 3. 100 ngs of LPS was used to stimulate the inflammatory response; additionally, pre-treating H9C2s with (+)-naloxone or (+)-naltrexone, followed by co-treatment with LPS and either TLR4 antagonist, was discovered to suppress NF-κB nuclear translocation.

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XIV: Statements of authorships (chapter 7)

Title of Paper	(+)-Naloxone and (+)-naltrexone does not improve cell viability in simulated ischaemic-reperfusion injury in H9C2s but does limits NF-κB nuclear translocation upon LPS stimulation
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Study 3 examined whether (+)-naloxone or (+)-naltrexone could suppress inflammation in LPS stimulated H9C2s. Immunohistochemistry was used to measure NF-κB nuclear translocation. RT-PCR in cell lysate were used to measure gene expression of TLR4, IL-6, IL-1β, TNF-α. Both compounds were also tested to see whether pre-treating either compound could protect H9C2s in simulated ischaemic-reperfusion injury. The neutral red cell assay was used to test cell viability.

Principal Author

Name of Principal Author	Mr Samuel Man lee		
Contribution to the Paper	Writing, proof-reading, editing, performed experiments and data analyses.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	11-01-2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	1-8-2018

Chapter 7: (+)-Naloxone and (+)-naltrexone does not improve cell viability in simulated ischaemic-reperfusion injury in H9C2s but does limits NF-κB nuclear translocation upon LPS stimulation.

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7.1: Abstract

Introduction: It is now known that myocardial inflammation plays an important role in the progression of tissue damage / repair during ischaemic-reperfusion injury. Toll-like receptor 4 (TLR4), an immunosurveillance receptor, plays an important role regulating inflammatory signalling during ischaemic-reperfusion injury. While the attenuation or removal of TLR4 limits infarct size, the proportion of TLR4 signalling mediated through cardiomyocytes remains poorly defined. This study investigated whether TLR4 antagonists, (+)-naloxone and (+)-naltrexone, suppresses myocardial inflammation and cell viability in H9C2 cells exposed to either lipopolysaccharide stimulation or simulated ischaemic-reperfusion injury.

Method: Immunocytochemistry (ICC): H9C2s were pre-treated with either (+)-naloxone or (+)-naltrexone (100 μM) for 1 hour before being co-treated with LPS (100 ng) and either drug (100 μM) for 30 minutes. H9C2s were then fixed and stained for NF-κB activity to study NF-κB cytosolic to nuclear translocation. RT-PCR: The same treatment protocol was also used for RT-PCR, but H9C2s were co-treated with LPS for 6 hours before being harvested and processed for cDNA. Simulated ischaemic-reperfusion injury: Cells were pre-treated with either compound for 1 hour (100 μM) before they were placed into a hypoxic incubation chamber and treated with a hypoxic buffer solution (2 hours). The cells were then removed from the chamber and the solution was replaced with a normoxic buffer solution [treated with or without (+)-naloxone / (+)-naltrexone] for 2 hours. Cells were then tested with the neutral red assay solution to determine cell viability.

Results: ICC: LPS triggered a significant increase in NF-κB translocation in H9C2s after 30 minutes of stimulation (cells with NF-κB nuclear translocation: 103/150 cells). Pre-and co-treatment of either (+)-naloxone (71/150) or (+)-naltrexone, at 50 μM (78/150) or 200 μM [(+)-naloxone-200 μM: 86/150, (+)-naltrexone-200 μM: 67/150], reduced NF-κB translocation. RT-PCR: Compared to veh-(+)-naloxone treated cells (0.4 ± 0.2), IL-1β gene expression was increased when stimulated with LPS (1.5 ± 0.2). However, LPS co-treatment with (+)-naloxone caused higher levels of IL-1β gene activity (2.4 ± 0.3) ($P < 0.05$). IL-6: Although no statistical difference was observed between veh-(+)-naloxone (1.0 ± 0.1) and LPS only (1.3 ± 0.1) groups, significance was observed between LPS-(+)-naloxone co-treated cells (1.7 ± 0.1) and vehicle-(+)-naloxone cells ($P < 0.05$). No changes were observed when TNF-α and TLR4 gene expression was examined. Simulated ischaemic-reperfusion protocol: H9C2s exposed to both buffers had a 40% reduction in cell viability (61.0 ± 8.3%) ($P<0.05$). When the cells were pre-treated with either (+)-naloxone or (+)-naltrexone, cell viability did not improve 2 or 24 hours later ($P > 0.05$).

Conclusion: (+)-Naloxone and (+)-naltrexone limits NF-κB nuclear translocation during acute LPS stimulation. Co-treating H9C2s with LPS and (+)-naloxone increased IL-1β and IL-6 production after a 6-hour treatment. Neither compound influenced cell viability when exposed to simulated ischaemic-reperfusion injury.

7.2: Introduction

Ischemic heart disease remains a significant cause of mortality throughout the developed world. In brief, the restriction or occlusion of blood flow deprives the myocardium of oxygen resulting in the initiation of cell stress pathways. The damage caused during ischaemia is exacerbated even further when blood flow is restored throughout the myocardium. Experimental models such as the isolated heart technique [1,2], or the left anterior descending artery ligation model [3,4], have allowed researchers to determine the exact causations of ischaemic-reperfusion injury. Although these techniques have been (and remain) invaluable within the cardiac field differences in animal models, age, gender, and methodology, can cause discrepancies [2][5][6]. It is also important to consider that the heart is not composed of just cardiomyocytes [7,8]; endothelial cells [9,10], fibroblasts [11] and interstitial cells [12] all play vital roles in myocardial function and pathology. The damage or activation of receptors expressed on these cell types during ischaemic reperfusion injury can have significant consequences on myocardial function and recovery. For instance, immunosurveillance receptors expressed on endothelial cells lining the vasculature are activated during reperfusion. During ischaemic-reperfusion injury, these immunosurveillance receptors recognise endogenous proteins which are either released from necrotic cells [13,14] or from white blood cells [15–17]. Termed as danger associated molecular pattern molecules (DAMPs), these endogenous proteins are recognised through genetically conserved molecular regions. One of the receptors known to play a major role in promoting myocardial inflammation during ischaemic-reperfusion injury is toll-like receptor 4 (TLR4). Aside from its main role in recognising lipopolysaccharide (LPS) [18–20], a structural component found in gram negative bacteria, TLR4 can also recognise DAMPs [14,21]. As reported by Nishimura *et al* [22], TLR4 is the most highly expressed TLR within the heart and

is predominantly expressed on coronary endothelial cells [23]. When endothelial cells or cardiomyocytes were exposed to LPS or given a myocardial infarctions, TLR4 expression is significantly upregulated [23]. Since these original findings, genetic [21,24,25] and pharmacological knockout studies [26] have shown that the suppression of TLR4 signalling reduces infarct size and myocardial inflammation. While these studies highlight the importance of TLR4 in myocardial inflammation, the proportion in which TLR4 signalling is mediated directly through cardiomyocytes remains poorly defined.

7.21: TLR4 signalling & TLR4 antagonists, (+)-naloxone and (+)-naltrexone.

Unlike other toll-like receptors (TLR), TLR4 has two main signalling networks; the MyD88 dependent and TRIF dependent pathways [27]. Of the two, the MyD88 signalling pathway is the first network to be activated and triggers myocardial inflammation. Innate immunity is the first response to be triggered resulting in a broad, rapid, and non-specific immunological response. TLR4 downstream signalling cascades lead to the activation of multiple mitogen activated protein kinases (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). MAPKs such as p38 and c-Jun N-terminal kinases (JNK) triggers the production of pro-inflammatory cytokines, such as interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α), as well as cell death pathways during reperfusion [28–32]. NF-κB on the other hand is an important inflammatory transcription factor which is located within the cytosol. Triggered by both pathways, activation of NF-κB leads to its translocation into the nucleus where it begins the de-novo synthesis of pro-inflammatory cytokines [18,33–35]. TLR4's alternative pathway, the TRIF pathway, is activated after MyD88 signalling and resolves innate immunity while simultaneously triggering adaptive immunity [20,27].

This study examined whether TLR4 antagonists, (+)-naloxone or (+)-naltrexone, could suppress inflammation in LPS stimulated H9C2s. Unlike the positive isoforms, the negative stereoisomers inhibit mu-opioid receptor activity and are often used to treat alcohol or opioid addiction [36–38]. Because of the evidence behind opioid receptors in triggering classical preconditioning [39,40], the positive stereoisoforms were selected to avoid conflicting variables. To determine whether (+)-naloxone or (+)-naltrexone could suppress myocardial inflammation, NF-κB cytosolic to nuclear translocation, and quantification of interleukin 1 beta (IL-1β), IL-6, TLR4 and TNF-α gene expression was examined. This study also investigated whether H9C2s pre-treated with (+)-naloxone or (+)-naltrexone showed improved cell viability when the cells were exposed to simulated ischaemic-reperfusion injury. H9C2s are cardiomyoblasts derived from the left ventricles of BDIX rats [41,42]. This cell-line was reported by Kuznetsoc *et al* [43] to be more responsive to hypoxic-normoxic conditions and were thus selected for this study.

7.30: Methods

7.31: Cell line / maintenance

H9C2(2-1) cells were purchased from ATCC (ATCC®CRL-1446) (ATCC, Virginia, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), Penicillin-Streptomycin (10,000 U/mL) and L-Glutamine (5 mM). Cells were split at sub-confluency between 80-90%.

7.32: Immunocytochemistry - NF-κB staining protocol

Cells were seeded onto poly-d-lysine coated coverslips (which were placed into 24 well plates) at 2×10^4 cells / ml and left to incubate overnight. Cells were then pre-treated with either 50 or 200 µM of (+)-naloxone or (+)-naltrexone (diluted in DMEM) for 1 hour at 37^{o}C. The media was then aspirated and replaced with fresh DMEM containing LPS (100 ng) (L2630) (Sigma-Aldrich, NSW, Australia) with or without (+)-naloxone or (+)-naltrexone (50 or 200 µM) for 30 minutes. After the final incubation, cells were fixed with 4% formaldehyde with 5% sucrose for 10 mins at room temperature (RT). The slides were washed once with ice cold PBS followed by a 10 mins blocking / permeabilization step with blocking buffer: 3% skim milk + 0.15% triton-X. Each well was then incubated with 1:1000 DonkeynRabbit NF-κB-p65 (diluted in blocking buffer) (Ab7970) (Abcam, Victoria, Australia), for 2 hours at RT. After incubation, cells were washed 3 times with ice cold PBS with 1 min wash periods. Cells were then treated with Alexa Fluor 488 (1:1000) in conjunction with DAPI (1:10,000) (both diluted with blocking buffer) for 1 hour, RT, in the dark. This was followed by another 3 washes with PBS before fixed onto mounting slides. These slides were then viewed using a confocal microscope at wavelengths 488 and 358 λ.

7.33: Immunocytochemistry - data analyses

Images from slides collected were quantified using ImageJ (NIH, Maryland, USA). Using the line tool, measurements were drawn across each cell. A custom macro script, used in conjunction with ImageJ, was used to generate data files from the measurements taken (discussed in the appendices). These files were then loaded into Rstudio (Rstudio, Massachusetts, USA) to generate histogram plots of NF-κB and DAPI staining intensity

(example figure 1k/1l). By overlapping the NF-κB and DAPI histogram plots, NF-κB translocation from the cytosol to the nucleus could be determined. 30 cells were counted for every sample (n = 5 per group), total number of cells counted per group was 150 cells.

7.34: Real time PCR – quantification of gene expression in LPS stimulated H9C2s treated with (+)-naloxone.

H9C2s were seeded onto 6 well plates at 4.5×10^4 cells / ml. Cells were pre-treated with 100 µM of (+)-naloxone for 1 hr followed by LPS treatment [with or without (+)-naloxone] at 100 ng for 6 hours. Cells were then harvested for RNA extraction using a Maxwell 16 LEV RNA extraction kit (AS1280) in conjunction with a Maxwell 16 LEV RNA machine (AS2000) (Promega Australia, NSW, Australia). RNA was then converted to cDNA using a High Capacity cDNA-RT kit (4368814) (Thermofisher, SA, Australia) and a MJ Research PTC-200 thermal cycler (8252-30-0001). Primers, Rpl13a (used as housekeeper), IL-6, IL-1β, TNF-α and TLR4 were designed through NCBI's primer design tool (NCBI, Maryland, USA) (table 1) and produced through Integrated DNA Technologies (IDT, Science Park, Singapore). 25 ng of cDNA was loaded into Biorad PCR plates with iTaq Universal SYBR green supermix (1725121), forward + reverse primers, and run through a Biorad C1000 touch thermal cycler + CFX96 real time system (Biorad, NSW, Australia) for 1 hour. All treatments were normalized to vehicle groups to reduce intergroup variability. The Livak method was used to determine the relative gene expression of interest after corrected with Rpl13a and the mastermix samples. Values presented as the normalized expression ($2^{-\Delta\Delta Cq}$) in the results section.

Name	NCBI Ref Sequence	Forward primer (5'-3')	Reverse primer (5'-3')
NF-κB	XM_006233360.3	GGACAACTATGAGATGAGCTCCG	ATCTCCAGTGAGGGACTCCG
IL-1β	NM_031512.2	TTGAGTCTGCACAGTTCCCC	TGTCCCGACCATTGCTGTTT
IL-6	NM_012589.2	CACTTCACAAGTCGGAGGCT	TCTGACAGTGCATCATCGCT
TLR4	NM_019178.1	TGGCAGTTTCTGAGTAGCCG	TCCCACTCGAGGTAGGTGTT
TNF-α	XM_008772775.2	GGAGGGAGAACAGCAACTCC	GCCAGTGTATGAGAGGGACG

Table 1: Primer designs of inflammatory markers

7.35: Simulated ischemic-reperfusion protocol

To replicate the physiological changes which occur during ischemic-reperfusion injury ischemic and normoxic buffers were used. Buffer recipes were taken from Gáspár *et al's* [44] study but with several modifications. Hypoxic buffer recipe: NaCI-119mM, KCI-5.4mM, NaH_2PO_4▪H_2O-1.2mM, $MgCl_2$▪$6H_2O$-0.5 mM, Hepes-5mM, $MgSO_4$▪$7H_2O$-1.3 mM, $CaCl_2$-0.9mM, $C_3H_5O_3Li$-20mM, BSA-0.1%, pH to 6.4. Normoxic buffer: NaCI-125mM, KCI-5.4mM, NaH_2PO_4▪H_2O-1.2mM, $MgCl_2$▪$6H_2O$-0.5 mM, Hepes-20mM, $MgSO_4$▪$7H_2O$-1.3 mM, $CaCl_2$-1mM, Glucose-15 mM, Taurine-5mM, Creatine monohydrate-2.5mM, BSA-0.1%, pH to 7.4. H9C2s were seeded at 3.0×10^4 cells/ml into 2 × 96 well plates and left to incubate overnight. To determine the effects of these buffers independently, four different groups were examined (table 2). Prior to hypoxic exposure, DMEM or hypoxic buffer (table 2) was added into the appropriate wells before the plates were placed into a hypoxic incubator chamber (STEMCELL Technologies Australia, Victoria, Australia). The chamber was then filled with high purity nitrogen (95% N_2 with 5% CO_2) to remove any O_2 before it was sealed and incubated at 37^oC. To determine whether the chamber was an independent factor which influenced cell viability, the 2nd plate was given the same treatments and placed into a cell incubator throughout the experiment. After 2 hours the plate was removed from the hypoxic chamber

and the DMEM / hypoxic buffer was replaced with either fresh DMEM, or normoxic buffer solution, for 2 hours in a cell incubator heated at 37^{o}C. Each treatment was performed in quadruplicates, n=6.

Treatment	**Hypoxia "ISCHAEMIA" (placed in hypoxic chamber for 2 hours)**	**Normoxia "REPERFUSION" (placed into incubator for 2 hours)**
DMEM only	DMEM (with 10% FBS)	DMEM (with 10% FBS)
Hypoxia + DMEM	Hypoxic buffer	DMEM (with 10% FBS)
DMEM + normoxia	DMEM (with 10% FBS)	Normoxic buffer
Hypoxia + normoxia	Hypoxic buffer	Normoxic buffer

Table 2: Schematic timelines of the 4 treatment groups examining hypoxic and / or normoxic buffer treatment exposure when placed into a hypoxic incubator chamber.

7.36: Neutral Red cell viability assay

After the simulated ischemic-reperfusion protocol was complete, H9C2s were treated with 100 µl of neutral red (N4638) (40 µg /ml diluted in DMEM) media for 2 hours. Each well was then washed with 150 µls of phosphate-based saline (PBS) followed by 150 µls of neutral red de-staining agent (96% ethanol, 49% deionized water and 1% glacial acetic acid). The plate was then placed onto a plate shaker to assist in the homogenous dispersion of the neutral red precipitate for 10 minutes. A Biotek microplate reader (Biotek, Vermont, USA) was then used to read the plate at 540 nm. Cell viability (%) was defined as [Intensity: treatment of interest / intensity: vehicle (DMEM only, incubator)] × 100%.

7.37: Simulated ischemic-reperfusion - (+)-Naloxone / (+)-naltrexone pre-treatment protocol.

Once the protocol was confirmed to have caused a significant reduction in cell viability, (+)-naloxone / (+)-naltrexone were tested. Before the hypoxic stage, H9C2s were pre-treated with, or without, (+)-naloxone or (+)-naltrexone for 1 hour (diluted in DMEM) at 100 µM. The cells were then exposed to ischaemic-reperfusion protocol outlined previously with only one modification. (+)-Naloxone or (+)-naltrexone was added into either DMEM or normoxic buffer solutions (100 µM) during the "reperfusion" stage. At the end of reperfusion, H9C2s were tested for cell viability using the neutral red assay immediately after the experiment or 24 hours later (during which treated cells were given fresh DMEM with FBS).

7.38: Data analyses

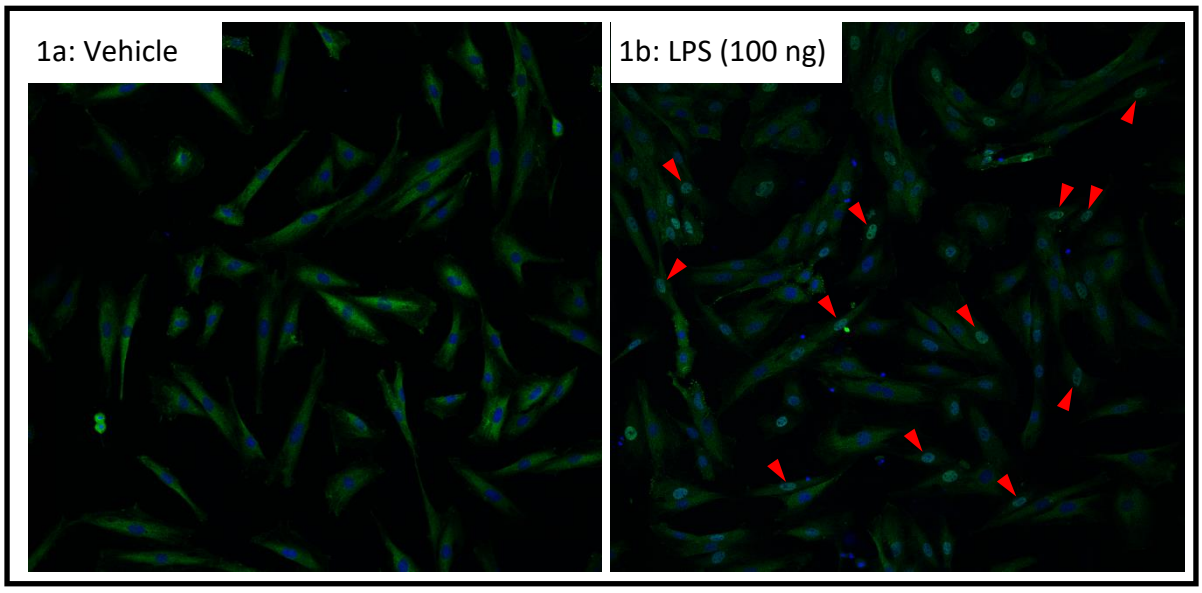
All data was formatted in Microsoft Excel (Microsoft, Washington, USA) before analysed with R in conjunction with Rstudio (Rstudio Team, MA, USA). Depending on the experimental design, Chi-square test with post-hoc analyses or multiway ANOVAs (with post-hoc TukeyHSD) were constructed. Data was graphed on Graphpad prism and presented as mean ± SEM.

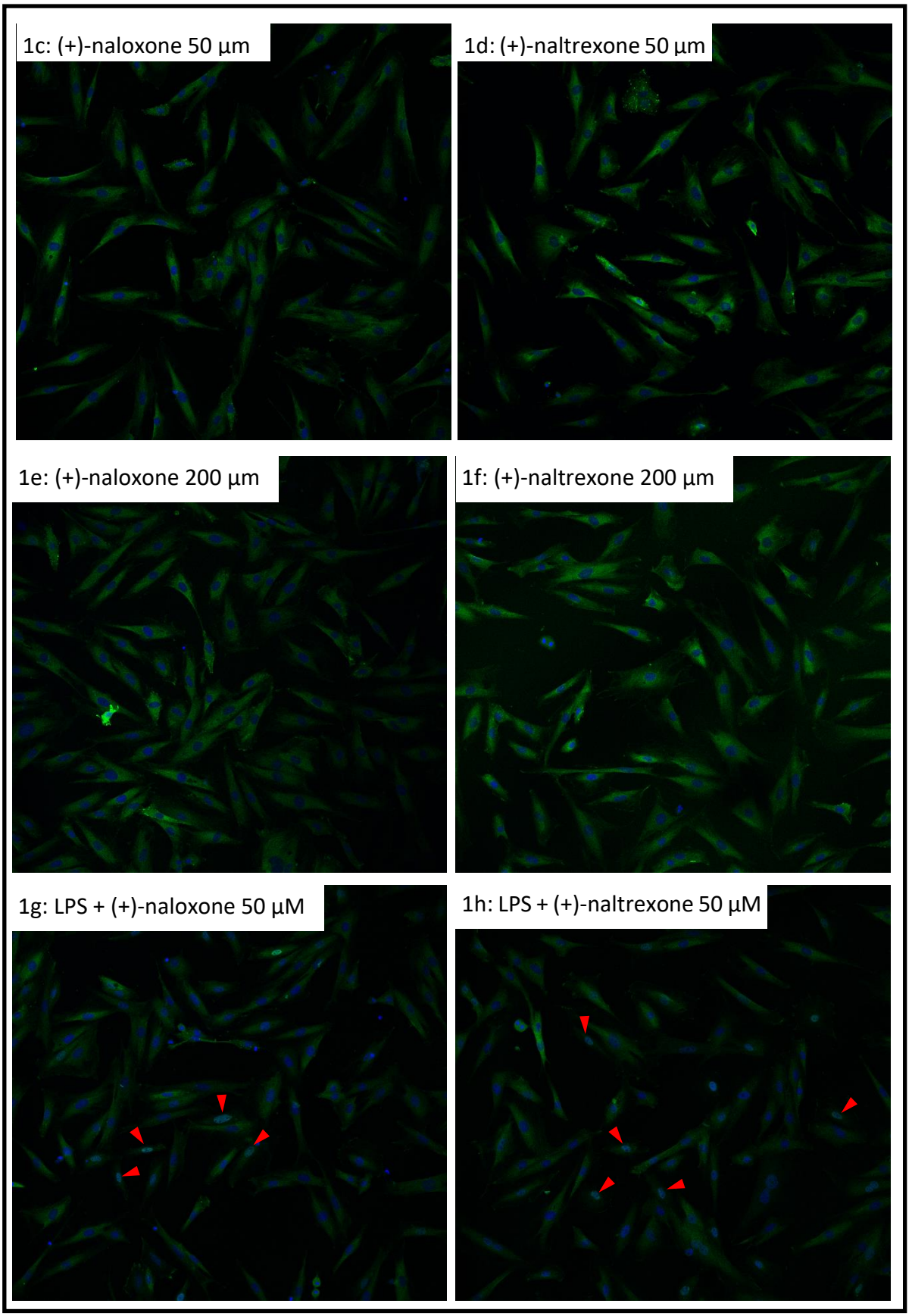
7.4: Results

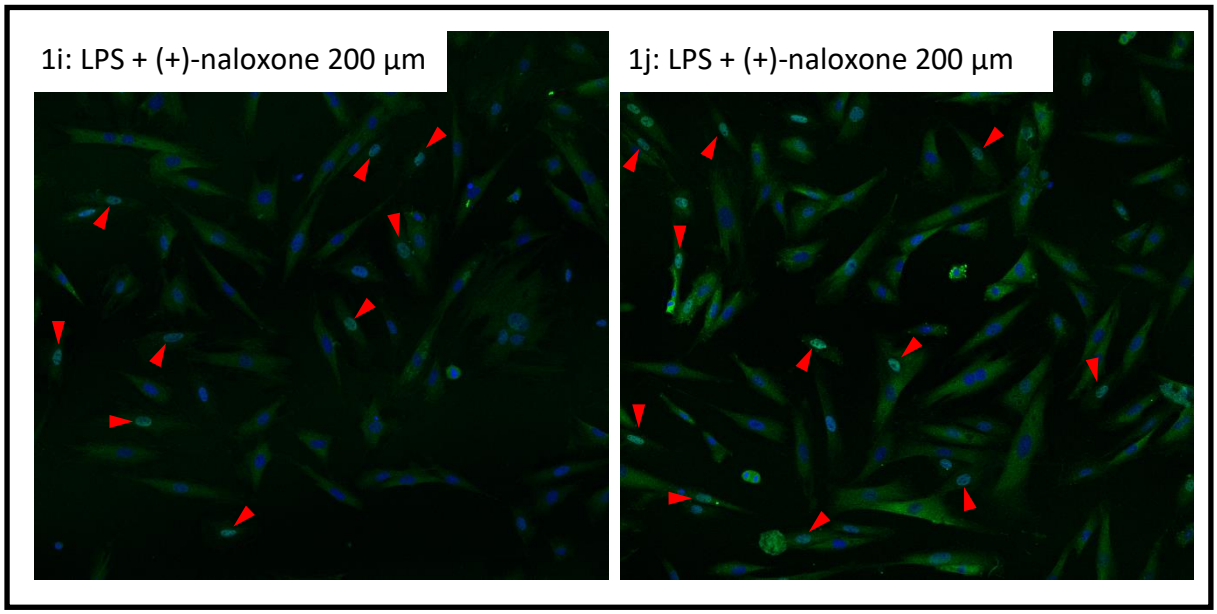
7.41: (+)-Naloxone and (+)-naltrexone limits nuclear translocation of NF-κB in LPS stimulated H9C2s.

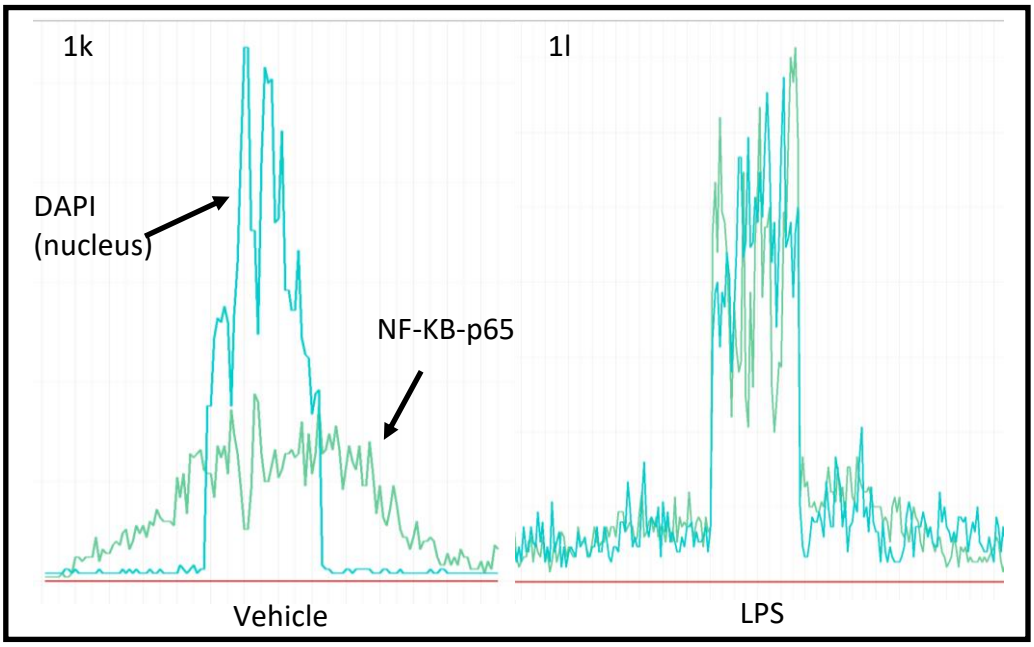
Cell counts revealed that LPS stimulation resulted in a significant increase in NF-κB nuclear translocation compared to the vehicle group [Cells with NF-κB nuclear translocation (Veh:

0/150 cells, LPS: 120/150, p < 0.05)] (figure 1a,1b & 1m). Without LPS stimulation, neither drug, regardless of dosage, caused NF-κB translocation (1m) [(naloxone-50: 0/150, naloxone-100: 0/150, naloxone-200: 0/150, naltrexone-50: 0/150, naltrexone-100: 0/150, naltrexone-200: 0/150, P > 0.05, 1c-1f)] (figure 1m). Pre-treatment of cells with (+)-naloxone or (+)-naltrexone at 50 µM limited LPS induced NF-κB nuclear translocation (LPS-naloxone-50: 71/150, LPS-natrexone-50: 78/150, figure 1g & 1h) (figure 1h). Increasing the concentration of (+)-naloxone or (+)-naltrexone to 200 µM (LPS-naloxone-200: 86/150, LPS-naltrexone-200: 67/150, p < 0.05, figure 1i & 1j) (figure 1n) also limited nuclear translocation of NF-κB. No dose dependent differences were observed between treatment groups (P > 0.05)




1c: (+)-naloxone 50 µm
1d: (+)-naltrexone 50 µm
1e: (+)-naloxone 200 µm
1f: (+)-naltrexone 200 µm
1g: LPS + (+)-naloxone 50 µM
1h: LPS + (+)-naltrexone 50 µM


1i: LPS + (+)-naloxone 200 µm
1j: LPS + (+)-naloxone 200 µm


1k
1l
DAPI
(nucleus)
NF-KB-p65
Vehicle
LPS

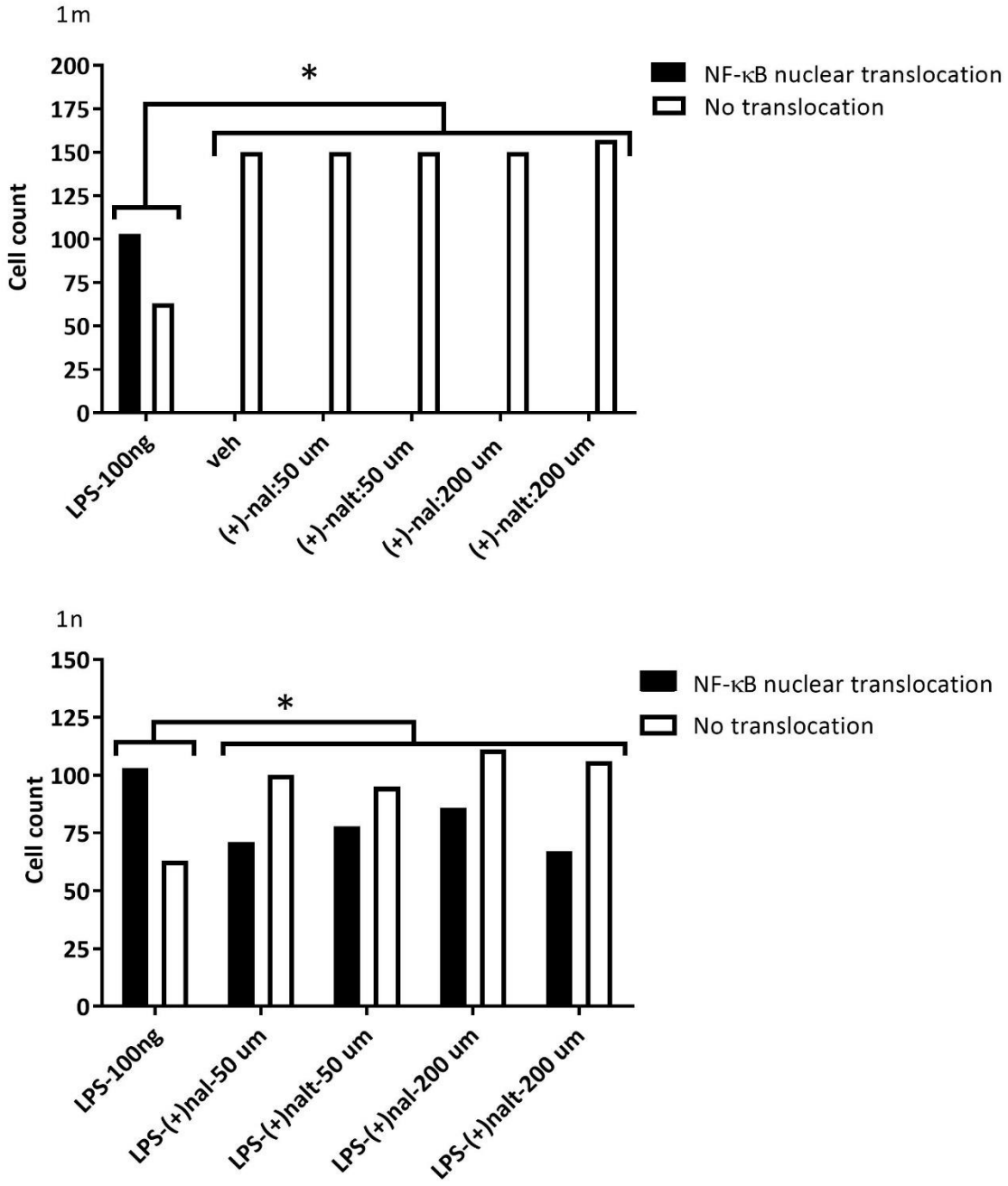


Figure 1: 1a-1j: Immunocytochemistry of LPS stimulated H9C2s treated with or without (+)-naloxone or (+)-naltrexone. ▲ Represents significant NF-κB nuclear translocation in marked cells; all H9C2s treated with or without LPS (100 ng). (+)-Naloxone or (+)-naltrexone were treated at either 50 or 200 µM with or without LPS. 1k-1l: Analysis of histogram plots were used to determine whether significant

NF-κB translocation into the nucleus had occurred. 1m-1n: Bar graphs representing cell counts of H9C2s which experienced NF-κB nuclear translocation when exposed to LPS. (+)-Naloxone and (+)-naltrexone treatments were examined with (1n) or without (1m) LPS stimulation. * represents the statistical significances observed between groups ($p < 0.05$), n = 6 per group.

7.42: (+)-Naloxone influences IL-1β and IL-6 gene expression in LPS stimulated H9C2s.

One-way ANOVAs, with post-hoc analyses, were constructed to determine whether (+)-naloxone influences gene expression of inflammatory markers studied in LPS stimulated H9C2s (figure 2). One-way ANOVA revealed interactions between groups when IL-1β (2a) or IL-6 (2b) was examined ($P < 0.05$). No effects were observed for either TNF-α (2c) or TLR4 (2d) gene expression ($P > 0.05$). Post-hoc analyses revealed that IL-1β expression increased in LPS treated H9C2s ($2^{-\Delta\Delta Cq}$: 1.5 ± 0.2) compared to cells treated only with (+)-naloxone (0.4 ± 0.2) ($P < 0.05$) (figure 2a). Statistical differences were also achieved between LPS treated cells and the (+)-naloxone + LPS co-treatment group which had higher IL-1β expression (2.4 ± 0.3) ($P < 0.05$). Although IL-6 expression increased in LPS treated cells, statistical significance was not achieved [vehicle + (+)-naloxone: 1.0 ± 0.1 vs LPS treatment: 1.3 ± 0.1] (2b). However, when LPS stimulated H9C2s were co-treated with (+)-naloxone, IL-6 expression significantly increased (1.7 ± 0.1) ($P < 0.05$). Finally, while LPS (2.5 ± 1.1) and LPS + (+)-naloxone cells (2.1 ± 0.8) had increased expression of TNF-α ($P < 0.05$), no statistical differences where observed when compared to vehicle + (+)-naloxone (1.4 ± 0.3) ($P > 0.05$) (2c).

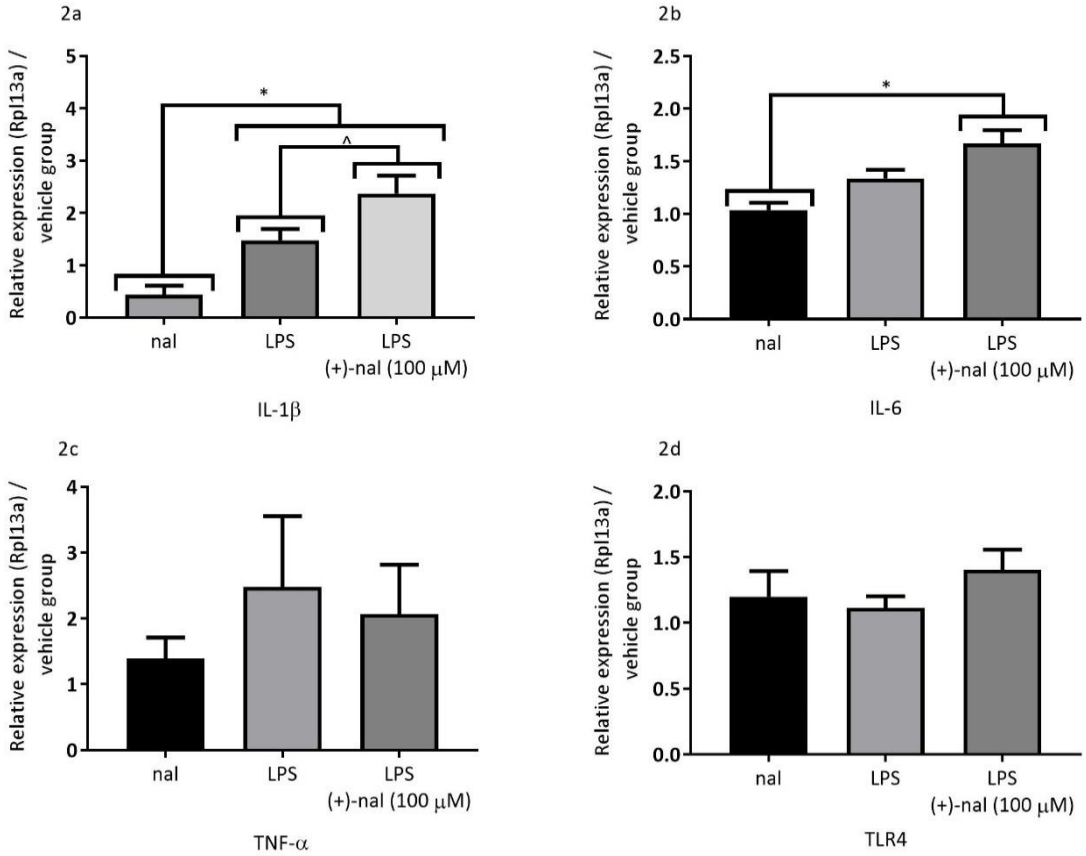


Figure 2: Gene expression levels of LPS stimulated H9C2s pre-treated with (+)-naloxone. Groups were normalized to vehicle groups to reduce intergroup variability. Relative expression of IL-1β (2a), IL-6 (2b), TNF-α (2c), TLR4 (2d). * = statistical difference between veh-(+)-naloxone & LPS-(+)-naloxone ($P < 0.05$). ^ = statistical difference of LPS + (+)-naloxone H9C2s compared to cells treated with (+)-naloxone ($P < 0.05$). (+)-nal = (+)-naloxone, Data presented as mean ± SEM, n = 5.

7.43: Simulated ischemic-reperfusion protocol reduces cell viability.

A three-way ANOVA was constructed to determine whether 1) hypoxic and normoxic buffers had any interactive effects and, 2) whether the use of a hypoxic incubator chamber influenced cell viability (figure 3a). Although hypoxic and normoxic treatments did not influence cell viability independently, an interactive effect was observed when H9C2s were exposed to both

buffers ($P < 0.05$). Furthermore, the use of a hypoxic chamber did not influence cell viability ($P > 0.05$). Post-hoc TukeyHSD revealed that when HC92s were exposed to both buffer treatments (Hypoxic+normoxic-incubator: 59.2 ± 12.1%, ± Hypoxic+normoxic-chamber: 61.0 ± 8.33%) there was a significant reduction in cell viability when compared to cells treated with only media (Media-incubator: 100%, media-chamber: 87.0 ± 3.60%) or hypoxic buffer + media (Hypoxic+media-incubator: 78.6 ± 8.78%, Hypoxic+media-chamber: 86.2 ± 7.93%) ($P < 0.05$) (figure 3a).

7.44: (+)-Naloxone and (+)-naltrexone does not influence cell viability in H9C2s exposed to simulated ischemic-reperfusion injury.

To test whether (+)-naloxone or (+)-naltrexone protects against cell death H9C2s were pre-treated with either compound prior to hypoxia and during the normoxic stage. Cell viability was measured after 2 and 24 hours of hypoxic-normoxic insult. The findings from this study suggest that neither compound improved cell survival ($P > 0.05$). Again, although hypoxic and normoxic buffer treatment significantly reduced cell viability during the 2-hour period (Media only incubator: 100%, Media only chamber: 79.6 ± 6.20% vs Hypoxic-normoxic-incubator: 63.1 ± 4.05%, Hypoxic-normoxic-chamber: 59.6 ± 7.09%) ($P < 0.05$), the hypoxic chamber did not influence cell viability ($P > 0.05$) (3b). When the cells were replaced with fresh DMEM for 24 hours, cell viability was restored to baseline levels (Hypoxic-normoxic buffer: $P > 0.05$) (3c). Interestingly, H9C2s which were placed in the hypoxic chamber had higher levels of cell viability compared to cells placed in the incubator (3c) (Hypoxic chamber: $P < 0.05$).

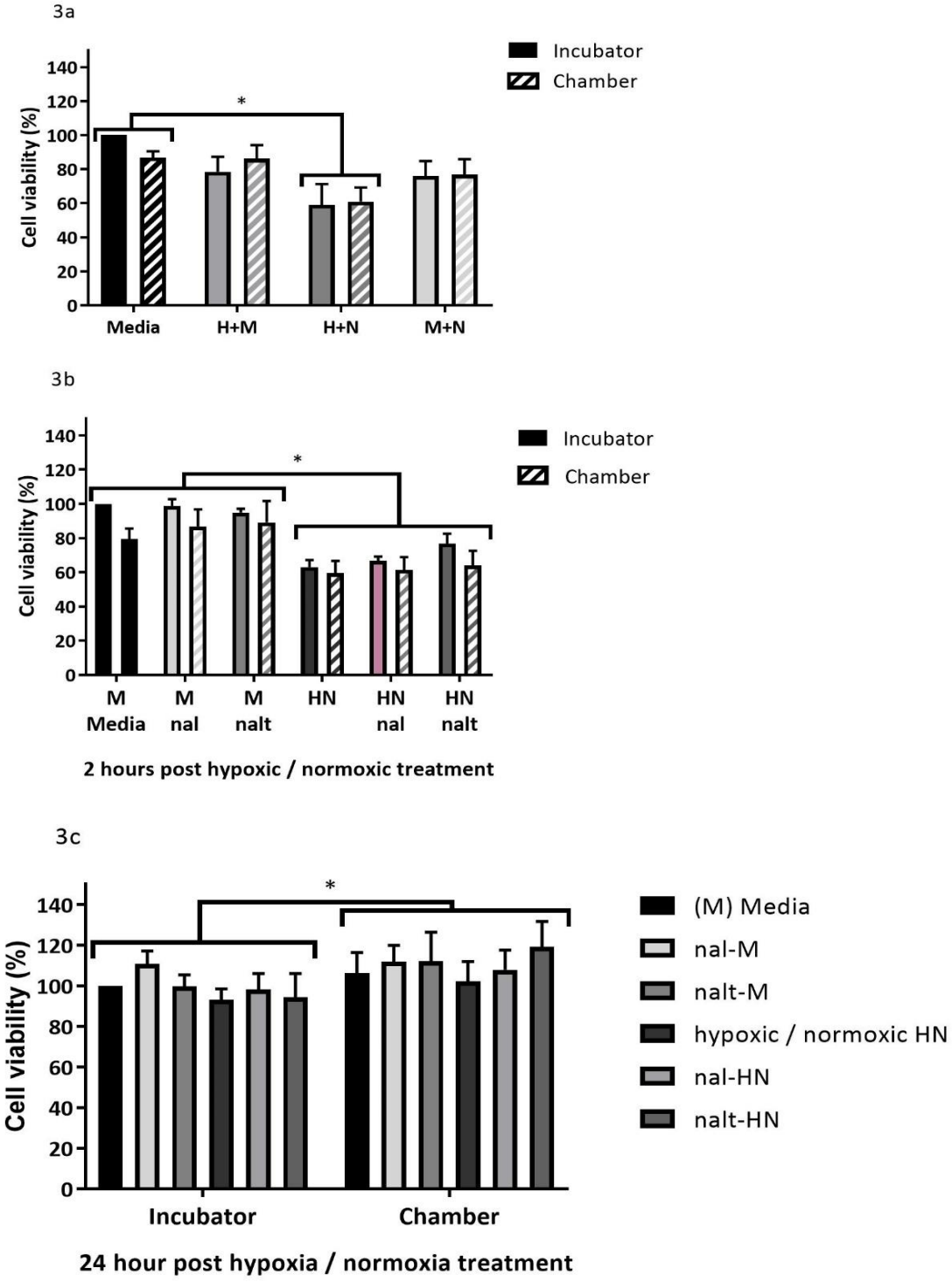


Figure 3: 3a) Cell viability of H9C2s examining the influence of hypoxic / normoxic buffer solutions and the use of a hypoxic chamber (n=6). M = media, H = hypoxic buffer, N = normoxic buffer. 3b-3c) H9C2s pretreated with (+)-naloxone or (+)-naltrexone before exposed to hypoxic-nomoxic injury. Cell viability

was tested at 2 (3b) or 24 (3c) hours later. M = media, HN = hypoxic-normoxic solutions, nal = (+)-naloxone (100 μM), nalt = (+)-naltrexone (100 μM). * = statistical significance ($P < 0.05$), data presented as mean ± SEM, n = 6.

7.5: Discussion

Since the early 2000s, TLR4 has been reported to play a significant role in promoting myocardial inflammation during ischaemic-reperfusion injury [24,25,45]. However, what remains unclear is what proportion of TLR4 signalling is mediated through cardiomyocytes. Compared to coronary microvascular endothelial cells, ventricular cardiomyocytes have been reported to express significantly lower levels of TLR4 [23]. Frantz *et al* [23] observed that when rat ventricular cardiomyocytes were treated with LPS, TLR4 expression was significantly upregulated 24 hours later. Furthermore, when rats were exposed to myocardial infarctions, TLR4 mRNA increased 4 days later [23]. This study attempted to address whether (+)-naloxone and (+)-naltrexone, both TLR4 antagonists, can limit inflammation and improve cell survival within the in-vitro setting. H9C2s, cardiomyoblasts derived from the left ventricles of rats [46,47], have been reported to exhibit cardiomyocyte properties when exposed to hypoxic injury and were selected for this study [42,43].

The negative stereoisomers of naloxone and naltrexone are commonly associated with the treatment of opioid or alcohol addiction due to their ability to inhibit mu-opioid receptors [36,37,48]. However this is stereospecific as (+)-naloxone and (+)-naltrexone have low binding affinity for the receptors [38]. While Hutchinson *et al* [48] and Wang *et al* [49] have both reported that (+)-naloxone and (+)-naltrexone can suppress TLR4 signalling their ability

to inhibit myocardial inflammation remains poorly defined. Finally, this study also examined whether either compound could protect against cell death when H9C2s were exposed to simulated ischaemic-reperfusion injury.

7.51: (+)-Naloxone and (+)-naltrexone limits the translocation of NF-κB into the nucleus of LPS stimulated H9C2s.

This study report that (+)-naloxone and (+)-naltrexone limited the translocation of NF-κB in LPS stimulated H9C2s. Located in cytosol, NF-κB is an important transcription factor which is involved in both early and late phase inflammation [50]. Its activation through TLR4 is particularly unique since TLR4's MyD88 and TRIF signalling pathways can both activate NF-κB [18,51,52]. Once LPS is recognised, the MyD88 signalling pathway is initially triggered leading to the activation of Transforming growth factor beta-activated kinase 1 (TAK1), a kinase which forms a protein complex with TGF-beta activated kinase 1 (MAP3K7) binding protein 1 (TAB1) and TGF-beta activated kinase 1 (MAP3K7) binding protein 2 (TAB2) [53]. TAK1 then stimulates the enzyme IκB kinase (IKK) which migrates to the inactive NF-κB complex [54]. NF-κB is activated when IKK phosphorylates IκBα, an inhibitory factor, causing it to detach from the transcription factor. This activates NF-κB causing it to migrate to the nucleus and begin the gene transcription of multiple pro-inflammatory cytokines. Through time-dependent studies conducted in our lab (data not shown), NF-κB translocation was reported as early as 30 minutes after LPS stimulation. Considering that TLR4-TRIF induced activation of NF-κB is a delayed response, (+)-naloxone / naltrexone's suppression of NF-κB is likely to be mediated through the MyD88 pathway. To our knowledge, this is the first study to report that

(+)-naloxone and (+)-naltrexone can suppress TLR4-MyD88 induced activation of NF-κB within the myocardial setting.

7.52: Co-treatment of LPS stimulated H9C2s with (+)-naloxone increased IL-1β and IL-6 gene expression.

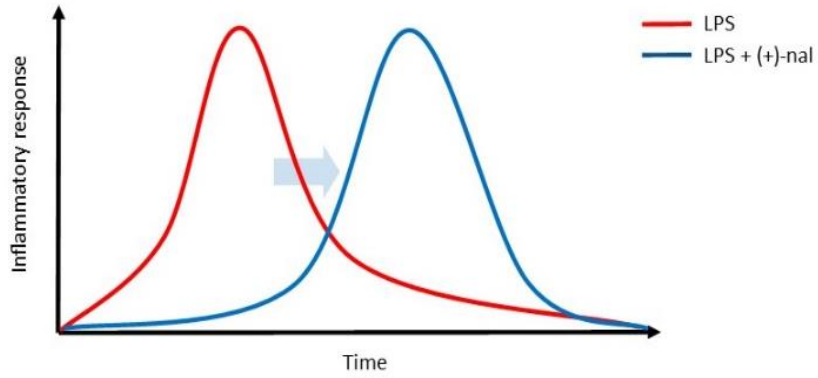
To determine whether (+)-naloxone suppressed inflammation, gene expression of pro-inflammatory cytokines was examined. RT-PCR analyses revealed that instead of suppressing gene activity in multiple inflammatory markers, (+)-naloxone, in-conjunction with LPS, increased IL-1β and IL-6 activity. These results do not support Hutchinson *et al* [48] findings who reported that (+)-naloxone suppressed TLR4 signalling and IL-6 / IL-1β gene expression in BV-2 cells. Considering that (+)-naloxone, by itself, did not increase IL-6 or Il-1β gene expression it is unlikely that the compound directly enhanced inflammation. These findings are even more conflicting considering that (+)-naloxone was reported to suppress the nuclear translocation of NF-κB. There are several possible theories as to why co-treatment between LPS and (+)-naloxone enhanced inflammation (figure 4). The most plausible theory (figure 4a) is that (+)-naloxone delayed the LPS response instead of attenuating TLR4 signalling. This may be possible considering H9C2s were exposed to 6 hours of LPS before they were harvested. Examining gene expression at earlier timepoints may determine whether (+)-naloxone is able to suppress innate immunity. The 2nd theory (figure 4b) is based on Wang's *et al* [49] study which suggests that (+)-naloxone blocks TLR4 signalling through the TRIF dependent pathway. Interferon-1 beta (IFN-β), a cytokine, and IFN regulatory factor 3 (IRF3), a transcription factor, were both reported to be downregulated when LPS-stimulated BV-2s were pretreated with (+)-naloxone [49]. These inflammatory mediators are activated through the TRIF pathway and

assist in the resolution of innate immunity while triggering adaptive immunity at the same time [18,55,56]. While it is uncertain as to whether (+)-naloxone can influence IFN-β / IRF3 activity within 6 hours, it may have influenced crosstalk between the MyD88 and TRIF dependent pathways [50]. Quantification of TRIF dependent end products or crosstalk regulators are possible targets of interest. The last possible theory (figure 4c) is associated with the half-life of naloxone itself. The compound has been reported to have a short half-life with animal / human studies suggesting that its bioavailability declines within 1-2 hours [36,40]. While LPS is quickly removed through the liver, or phagocytosed by macrophages / hepatocytes [57], the ability of H9C2s to respond to LPS remains poorly defined. It is possible that the bioavailability of (+)-naloxone declines over the 6-hour period while free LPS is still available. Testing this theory would require studying the pharmacokinetics of (+)-naloxone and the duration / degree of LPS signalling.

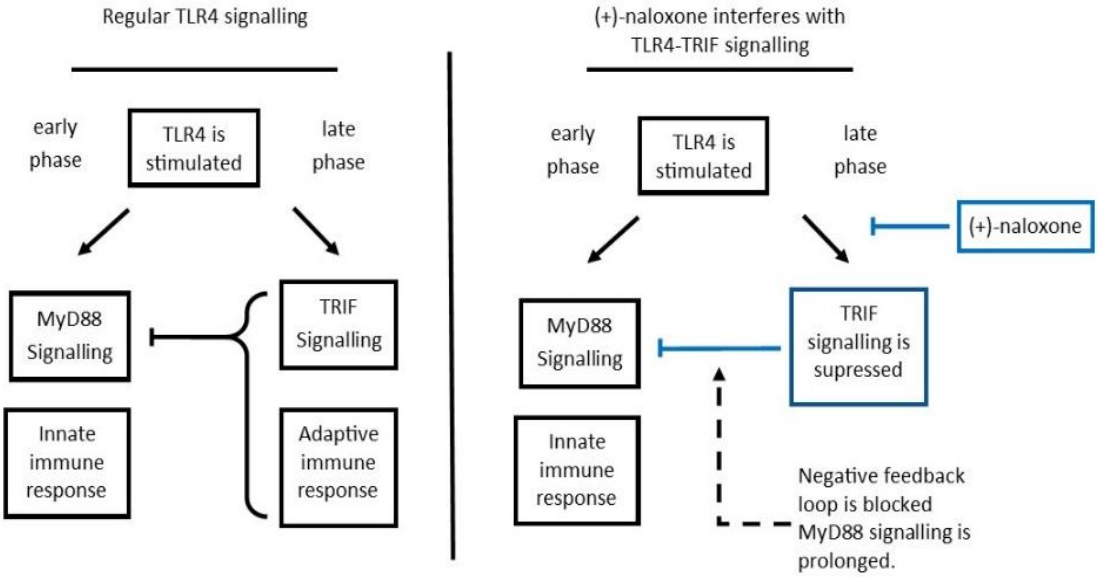
7.53: (+)-Naloxone and (+)-naltrexone does not influence H9C2s survival when exposed to simulated ischaemic-reperfusion injury.

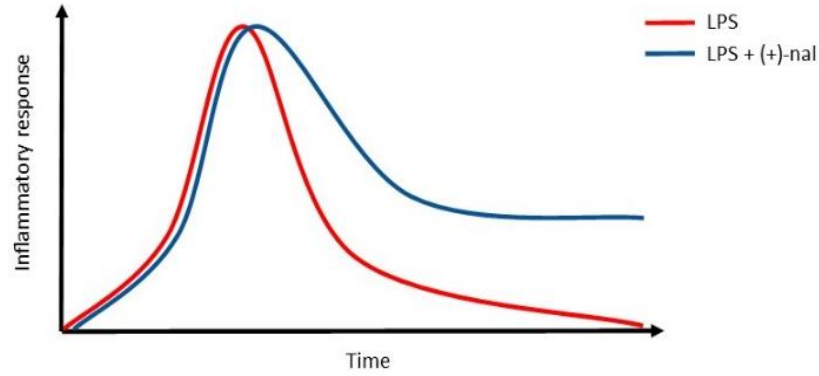
Previous experiments conducted in our lab suggest that (+)-naloxone and (+)-naltrexone can influence contractile recovery in ischaemic-preconditioned isolated hearts (refer to chapter 5, study 2). Although neither compound improved contractile recovery in non-preconditioned hearts, this study was designed to see whether H9C2 survival, when exposed to simulated ischaemic-reperfusion injury, could be improved by (+)-naloxone or (+)-naltrexone pre-treatment. The findings from this study support our previous findings which suggests that neither compound directly influences cell survival. When it was discovered that both compounds limited early NF-κB activity, it was originally assumed that MyD88 signalling was

4a: Theory 1 - Delayed LPS response



4b: Theory 2 - (+)-naloxone supresses TLR4-TRIF signal-





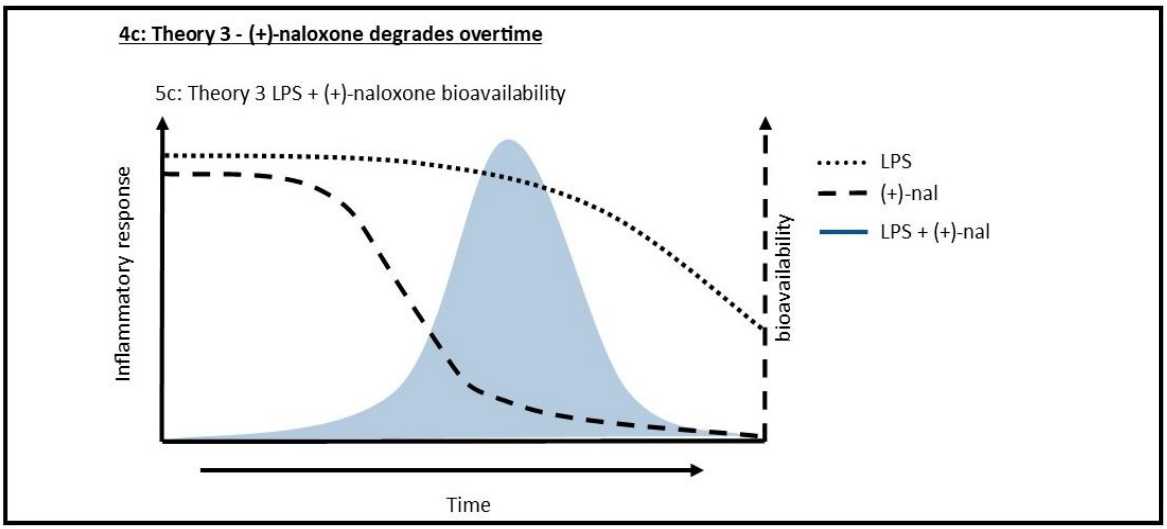


Figure 4: Figures outlining the possible reasons as to why IL-6 and Il-1β gene expression was enhanced in LPS stimulated H9C2s pre-and co-treated with (+)-naloxone. 4a) Theory 1: The innate immune response curve after LPS stimulation. LPS co-treatment with (+)-naloxone causes the response timing to shift to the right and thus delay the innate immune response. 4b) Schematic figures of the TLR4 signalling network and how (+)-naloxone may interfere with TRIF dependent negative feedback signalling. Loss of this regulatory feedback prolongs MyD88 signalling and thus innate immunity is maintained. 4C) Theory 3: (+)-Naloxone's short half-life means that the compound may have degraded over the 6-hour co-treatment period with LPS.

completely suppressed. Since the MAPK family and NF-κB both require the same signalling kinases (such as the TAK1 signalling complex [51–53]) it was hypothesized that (+)-naloxone and (+)-naltrexone would also suppress cell death signalling. Considering the discrepancies from this study, the next step is to determine whether MyD88 blockade is partial or complete. It is also possible that cell-survival, induced through TLR4 inhibition, is dependent on the cell type. Considering that a large portion of TLR4 activity is mediated through endothelial cells [23], studying the coronary vasculature would perhaps show a more prominent effect in

regards to TLR4 ligand induced activity. Endothelial cells play an important role in the recruitment and attachment of white blood cells circulating throughout the bloodstream. Finally, it is important to note that white blood cells, such as macrophages [58], mast cells [59], dendritic cells [60] and neutrophils [61], all express TLR4. The recruitment of these cell types into the ischaemic myocardium has double-edged sword effect; tissue damage is exacerbated through the recruitment of neutrophils while macrophages phagocytose cellular debris and facilitate angiogenesis. The interactions of all these cell types (endothelial, white blood cells and cardiomyocytes), in context of TLR4 signalling, remain poorly defined and requires additional investigation.

7.6: Conclusion

These findings suggest that (+)-naloxone and (+)-naltrexone limit the translocation of NF-κB in the nucleus of LPS stimulated H9C2s. Although these findings are contradicted by the RT-PCR results, there may multiple reasons as to why this occurred. This study also showed that neither compound alone can improve H9C2 survival when exposed to simulated ischaemic-reperfusion injury. Considering these findings, it may be possible (+)-naloxone / (+)-naltrexone has a more prominent effect in the in-vivo setting where TLR4 is present on other cell types. Future studies will examine with either compound can limit infarct size when infused during reperfusion.

7.7: Acknowledgements

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XV: Statements of authorships (chapter 8)

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Principal Author

Name of Principal Author	Mr Samuel Man lee		
Contribution to the Paper	Writing, proof-reading, editing, performed experiments and data analyses.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	11-01-2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Professor David Saint		
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Chapter 8: (+)-Naloxone / (+)-naltrexone does not directly influence cell viability but does manipulate TLR4 signalling.

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8.1: Introduction

As mentioned in chapter 6, the scope of this thesis was revised to focus on the potential benefits of (+)-naloxone and (+)-naltrexone in the clinical setting. Before beginning any animal work, we decided to address two questions which were not examined in study 1 and 2. That is, can (+)-naloxone and (+)-naltrexone protect against cardiomyocytes exposed to simulated ischaemic-reperfusion injury? And can either compound suppress myocardial inflammation when stimulated with lipopolysaccharide (LPS)? Hence study 3 was an in-vitro study designed to examine whether (+)-naloxone or (+)-naltrexone can influence H9C2 cells (cardiomyoblasts with cardiac-like properties) when exposed to LPS or simulated ischaemic-reperfusion injury. Study 3's findings discovered that while (+)-naloxone and (+)-naltrexone can suppress H9C2 induced inflammation, neither compound protects against cell death.

These negative results suggest that the suppression of toll-like receptor 4 (TLR4) signalling alone in cardiomyocytes cannot attenuate cell death. Considering that TLR4 has been reported to be expressed at low levels in cardiomyocytes [1] these findings are perhaps unsurprising. It is likely that TLR4 signalling mediated through endothelial cells [1] and white blood cells [2–4] generate a more potent inflammatory response. Furthermore, care should be taken into interpreting study 3's results as H9C2s are cardiomyoblasts, not cardiomyocytes. Repeating this study using primary left ventricular cardiomyocytes, or examining coronary endothelial cells, may yield different results. The immunohistochemistry and RT-PCR results were also conflicting. While both compounds suppressed acute nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) nuclear translocation in LPS stimulated H9C2s, co-treating with (+)-naloxone and LPS (for 6 hours) enhanced interleukin-

6 (IL-6) and (interleukin-1 beta) IL-1β gene expression. As discussed in study 3, I believe that this is due to the timepoint in which LPS stimulated cells were harvested. Examining gene expression at earlier periods may show more promising results and will be performed in the future. Considering the timing in which NF-κB translocation was suppressed, it is likely that (+)-naloxone and (+)-naltrexone suppresses MyD88 signalling. This suggests that that both compounds could potentially block TLR4 signalling during acute reperfusion. On the other hand, the enhanced activity of IL-6 and IL-1β gene expression may suggest that (+)-naloxone blocked TRIF induced signalling which itself is a negative regulator of innate immunity (this is discussed in further detail in chapter 7). While study 3 did not examine TRIF dependent signalling, additional experiments will be conducted in the future to test TRIF related markers (i.e. interleukin-10m interferon gamma and interferon regulatory factor 3) [5]. Although the findings from study 1, 2, and 3, have provided some insight into the effects of (+)-naloxone and (+)-naltrexone, their role in the in-vivo setting has not been examined.

8.2: Study 4 - Do (+)-naloxone and TAK242 attenuate myocardial inflammation and reduce infarct size in rats?

The final study of this thesis used an in-vivo infarct recovery model to examine how novel TLR4 blockers could influence myocardial inflammation and infarct size. It should be noted that at the time of this study (+)-naltrexone was no longer available and could not be tested. Using the left anterior descending artery ligation technique, rats were given myocardial infarctions for 30 minutes and then allowed to recover for 48 hours. This final study addressed several limitations of study 1,2 and 3. Firstly, study 4 allowed me to observe the full inflammatory cascade in a long-term recovery model. As described in chapter 3, the innate

and adaptive immune response is a highly complex system [5,6][6]. From the activation of immunosurveillance receptors on multiple cell types, to the recruitment of circulating white blood cells from both the lymph and circulatory system; the removal of any component can significantly depress the immune response [5–8]. For instance, one of the limiting factors of the isolated heart technique, in the context of inflammation, is the lack of blood flow. Without this, the ischemic myocardium is not exposed to circulating white blood cells or the complement system (part of innate immunity) which are both important components of innate immunity [6]. It is also important to remember that the isolated heart technique can only measure acute contractile recovery for 1-2 hours as left ventricular function will progressively decline [9]. Finally, the role of inflammation (specifically TLR4) in wound healing has not been examined in this thesis. If Wang *et al* [10] findings also apply here, then suppression of TRIF signalling will influence how inflammation contributes to wound healing after ischaemic injury. Whether (+)-naloxone / (+)-naltrexone's blockade of TLR4 is beneficial or detrimental during recovery from a myocardial infarction is still uncertain. This study also tested a secondary TLR4 antagonist, TAK-242 (or resatorvid). A more recent TLR4 antagonist, significant research into the pharmacological properties of this compound has shown it to be a potent TLR4 antagonist which blocks both MyD88 and TRIF signalling [11–14]. Although TAK-242 has been reported to be protect against cerebral ischaemic-reperfusion injury [15], no study has examined whether it reduces infarct size in the myocardial setting.

Study 4 was also aimed at testing the benefits of suppressing TLR4 in a more clinically relevant model. Instead of pre-treatment, a TLR4 antagonist was administered through an intravenous bolus injection before the LAD ligature was released. Because a large proportion of damage

is observed during early reperfusion, pharmacological invention during or immediately after ischaemia can significantly minimise tissue damage and improve post-op recovery. Furthermore, because of (+)-naloxone's short half-life [16,17] in the in-vivo setting, I felt that administering the TLR4 antagonist prior to reperfusion would be the more effective than pre-treatment. Using the Evan's blue staining technique [18], infarct size was measured to determine whether (+)-naloxone or TAK-242 protected against ischaemic-reperfusion injury. Finally, all groups were repeated to collect the left ventricles and quantify IL-1β activity and NF-κB nuclear translocation using western blots. Caspase-3, a cell death effector [19–22], and matrix-metalloproteinase 9 (MMP-9) [23–25], which are important regulators in tissue damage, were also examined. Finally, gene expression was quantified for IL-6, IL-1β, TLR4, tumour necrosis factor alpha (TNF-α) and NF-κB using RT-PCR. If these TLR4 antagonists (when administered rapidly) can block TLR4 during early reperfusion then it is expected that infarct size and myocardial inflammation will be reduced.

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XVI: Statements of authorships (chapter 9)

Title of Paper	TLR4 antagonists, (+)-naloxone and TAK-242, limits infarct size through the suppression of caspase-3, MMP-9, and myocardial inflammation.
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Study 4 examined whether (+)-naloxone or TAK242 can suppress infarct size and myocardial inflammation using an in-vivo infarct model. Heart were stained for infarct size. Left ventricles were harvested to quantify the activity of damage markers, caspase-3 and MMP-9. Additionally, IL-1β and NF-κB activity was also examined using western blots. Finally gene expression of TNF-α, IL-6, IL-1β, TLR4 and NF-κB was also quantified.

Principal Author

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Contribution to the Paper	Writing, proof-reading, editing, performed experiments and data analyses.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Professor David Saint		
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Name of Co-Author	Dr Kenner Rice		
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Name of Co-Author	Dr Dan Donner
Contribution to the Paper	Provided training for the in-vivo LAD rat ligation technique
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Chapter 9: TLR4 antagonists, (+)-naloxone and TAK-242, limits infarct size through the suppression of caspase-3, MMP-9, and myocardial inflammation.

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9.1: Abstract

Introduction: In recent years myocardial inflammation has drawn increasing interest as a potential therapeutic target against cardiovascular disease (CVD). Although pro-inflammatory cytokine specific blockers have been investigated in the clinical CVD setting, it is uncertain if suppressing immunosurveillance receptors would be more effective. This study investigated whether two TLR4 antagonists, (+)-naloxone and TAK242, when administered prior reperfusion, could limit myocardial infarct size and inflammation in an in-vivo infarct model.

Method: Sprague Dawley rats (300-350 g) were anaesthetized with ketamine + domitor before the left jugular vein was catheterized with polyethylene tubing for drug infusion. The left ventricle was accessed via the 5th intercostal region, the left anterior descending (LAD) artery was ligated with 5-0 proline suture for 30 minutes. 2 minutes before release of the ligature, (+)-naloxone (10 mg/ml) or TAK242 (1 mg/ml), was administered through an intrabolus injection using the infusion line. The animals were then allowed to recover for 48 hours before the heart was removed for Evan's blue infarct staining. For protein analyses, caspase-3, MMP-9, IL-1β and NF-κB activity was examined in the left ventricles using western blots (presented as 'arbitrary units'). Gene expression of inflammatory mediators (IL-1β, IL-6, TLR4, NF-κB and TNF-α) was also studied using RT-PCR (data presented as $2^{-\Delta\Delta Cq}$).

Results: (+)-Naloxone (nal) and TAK242 both suppressed infarct size when rats were treated with either drug (infarct-veh: 28.1 ± 1.1% vs nal:17.2 ± 2.3% or TAK242: 18.2 ± 2.0%) before reperfusion was restored. Capase-3 (infarct-veh: 2.6 ± 0.8 vs nal: 1.2 ± 0.10 or TAK242: 0.8 ±

0.2) and MMP-9 (infarct-veh: 2.9 ± 0.3 vs nal: 2.0 ± 0.4 or TAK242: 1.5 ± 0.2) activity was both suppressed by either TLR4 antagonist in infarct hearts ($P < 0.05$). (+)-Naloxone, but not TAK-242 (2.2 ± 0.6), suppressed the translocation of NF-κB protein from the cytosol to the nucleus (infarct-veh: 1.6 ± 0.3 vs nal: 1.0 ± 0.1) ($P < 0.05$). RT-PCR revealed that (+)-naloxone suppressed IL-1β (infarct-veh: 2.1 ± 0.5 vs nal: 0.9 ± 0.1) and TNF-α (infarct-veh: 1.4 ± 0.3 vs nal: 0.5 ± 0.1) production ($P < 0.05$). However, no statistical differences for TNF-α were observed between sham and infarct hearts (sham: 0.7 ± 0.2 vs infarct: 1.4 ± 0.3) ($P > 0.05$). TAK-242 did not influence either IL-1β or TNF-α mRNA expression ($P > 0.05$). Analysis of TLR4, IL-6, and NF-κB mRNA showed no difference between groups ($P > 0.05$).

Conclusion: (+)-Naloxone and TAK242 limited infarct size. These findings are supported by the suppression of caspase-3 and MMP-9 activity. Finally, (+)-naloxone, but not TAK242, attenuated myocardial inflammation.

9.2: Introduction

Cardiovascular disease (CVD) remains the most prevalent cause of mortality throughout the western, and now eastern, developed world. Poor diets, increased sedentary lifestyles, and a rising elderly population have all contributed to the rise in CVD [1]. Reactive oxygen species (ROS), mitochondria permeability transitional pore (mPTPs) formation, and intracellular Ca^{2+} overload are all well-known factors which promote early reperfusion injury [2]. Because infarct size is directly proportional to the area and duration of ischemia, immediate surgical or pharmacological intervention is crucial in limiting reperfusion injury. Over the past decade, inflammation has drawn increasing interest as a potential therapeutic target against CVD [3,4]. Myocardial inflammation can be triggered when endogenous proteins are released, or secreted, into the extracellular environment [5,6]. Defined as danger associated molecular pattern molecules (DAMPs), these proteins bind onto immunosurveillance receptors and thereby trigger innate immune signalling [5,7,8]. Depending on the receptor which is triggered, signalling kinases such as the mitogen activated proteins kinase (MAPK) family enhance ischaemic-reperfusion injury by promoting cell death signalling and activation of pro-inflammatory mediators. This triggers the production / release of pro-inflammatory cytokines such as the interleukin family [such as interleukin 1 beta (Il-1β), interleukin 6 (IL-6) or interleukin 8 (IL-8)] or tumour necrosis factor-alpha (TNF-α) [3],[9]. The damage caused by myocardial inflammation during the early stages of reperfusion can lead to a depression in contractile function, vasculopathy, and fibrosis [10],[2]. On the other hand, inflammation is also responsible for triggering wound healing and angiogenesis throughout the infarct region.

9.21: Toll-like receptor 4 (TLR4): Its role in myocardial ischaemic-reperfusion injury and TLR4 antagonists: (+)-naloxone and TAK-242.

While pharmacological treatments such as, aspirin [11] or statins [12], have been reported to suppress myocardial inflammation, no specific inflammatory blockers have been approved for clinical use [4]. This year, the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) trial [13], a phase III clinical trial examining the cardioprotective effects of Canakinumab (an IL-1β antagonist), was finally completed . Ridker *et al* [13] concluded that while Canakinumab caused a 15 % risk reduction of inheriting CVDs, overall mortality was not reduced. Considering the broad spectrum of mediators involved in the innate immune response, blockade of an upstream inflammatory signalling mediator may be more beneficial. To date, no clinical trial has been conducted examining whether the suppression of immunosurveillance receptors protects against CVDs. One of these receptors, Toll-like receptor 4 (TLR4), is well-known to trigger myocardial inflammation during ischaemic reperfusion injury [8,14–19]. Although its main role is to detect gram negative bacteria by recognising lipopolysaccharide (LPS), TLR4 can also recognise DAMPs [20,21] through the recognition of conserved molecular patterns. Myocardial studies have shown that high mobility group box one (HMGB1) [6,7,22], heat shock protein 60 (HSP60) [23,24], and multiple members of the S100 family [6,25] are recognised by TLR4 and trigger inflammation. Unlike its family members, TLR4 has two distinct signalling networks which allows it to regulate innate and adaptive immunity. When the TLR4 receptor complex recognises a ligand, the MyD88 dependent pathway is triggered leading to the phosphorylation of members of the MAPK family; p38, JNK (c-Jun N-terminal kinases) and ERK 1/2 (extracellular signal-regulated kinases 1/2) [20,26]. MyD88 signalling also activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), an important inflammatory transcription factor

[15,18,21,27]. The translocation of NF-κB into the nucleus triggers the de-novo synthesis of interleukin 6 (IL-6) [23,28], tumour necrosis factor-alpha (TNF-α) [14,29] and interleukin 1 beta (IL-1β) [3,28]. This phase of inflammation is known as innate immunity and is defined as a rapid, non-specific, inflammatory response. Aside from enhancing inflammation, specific pro-inflammatory cytokines have been shown to promote cell death. TNF-α, for example, binds onto tumour necrosis factor receptor 1 (TNFR1) triggering apoptosis through the cell death effector caspase-3 [30],[31]. TLR4 also promotes the release of chemokines [3,32] and adhesion molecules [33], which recruits white blood cells, such as neutrophils [17,33,34] or macrophages [3,32], into the ischaemic myocardium. While macrophages phagocytose apoptotic bodies and DAMPs, neutrophils releases collagenases, such as matrix-metalloproteinases-9 (MMP-9), which degrades the extracellular matrix [35,36]. MMP-9 can be particularly harmful within the ischemic myocardium and increases the risk of ventricular rupture and depressed contractile function [37,38]. Finally, TLR4's alternative network, the TRIF pathway, is involved in the simultaneous resolution of innate immunity and activation of adaptive immunity [26]. Emerging evidence suggests that lymphocytes, another class of white blood cells, are recruited into the myocardium through adaptive immune signalling during reperfusion. Specifics subsets of T / B cells are recruited into the ischaemic myocardium and either exacerbate or protect against tissue damage [39–41]. Currently, TLR4's role in the recruitment of these lymphocytes during myocardial ischaemic-reperfusion injury remains poorly defined.

Though TLR4 genetic knockout studies [16,17,23,42] have shown that the suppression of TLR4 reduces infarct size, improves survival rates, and suppresses myocardial inflammation, the

pharmacological evidence is limited [18]. Shimamoto *et al* [18] reported in an in-vivo infarct model that the infusion of Eritoran (a TLR4 antagonist), prior to ischaemic insult, reduced infarct size after 2 hours of reperfusion. Suppression of TLR4 signalling was confirmed by reduced NF-κB nuclear translocation and synthesis of TNF-α, IL-1β and IL-6 mRNA production [18]. While these findings are promising, pretreating animals with a pharmacological antagonist does not replicate clinical conditions as myocardial infarctions cannot be readily predicted. Furthermore, in the context of myocardial inflammation, the short reperfusion protocol used by Shimamoto *et al* [18] meant that the full spectrum of inflammation was not properly explored. This study examined whether (+)-naloxone and TAK242 (or resatorvid), two TLR4 antagonists, could limit infarct size and myocardial inflammation in a left anterior descending artery (LAD) ligation and recovery model. To replicate clinical conditions, either compound was infused during the ligation period before reperfusion was restored.

9.22: (+)-Naloxone and TAK242

Naloxone is a mu-opioid receptor antagonist commonly used to treat alcohol and opioid addiction [43]. While negative and positive stereoisomers exist, (+)-naloxone does not suppress opioid receptor signalling [43,44]. In the late 2000s, Hutchinson *et al* [44] discovered that naloxone, regardless of the stereoisomer, suppressed IL-1β and IL-6 mRNA production in LPS treated rats. Prior to Hutchinson's *et al* [44] findings, researchers examined the opioid blockade effects of (-)-naloxone in myocardial ischaemic-preconditioning [45,46]. During the 1990s, the cardioprotection mediated through ischaemic-preconditioning was hypothesized to be partially mediated through the stimulation of opioid receptors [46,47]. By administrating (-)-naloxone, the cardioprotective effects of ischaemic-preconditioning was abolished [45,46,48]. While this blockade of ischaemic-preconditioning was hypothesized to

be due to the suppression of opioid receptors, authors at the time were unaware of (-)-naloxone's TLR4 antagonistic properties. Although this study reported herein did not investigate ischaemic-preconditioning, (+)-naloxone was selected to avoid any potential conflicting variables. Furthermore, it is still uncertain as to whether (+)-naloxone suppresses MyD88 or TRIF induced signalling. Recently, Wang *et al* [49] reported in BV-2 cells that (+)-naloxone blocked the production of TRIF, but not MyD88, dependent markers. Interferon gamma (IFN-γ) and interferon regulatory factor 3 (IRF-3) were reported to be attenuated by (+)-naloxone. Whether (+)-naloxone suppresses TRIF induced signalling and influences in-vivo infarct size is unknown. The other TLR4 antagonist which was examined in the present study is TAK242 (resatorvid), a more recently developed inflammatory blocker. TAK242 has been reported to block upstream adaptor proteins, TIRAP [toll-interleukin 1 receptor (TIR) domain containing adaptor protein] and TRAM (TRIF-related adaptor molecule), from interacting with TLR4 [27,50,51]. This causes the suppression of MyD88 and TRIF signalling and thus abolishes TLR4 signalling. Although TAK-242 has been examined in several pathological models of injury, it remains to be seen as to whether this TLR4 antagonist limits infarct size in a myocardial in-vivo infarct model [52,53]. To determine if either compound is cardioprotective, hearts were stained for infarct size. Protein analysis of caspase-3 and MMP-9, both mediators of tissue damage, in the left ventricular was examined. Furthermore, inflammation protein markers were quantified for NF-κB nuclear translocation and IL-1β activity. Using RT-PCR, TLR4, NF-κB, IL-6, IL-1β, TNF-α gene expression was also measured.

9.31: Methods

9.32: Animal ethics

Sprague Dawley male rats (300-400g) were selected for this study. Animals were given ad libitum access to food and water prior to and after surgery. The project was approved by the University of Adelaide's animal ethics committee, approval number: M-2016-039b.

9.33: Procedure set up and right jugular cannulation

Rats were anaesthetized with ketamine (100 mg/kg) and domitor (0.5 mg/kg) followed by the paw / reflex test to confirm anaesthesia. Animals were then shaved at the sites of surgery, intubated, and ventilated at a rate of 55 BPM with a volume of 4.0 cm^3. All animals were ventilated with humidified medical grade oxygen while body temperature was maintained at 37^oC with a heat mat throughout surgery. The shaved neckline was sterilized with 70% ethanol + betadine before blunt dissection was performed to isolate the right jugular vein. The vein was then cannulated with polyethylene tubing connected to a heparinized saline filled syringe which was attached to a syringe pump (Harvard Apparatus, Massachusetts, US). The surgical site was covered with a saline soaked gauze. Holter electrodes were placed onto the fore and hindlegs to monitor ECG and BPM using a Powerlab 4/20T (ADinstruments, NSW, Australia) in conjunction with Labchart 5 (ADinstruments, NSW, Australia).

9.34: Left anterior descending artery ligation

Once the surgical area was swabbed in 70% ethanol + betadine, the heart was accessed via the 5th intercostal region. The pericardium was then removed, and the LAD was ligated with

5-0 prolene suture (Ethicon, NSW, Australia). Fine tubing was then threaded between both ends of the suture and held in place with needle holders to compress the LAD. Ligation was confirmed by cyanosis of the left ventricle and increased QRS complexes. The surgical site was covered in saline soaked gauzes and the ligation was maintained for 30 minutes. 2 minutes prior to reperfusion, an intravenous bolus injection of (+)-naloxone (10 mg/ml) or TAK242 (1 mg/ml) (both diluted in saline) was infused through the jugular vein. Another 0.2 ml of saline was then infused through the line to ensure complete infusion of the drug. Upon the 30th minute, the ligation was released, and the ribcage / surgical site was closed with 3-0 silk suture. The prolene suture used for the ligation was left in the ventricle for infarct staining. A 50-ml syringe connected to fine tubing was used to drain the cavity of any fluid and to restore negative pressure within the chest cavity. Surgical sites were swabbed with betadine and each animal was given 10 mg / kg of Baytril. Domitor was reversed by the administration of antisedan before the rat was returned to its cage. Buprenorphine was administered (50 µg/kg) every 24 hours. After 48 hrs the hearts were either stained for infarct size or flash frozen for molecular work.

9.35: Infarct stain

Bohl *et al* [54] infarct staining protocol was adapted for this study with several modifications. Post-surgical rats were anaesthetized with ketamine (100 mg/kg) and domitor (0.5 mg/kg) before cardiac punctures were performed for blood collection. The hearts were then rapidly excised and submerged into ice cold saline to reduce metabolic activity. Upon successful cannulation of the ascending aorta, Krebs Henseleit buffer (Recipe: 130 mM NaCl, 4 mM KCl, 0.6 mM MgCl, 24 mM $NaHCO_3$, 24 mM NaH_2PO_4, 12 mM D-Glucose, 1 M of $CaCl_2$, heated at

37˚C, and gassed with carbogen) was perfused into the hearts using a constant flow langendorff system. Once all intracavitary / coronary blood was cleared, the LAD was re-ligated and stained with a 1 ml infusion of 0.1% Evan's blue (diluted in saline). Hearts were then removed from the apparatus, dried, wrapped in gladwrap, and placed into a -20^{o}C freezer for 1 hour. The semi frozen tissue was then sectioned off into 1-2 mm slices, allowed to thaw, and incubated at 37^{o}C in 1% Triphenyl tetrazolium chloride (diluted in saline). Once dried, the slices were immersed in 10% formalin at RT for 20 minutes. Excess formalin was blotted from the slices and they were scanned with a portable scanner. Heart slices were scanned with a HP1315 commercial scanner (HP, California, USA) at 2400 dpi. Image analysis was performed using ImageJ (National Institutes of Health, Maryland, USA) to measure area of necrosis (AON) which was expressed as a percentage of area at risk (AAR).

9.36: Tissue protein / RNA homogenization

Protein isolation was performed using the cell fractionation protocol described by Baghirova *et al* [55] with several modifications. Frozen heart samples were crushed initially under a heavy weight before being pulverised in a mortar and pestle containing liquid nitrogen. Equal samples were transferred between two tubes containing either protein lysis buffer A (recipe described by Baghirova *et al* [55]) or RNA homogenization buffer from a 16 RNA LEV Promega kit (Promega, NSW, Australia). Tissue samples designated for protein quantification were homogenized using an Ystral E-X10 (Ystral Asia, Singapore) homogenizer for 3 × 10 seconds bouts on ice. For RNA isolation, the tissue samples were sonicated using a Labsonic 1510 sonicator (Sartorius, Goettingen, Germany) for 3 × 10 seconds bouts on ice. Cytosolic, membrane and nuclear protein fractions were isolated according to Baghirova *et al* [55]. Promega's 16 RNA LEV Promega kit was used according to the manufacturer's instructions.

RNA samples were then processed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Thermofisher Scientific, SA, Australia) in conjunction with a MJ Research PTC-200 Thermal Cycler (MJ Research, Quebec, Canada). All protein and cDNA samples were stored in a -80^{o} freezer.

9.37: Western blots

Protein samples were quantified using a Pierce BCA protein assay kit (Thermofisher Scientific, SA, Australia). Depending on whether the samples tested were cytosolic or nucleus, the respective lysis buffers used to isolate the fraction was used as a diluent. 5 X Sample buffer recipe (Tris - 1.9 g, glycerol - 25 mls, DTT - 1.95g, SDS - 5.75g, 25 mls dH_2O, pH to 6.8) was used for sample preparation. Prior to gel electrophoresis samples were heated at 85^{o}C for 5 minutes. Gel electrophoresis and gel transfer was performed using mini gel tank and blot module sets (Thermofisher Scientific, SA, Australia). 25 µl of samples (at 1 µg /µl) were then loaded into either 4-12% or 12% Bis-Tris BOLT 12 well gels (Thermofisher Scientific, SA, Australia). Electrophoresis was run initially at 180 volts for 15 mins followed by 30-35 mins at 200 volts at 4^{o}C. Gels were then transferred onto nitrocellulose membranes at 20 V for 1 hr, RT. Membranes were then blocked with 5% skim milk diluted in tris-based saline (TBS) for 2 hrs followed by an overnight incubation (at 4^{o}C) with the following antibodies: Housekeepers: rabbit alpha tubulin (ab4074, 1:5000) or rabbit TATA TBP (ab63766, 1:1000), targets: rabbit caspase-3 (ab4074, 1:1000), rabbit IL-1β (ab9722, 1:1000), rabbit MMP-9 (ab76003, 1:1000) or rabbit NF-κB (ab7970, 1:1000) (Abcam, Victoria, Australia). Membranes were then washed with TBS + Tween 0.1% (TBS.T) and incubated with DonkeynRabbit 680 for 1hr, RT, in the dark (LCR-925-68073, 1: 10,000) (Licor, Nebraska, U.S). After multiple TBS.T washes, membranes

were read on a Licor Odyssey scanner (Li-cor, Lincoln, USA) at either 700 or 800 λ and quantified using ImageJ (National Institutes of Health, Maryland, USA).

9.38: RT-PCR

ITaq Universal SYBR green mix (Biorad, NSW, Australia) was used to quantify cDNA samples through RT-PCR. Primers were designed through NCBI's primer design tool (NCBI, Bethesda, USA) and produced through Integrated DNA technologies (Integrated DNA technologies, Iowa, USA) (table 1). Target gene primers designed: TLR4, NF-κB, IL-6, IL-1β, TNF-α, Rpl13a (table 1). Samples were placed into Hard-Shell® 96-Well PCR Plates (Biorad, NSW, Australia) at a final concentration of 25 ng (8 µl per well) with working primers (forward and reverse both a 1 µl) at concentrations of 300 nm. 10 µl of iTaq was added into each well at a 1:1 ratio. Plates were placed onto a plate shaker for 30 seconds, centrifuged for 20 seconds and placed into a C1000 + CFX96 RT Biorad PCR system (Biorad, NSW, Australia).

Name	**NCBI Ref Sequence**	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
NF-κB	XM_006233360.3	GGACAACTATGAGATGAGCTCCG	ATCTCCAGTGAGGGACTCCG
IL-1β	NM_031512.2	TTGAGTCTGCACAGTTCCCC	TGTCCCGACCATTGCTGTTT
IL-6	NM_012589.2	CACTTCACAAGTCGGAGGCT	TCTGACAGTGCATCATCGCT
TLR4	NM_019178.1	TGGCAGTTTCTGAGTAGCCG	TCCCACTCGAGGTAGGTGTT
TNF-α	XM_008772775.2	GGAGGGAGAACAGCAACTCC	GCCAGTGTATGAGAGGGACG

Table 1: Primer designs of inflammatory markers.

9.39: Data analyses

All data was formatted into Microsoft Excel (Microsoft, Washington, USA) and analysed through R-64 (R: A Language and Environment for Statistical Computing, 2016, Austria) in tandem with Rstudio (Rstudio, Massachusetts, USA). Multiway ANOVAs with post-hoc TukeyHSDs were constructed for this study. Infarct size was analysed as infarct/AAR per heart. For western blots, the relative expression of caspase-3, MMP-9 and IL-1β in the cytosolic fraction was compared to the house-keeper alpha tubulin. NF-κB protein levels were analysed in both the cytosolic and nuclear tissue fractionate; TATA TBP was used as the nuclear housekeeper for nuclear fractions. To control for intergroup variability, cytosolic NF-κB was divided by the respective nuclear NF-κB value for each sample. For quantifying gene expression, the Livak method was used to analyse all pro-inflammatory mediators. Data was presented as $2^{-\Delta\Delta Cq}$ after controlled by the housekeeping gene (Rpl13a) and the mastermix sample. Data is presented as mean ± SEM, $p < 0.05$ was defined as statistical significance. Graphs were produced through Graphpad Prism (Graphpad Software Inc, California, USA).

9.4: Results

9.41: (+)-Naloxone and TAK242 reduced infarct size

Sham hearts, from visual inspection, revealed that without ligating the LAD, the procedure itself did not contribute to infarct size (figure 1b). One-way ANOVA revealed that infusion of either TAK242 or (+)-naloxone prior to reperfusion, suppressed infarct size ($p < 0.05$) (figure 1a). Compared to infarct vehicle hearts (28.1 ± 1.14%) there was 40% reduction in infarct size compared to (+)-naloxone treated hearts (17.2 ± 2.25%) (figure 1b) ($P < 0.05$). TAK242 also limited infarct size with a 35% reduction (18.2 ± 1.95%) when again compared to infarct

vehicle hearts (P < 0.05). No statistical difference was observed between (+)-naloxone and TAK242 treated hearts (p > 0.05). No differences were observed between groups for either AOR or AON when examined independently (p > 0.05)

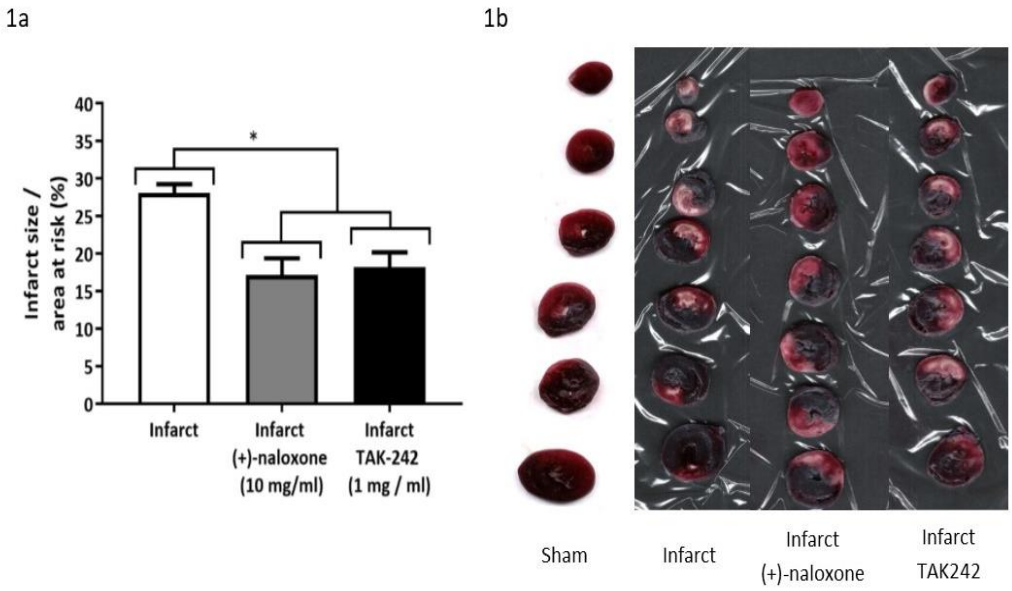


Figure 1: 1a) Infarct size / area of risk measurements of hearts treated with either (+)-naloxone (10 mg / ml) or TAK242 (1 mg / ml). 1b) Representative images of infarct sizes of rat hearts collected 48 hours after ligation of the left anterior descending artery. Data presented as mean ± SEM, * =, P < 0.05, n = 6.

9.42: (+)-Naloxone and TAK-242 suppresses caspase-3 activation in hearts exposed to myocardial infarctions.

Caspase-3 is a pro-apoptotic factor which requires cleavage for activation. Both full and cleaved forms (the active form) of caspase-3 were examined (figure 2). Although ligation of the LAD, irrespective of treatment, reduced the levels of full capase-3, no differences were

observed between sham and infarct hearts [full caspase-3: sham: 1.90 ± 0.57 vs infarct-veh: 1.23 ± 0.29, (P > 0.05)] (figure 2a). However, a treatment effect was observed revealing that TAK242 (0.43 ± 0.10), but not (+)-naloxone (0.72 ± 0.23), reduced full caspase-3 expression compared to infarct vehicle hearts (P < 0.05). When the cleaved form of caspase-3 was examined, an infarct and treatment effect was observed (P < 0.05) (figure 2b). Infarct vehicle (2.56 ± 0.75) treated hearts had significantly higher levels of active caspase-3 when compared to sham hearts (1.51 ± 0.18) suggesting increased cell death (P < 0.05). Caspase-3 activation was significantly reduced in infarct hearts when TAK-242 (0.84 ± 0.15) or (+)-naloxone (1.21 ± 0.09) was administered (P <0.05). These findings suggest that both compounds limited apoptotic cell death.

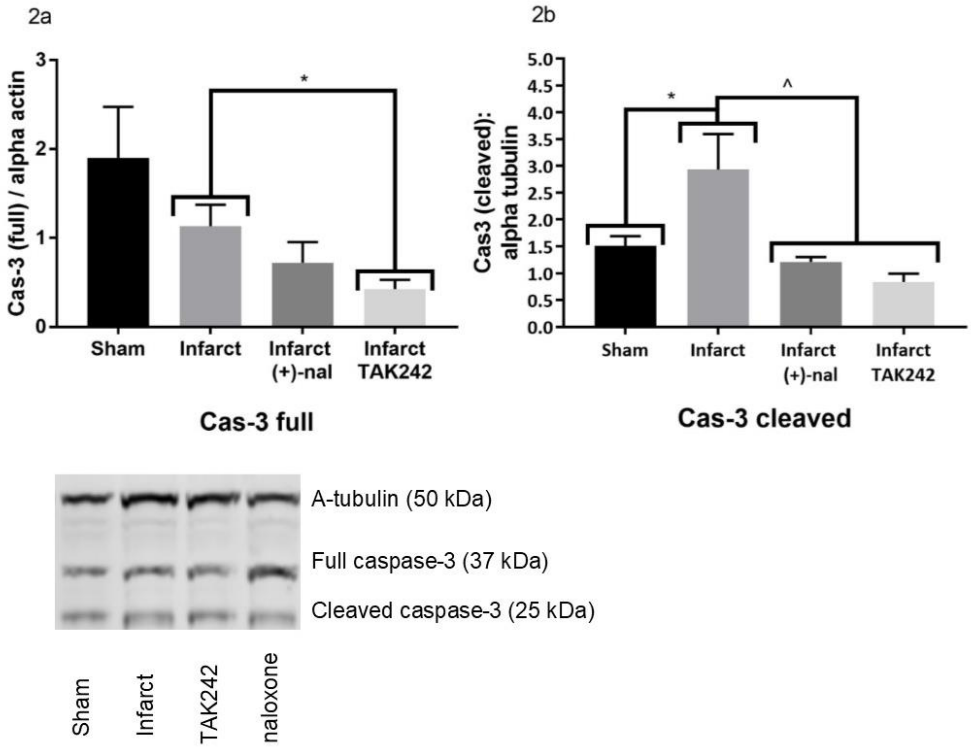


Figure 2: Protein analysis of caspase-3 activity. 2a) Caspase-3 full. 2b) Caspase-3 cleaved (active form). * = represent statistical differences observed between sham vs infarct, ^ = represents infarct vs drug treatment ($P < 0.05$). Data presented as mean ± SEM, n=6.

9.43: (+)-Naloxone suppressed the translocation of NF-κB in the nucleus of hearts exposed to myocardial infarctions.

NF-κB is an important pro-inflammatory transcription factor which influences both innate and adaptive immunity (figure 3). Activation of NF-κB from the cytosol causes it to enter the nucleus and begin the de-novo synthesis of inflammatory mediators. This study investigated both cytosolic and nuclear content of NF-κB to determine whether the inflammatory marker was influenced by either compound. Examination of the cytosolic fraction revealed no differences between sham and infarct treated groups ($P > 0.05$) (figure 3a). Furthermore, when the nuclear fraction was examined, no infarct or treatment effect was observed (figure 3b). Infarct + (+)-naloxone treated hearts (1.00 ± 0.09) had lower levels of NF-κB compared to infarct vehicles (1.39 ± 0.57), no statistical significance was reported ($P > 0.05$) (figure 3b). TAK242 in infarct treated hearts (1.92 ± 0.35) also showed that NF-κB nuclear translocation was not suppressed ($P > 0.05$). To control for intergroup variability, this study then examined the ratio between NF-κB cytosolic: nuclear content (figure 3c). Analysing this ratio revealed an infarct and treatment effect ($P < 0.05$). When hearts were exposed to myocardial infarctions the proportion of NF-κB translocation (1.63 ± 0.27) was significantly higher compared to sham hearts (0.91 ± 0.05) ($P > 0.05$) (figure 3c). Although the administration of (+)-naloxone suppressed NF-κB cytosolic to nuclear translocation (1.03 ± 0.10) ($P < 0.05$), no changes were observed in TAK242 treated hearts (2.16 ± 0.59) ($P < 0.05$).

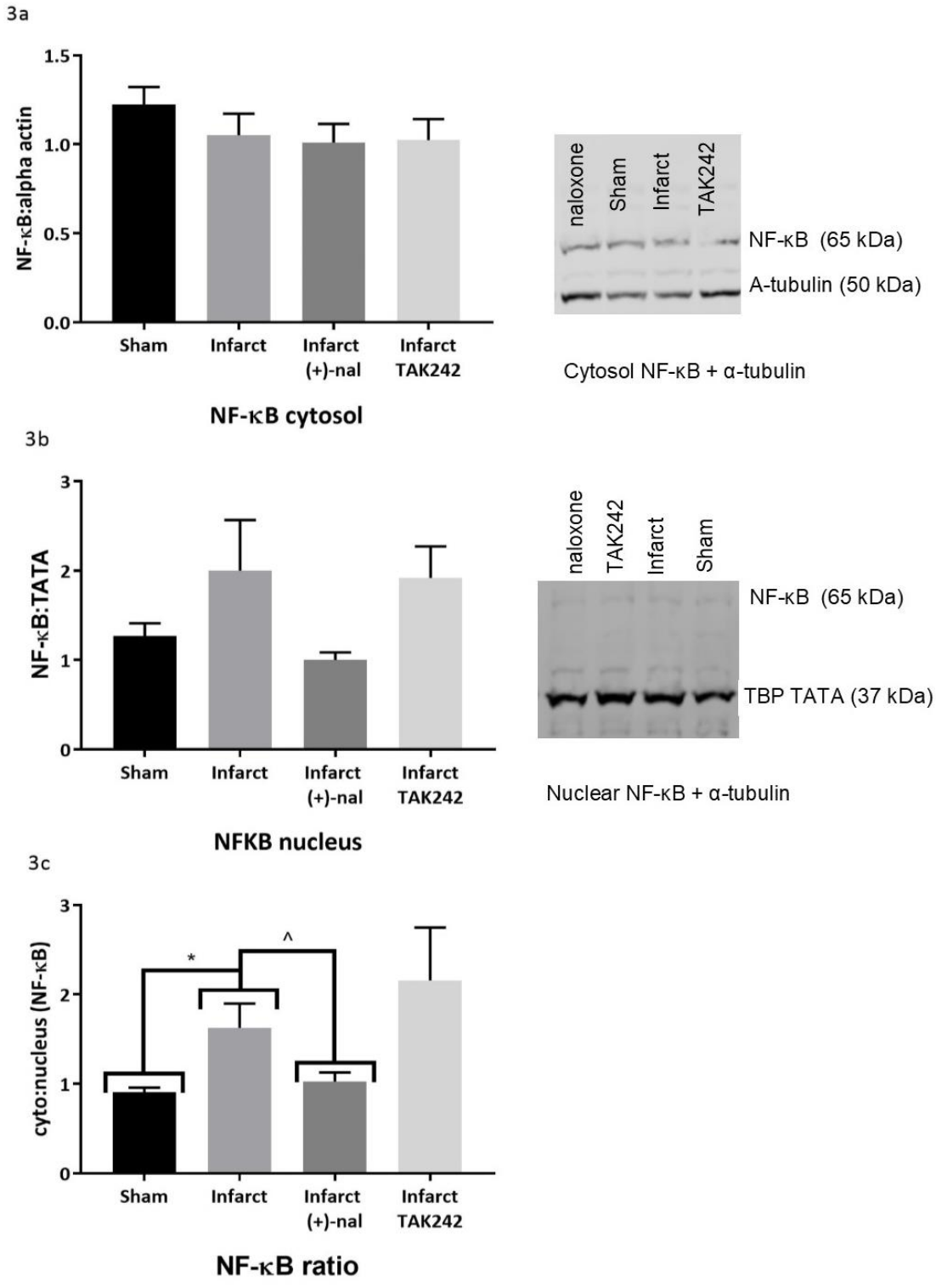


Figure 3: Protein analysis of NF-κB translocation; 3a) NF-κB cytosolic content, 3b) NF-κB nuclear content, 3c) proportion of cytosolic: nuclear NF-κB translocation. * = represent statistical differences

observed between sham vs infarct, ^ = represents statistical significance between infarct vs drug treatment ($P < 0.05$). Data presented as mean ± SEM, n=6.

9.44: (+)-Naloxone and TAK242 suppresses the activation of MMP-9

Residing in the cytosol, the full form of MMP-9 (or pro MMP-9) requires cleavage in order to activate the collagenase. Additionally, the cleavage of MMP-9 is known to produce two active forms at 65 or 82 kDA (figure 4). While western blot runs could detect pro MMP-9 and MMP-9-65 kDA, the 82 kDA isoform was not detected. Two-way ANOVA showed that myocardial infarctions and drug treatments did not influence protein expression of pro MMP-9 ($P > 0.05$) (figure 4a). Compared to sham hearts (0.85 ± 0.33), pro MMP-9 levels were increased in all groups given myocardial infarctions [infarct: 1.75 ± 0.56, infarct + (+)-naloxone: 2.16 ± 0.41, infarct + TAK242: 2.37 ± 0.81]; however, no statistical differences were observed between groups ($P > 0.05$). When the active form of MMP-9 (65 kDa) was examined, an infarct and treatment effect was apparent ($P < 0.05$) (figure 4b). Interestingly, sham hearts had higher levels of active MMP-9 (5.33 ± 1.13) when compared to infarct treated hearts (2.96 ± 0.31, $P < 0.05$). However, the administration of (+)-naloxone (1.97 ± 0.40) or TAK242 (1.46 ± 0.22) both reduced active MMP-9 65 kDa levels when compared to infarct vehicle hearts, $P < 0.05$).

9.45: Heart with myocardial infarctions had reduced pro-IL-1β levels compared to shams

The cytosolic levels of the inactive (pro-1L-1β) and active form (cleaved-IL-1β) of IL-1β was examined (figure 5). Two-way ANOVA showed that regardless of treatment, pro-1L-1β was reduced in hearts given myocardial infarctions (sham: 2.58 ± 0.35 vs infarct: 1.76 ± 0.28,

infarct + (+)-naloxone: 1.10 ± 0.31, infarct + TAK242: 1.28 ± 0.49) (P < 0.05) (figure 5a). Between hearts exposed to myocardial infarctions, neither (+)-naloxone or TAK242 influenced pro-IL-1β levels (P > 0.05). Differences between sham (1.45 ± 0.34) and infarct treated hearts (1.60 ± 0.31) was lost when the cleaved form of IL-1β was examined (P > 0.05) (figure 5b). Finally, no differences were observed for (+)-naloxone (1.17 ± 0.21) and TAK-242 treated hearts compared to infarct vehicles (1.70 ± 0.58) (P > 0.05).

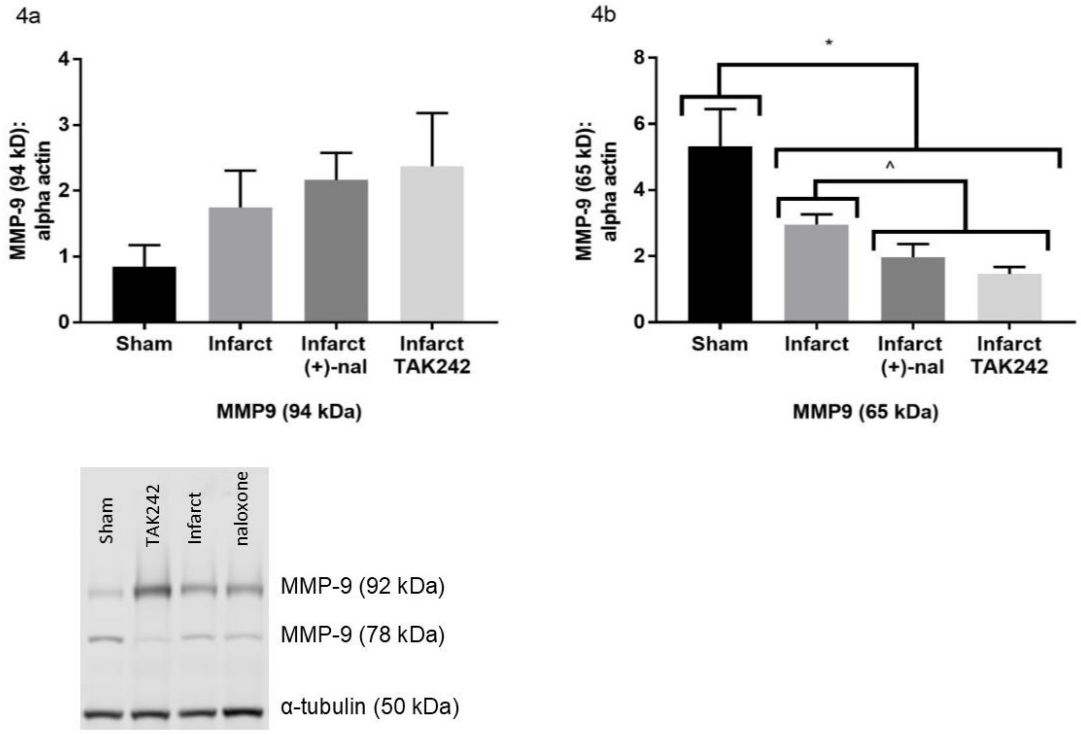


Figure 4: Protein analysis of MMP-9 4a) pro MMP-9 (95 kDa) and 4b) MMP-9 active (65 kDa) form. * = represent statistical differences observed between sham vs infarct, ^ = represents statistical significance between infarct vs drug treatment (P < 0.05). Data presented as mean ± SEM, n=6.

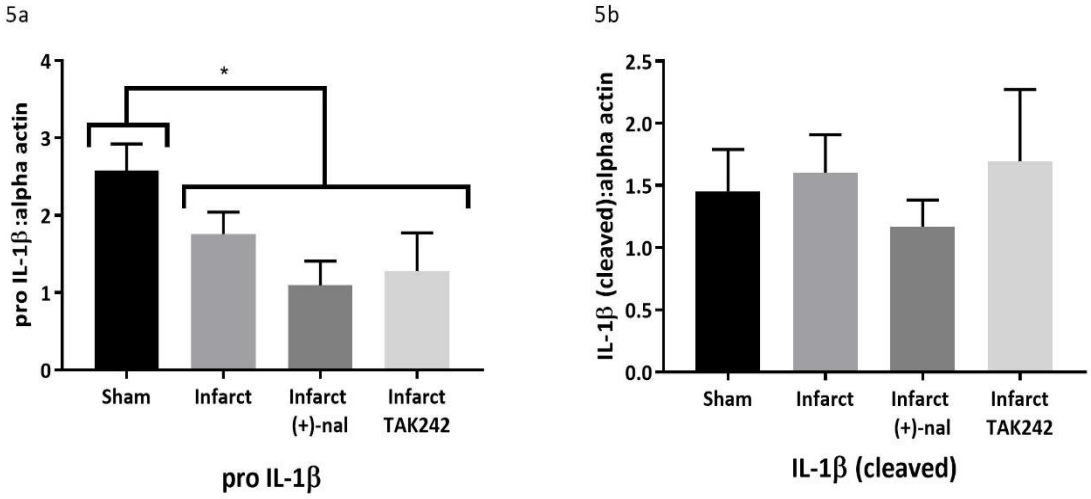


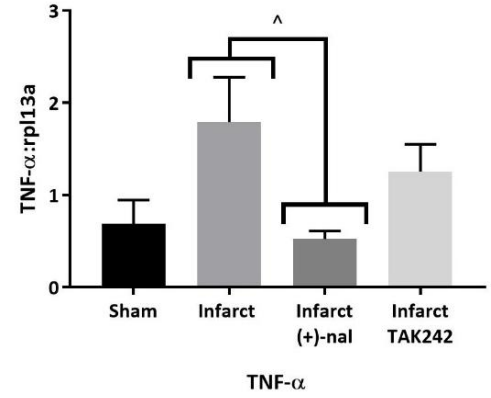
Figure 5: Protein analysis of IL-1β 5a) pro IL-1β and 5b) IL-1β cleaved (active). * = represents statistical differences observed between sham vs infarct. Data presented as mean ± SEM, n=6.

9.46: (+)-Naloxone, but not TAK242, suppresses TNF-α and IL-1β mRNA production

Gene expression of inflammatory markers, TNF-α, NF-κB, IL-1β, IL-6 and TLR4, was also examined in this study (figure 6). Two-way ANOVAs observed interactive effects for TNF-α and IL-1β ($P < 0.05$). A treatment, but no infarct, effect was observed for TNF-α ($P > 0.05$) (figure 6a). While TNF-α levels were higher in infarct hearts (1.39 ± 0.27) compared to shams (0.77 ± 0.18) statistical significance was not achieved ($P > 0.05$). When drug treatments were compared, post-hoc TukeyHSD showed that (+)-naloxone (0.52 ± 0.09), but not TAK242 (1.32 ± 0.24), significantly limited TNF-α gene expression ($P < 0.05$). IL-1β was also reported to have increased in hearts exposed to myocardial infarctions (2.07 ± 0.46) compared to the sham group (0.74 ± 0.08) (figure 6b). Again (+)-Naloxone treated hearts were observed to have suppressed IL-1β activity (0.93 ± 0.14) when compared to infarct vehicle hearts ($P < 0.05$). While TAK242 (1.76 ± 0.37) also reduced IL-1β gene expression statistical significance was not

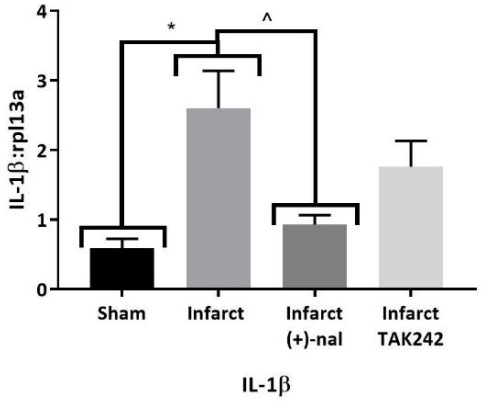
achieved (P > 0.05). Two-way ANOVA for TLR4, NF-κB and IL-6 did not show statistical significance for either infarct or drug treatments (P > 0.05) (figure 6c-6e).

6a



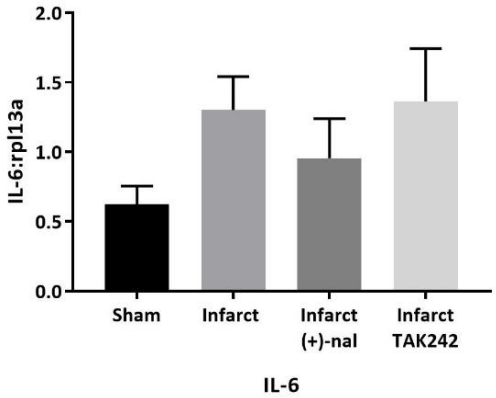
TNF-α

6b



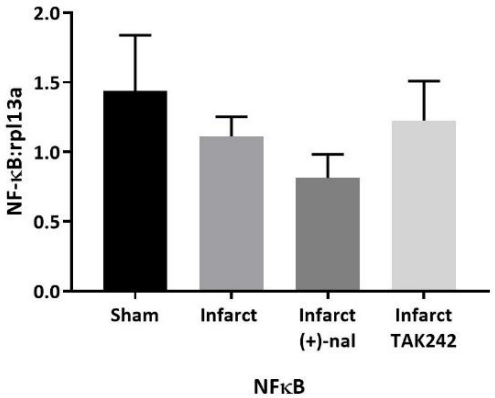
IL-1β

6c



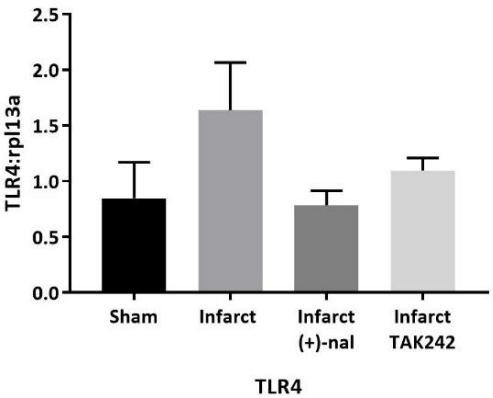
IL-6

6d



NFκB

6e



TLR4

Figure 6: Gene expression of inflammatory markers; 6a) TNF-α, 6b) IL-1β, 6c) IL-6, 6d) NF-κB and 6e) TLR4. * = represents statistical differences observed between sham vs infarct, ^ = represents statistical significance between infarct vs drug treatment ($P < 0.05$). Data presented as mean ± SEM, n=6.

9.5: Discussion

Myocardial inflammation has drawn increasing interest as a potential therapeutic target against ischemic-reperfusion injury. Cytokine specific blockers such as Canakinumab, Methotrexate, and Colchicine, have shown promising results and are currently in CVD clinical trials [4]. Recently, the Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS) study, a large scale phase III clinical trial examining the benefits of using an IL-1β blocker in patients suffering from CVDs, was completed [13]. Although, Ridker *et al* [13] findings concluded that Canakinumab significantly reduced the risk of CVDs by 15%, mortality was not reduced. While these findings are promising, innate immunity is mediated by a broad range of inflammatory mediators and may explain why Canakinumab did not improve mortality rates. Furthermore, it is still uncertain as to whether blockade of multiple inflammatory mediators is more beneficial in treating CVD. This study examined whether blockade of the immunosurveillance receptor, TLR4, could limit infarct size in rat hearts exposed to myocardial infarctions. Although Shimamoto *et al* [18] reported that the pharmacological inhibition of TLR4 protects against ischaemic-reperfusion injury, the antagonist was administered before ischaemic insult. To replicate a more clinically relevant setting, this study investigated whether TLR4 antagonists, (+)-naloxone or TAK-242, could limit infarct size when administered during the ischaemic phase immediately prior to reperfusion. (+)-Naloxone, a TLR4 antagonist, has been previously studied in our lab to

determine whether the compound has any beneficial properties in the cardiovascular setting (see study 3). Because of its ability to suppress mu-opioid receptors (-)-naloxone [46,48,56] is normally used to treat opioid and alcohol addiction. While (-)-naloxone is also known to suppress TLR4, (+)-naloxone, which cannot suppress mu-opioid receptor signalling [45,46], was selected for this study to avoid conflicting variables. To date, it is still uncertain if the TLR4 suppressive effects of (+)-naloxone is mediated through MyD88 or TRIF dependent signalling [44,49]. TAK242 is a more recently developed TLR4 antagonist reported to suppress both MyD88 and TRIF dependent pathways [27,50]. Though TAK242 has been reported to be cardioprotective [52], there is still no evidence to suggest that it limits infarct size. Aside from staining for infarct size, caspase-3, an apoptotic cell death protein, was also quantified using western blot analyses. MMP-9, a collagenase involved in tissue damage and angiogenesis [36], was also examined. Finally, to measure myocardial inflammation, protein and gene expression of inflammatory markers such as IL-1β, IL-6, TNF-α, NF-κB and TLR4 was examined.

9.51: (+)-Naloxone and TAK242 limits the development of infarct size and suppresses caspase-3 activity.

The findings from this study report that (+)-naloxone and TAK242 both limit infarct size and suppressed caspase-3 activity. To our knowledge, this is the first study to report the cardioprotective benefits of these TLR4 antagonists in an in-vivo infarct model. This study also showed that the rapid administration of either TLR4 blocker, prior reperfusion, can still reduce infarct size. These findings are promising and suggest that either compound could be used in the surgical setting where early pharmacological intervention is required to minimize ischaemic-reperfusion injury. Considering that the hearts were harvested 48 hours after

ischaemic insult, it would be interesting to see whether the TLR4 blockers minimized acute reperfusion injury. Collecting blood samples (for cardiac biomarkers) after reperfusion or measuring the hemodynamics could answer this question without sacrificing the animal. Alternatively, hearts could be examined 1-2 hours after reperfusion to quantify MAPK activity, neutrophil recruitment, or ROS levels all of which are enhanced during the acute phase of reperfusion [2,3]. Wang *et al* [49] recently reported that (+)-naloxone can suppress ROS and nitric oxide (NO) levels in LPS stimulated BV-2s. Known to contribute to cellular damage, high levels of ROS released during reperfusion enhance early reperfusion injury [57]. While not a direct mediator of cellular damage, NO is known to be converted to the highly reactive ROS, peroxynitrite, under conditions of ischaemia and reperfusion [58,59]. Hence examining whether (+)-naloxone, or perhaps even TAK242, can limit ROS production during early reperfusion is another potential target in understanding TLR4's role in reperfusion injury.

This study also reported that both compounds suppressed cytosolic caspase-3 activity. Like its family members, caspase-3 is composed of a small and large subunit which is joined through a pro-domain [31]. Cleavage of the pro-domain releases the large subunit, which is the active form of caspase-3 [60]. This activation process can be triggered through either extrinsic (by transmembrane receptor activation) or intrinsic (through intracellular signalling) pathways depending on the stimuli. In the context of TLR4, suppression of caspase-3 cleavage may have occurred through the suppression of TNF-α. Aside from its role as a pro-inflammatory marker, TNF-α can also trigger cellular apoptosis by binding onto the death receptor, TNFR1 [30]. While this study did not measure TNF-α protein levels, the suppression of caspase-3 activity in (+)-naloxone treated hearts is supported by the reduction in infarct

size and TNF-α gene expression. Interestingly, while TAK242 also limited infarct size and caspase-3 activity, TNF-α was not suppressed. Considering that caspase-3 can be activated by several different pathways [31] TAK242 may have reduced caspase-3 cleavage without suppressing innate immunity.

9.52: (+)-Naloxone but not TAK242 suppressed NF-κB nuclear translocation and IL-1β gene expression.

This study also reported that (+)-naloxone, but not TAK242, partially suppressed inflammation in hearts exposed to myocardial infarctions. The reduction in NF-κB nuclear translocation and IL-1β gene expression suggest that the infarct-limiting effects of (+)-naloxone was mediated through the suppression of TLR4 signalling. These findings are supported by previous literature which suggests that NF-κB [29], [61] and IL-1β [15,28] are upregulated in hearts exposed to myocardial infarctions. While (+)-naloxone suppressed TNF-α in infarct treated hearts, statistical significance was not achieved between infarct and sham hearts. The lack of statistical significance may be due to innate immunity resolving around 48 hours with TNF-α beginning to return to baseline [3]. This theory may explain why lower (though not statistically significant) values of IL-1β protein and IL-6, NF-κB, and TLR4 gene expression was observed in (+)-naloxone treated hearts. Examining these inflammatory markers at earlier timepoints may show a more significant difference between groups. While the anti-inflammatory effects of (+)-naloxone are promising, there is still some confusion in whether the TLR4 antagonist attenuates MyD88 or TRIF signalling. It was originally hypothesized that, because of its short half-life [48,56], (+)-naloxone's greatest potency would be observed when administered prior to reperfusion. Considering that MyD88 signalling is triggered before TRIF activity, (+)-

naloxone is likely to have attenuated MyD88 activity during the early phase of reperfusion. To test this theory, blood and hearts samples should be analysed between 1-12 hours after reperfusion. However, the findings from this study should not be taken for granted as it shows that interventional treatment with (+)-naloxone is still able to influence myocardial inflammation during the later phases of reperfusion.

The other theory is based on evidence which suggests that (+)-naloxone suppresses TLR4 in a TRIF dependent manner. As mentioned previously, Wang *et al* [49] reported that (+)-naloxone suppressed TRIF dependent inflammatory regulators IRF3 and IFN-γ. While this study did not examine these markers, NF-κB is also known to be activated during TRIF dependent signalling [21]. Recently, Chen *et al* [62] explored TLR3 and TRIF dependent signalling in genetic knockout in-vivo infarct models. TLR3, which only activates TRIF dependent signalling, is known to recognise extracellular RNA released from necrotic cells [62]. The study reported that the genetic knockout of either TLR3 or TRIF induced signalling reduced infarct size and caspase-3 activity. However, when protein and mRNA analyses of TRIF dependent inflammatory markers were examined, no differences were reported. This poses the question of whether TRIF signalling is involved in reperfusion injury and how it regulates innate immunity within the myocardium. On the other hand, it should be noted that Chen *et al* [62] did not examine whether suppression of TRIF influences B / T cell activity. Recently, multiple studies have reported that specific subsets of B [63] and T cells (i.e. regulator T cells [39,40] and Natural killer T cells [41]) have beneficial / detrimental effects on reperfusion injury . How TLR4-TRIF signalling influences these lymphocyte subsets are poorly defined and requires additional investigation.

This study also reported that while TAK242 reduced infarct size, the inflammatory markers studied were not suppressed. While TNF-α, IL-1β and TLR4 gene expression was reduced in TAK242 treated infarct hearts, statistical significance was not achieved. Again like (+)-naloxone, this may be associated to the timing in which the hearts were harvested. However, the other possible theory of why inflammation was not suppressed is because of the attenuation of TRIF signalling. As reported by Matsunaga *et al* [27], TAK242 has been shown to suppress the MyD88 and TRIF induced signalling by suppressing upstream adaptor proteins TRAM and TIRAP. Considering its regulatory role on innate and adaptive immune signalling, it may be possible that the suppression of TRIF also blocked the negative feedback on MyD88. For instance, TRIF induced signalling is known to synthesize IL-10 [64], a potent anti-inflammatory cytokine, which suppresses innate immunity. However, the fact that this study showed that TAK242 was still able to limit infarct size suggests that either 1) TAK242 suppresses non-inflammatory components involved in ischaemic-reperfusion injury, or b), that most of the damage caused through TLR4 induced signalling is through MyD88 activity. Again, collecting blood / heart samples during the early stages of reperfusion may determine whether TAK242 suppresses myocardial inflammation during the blockade of MyD88 signalling.

9.53: (+)-Naloxone and TAK242 suppresses active MMP-9 (65 kDa) levels in hearts exposed to myocardial infarctions.

Part of the matrix-metalloproteinase family, MMP-9 plays an important role in both the progression of myocardial pathogenesis [35,37,38,65] and promoting angiogenesis [36,66] . Like its family members, MMP-9 is involved in the breakdown of ECM and activation of

signalling kinases such as MAPK and PI3K / Akt [36,66]. Ducharme *et al* [67] was the first to report that the genetic knockout of MMP-9 significantly reduced the dilation of the left ventricle when mouse hearts were exposed to myocardial infarctions. The precursor of pro MMP-9 (95 kDa) resides in the cytosol and is activated when pro MMP-9 is cleaved into the active forms, 65 or 82 kDa, and released into the extracellular environment [68]. While both isoforms have similar roles, the 65 kDa derivative lacks a C-terminal domain. This difference means that TIMP metallopeptidase inhibitor 1 (TIMP-1), an inhibitor of MMP-9, does not recognise the active 65 kDa isoform [68],[69]. Aside from pro MMP-9, this study was only able to detect 65 kDa. It is unknown as to why this study was unable to detect the 82 kDa derivative though it may be related to the tissue processing protocol used [55]. Though not statistically significant, the relative increase of pro MMP-9 in all hearts exposed to myocardial infarctions, is supported by previous literature. For instance, clinical studies have reported that pro MMP-9 is upregulated in patients suffering from myocardial infarctions [36]. This is also supported by studies which have tracked MMP-9 expression in animal infarct tissue reporting an increase around 2-3 days [70,71].

While an infarct effect was observed, MMP-9 65 kDa was reported to be higher in sham groups compared to hearts treated with myocardial infarctions. Interestingly, Bellini *et al* [69] reported that MMP-9 82 and 65 kDa isoforms could be detected in the serum of healthy patients (however, pathological groups were not examined). It is unknown as to why MMP-9 65 kDa levels were expressed at higher levels in sham hearts. Although MMP-9 is active in non-pathological roles, it was hypothesized that myocardial infarctions would increase MMP-9 activity. Furthermore, sham hearts were reported to show no signs of developing myocardial infarctions, so these results require additional investigation. While MMP-9 65 kDa

levels were lower in infarct vehicle hearts compared to shams, (+)-naloxone and TAK-242 treatment still suppressed MMP-9 65 kDa activity. It is interesting to note aside from the fact that TIMP-1 cannot suppress MMP-9 65 kDa, the differences between 82 and 65 kDa remains poorly defined. Whether these isoforms have different roles in ischaemic heart disease requires additional investigation. However, from the literature that has been published, increased MMP-9 activity in the ischemic myocardium / interstitium is correlated to thinning of the left ventricular wall and increased risk of its rupture [36,37,65]. As a collagenase, MMP-9 can degrade parts of the extracellular matrix which supports the myocardium. This can be particularly detrimental in the ischaemic myocardium if the supporting extracellular matrix is damaged. Evidence suggests that MMPs can be regulated through inflammation mediators [36]. For instance, TNF-α and NF-κB have been reported to upregulate MMP-9 levels / activity in multiple cells types (neutrophils and endothelial cells) [72,73],[36]. It may be possible that the suppression of MMP-9 in this study may be correlated to (+)-naloxone's suppressive effects on innate immunity. Although this theory is supported by the changes observed in the (+)-naloxone treated hearts the same cannot be said for TAK242. Finally, it is uncertain as to whether (+)-naloxone and TAK-242 is directly acting on the heart or through white blood cells to suppress the release of MMP-9.

9.6: Conclusions

This study has shown that (+)-naloxone and TAK-242 suppressed infarct size, a predictor of ischaemic-reperfusion injury, in Sprague Dawley rats. These findings are supported by the attenuation of caspase-3 and MMP-9 activity which are upregulated in infarct hearts. Finally (+)-naloxone, but not TAK-242, was reported to suppress NF-κB nuclear translocation and IL-

1β / TNF-α gene expression. It is uncertain as to whether TAK-242's inability to suppress inflammation is associated to the experimental design (i.e. timing / dose) or because the TLR4 antagonist suppressed TRIF dependent signalling. In conclusion, this study suggests that (+)-naloxone and TAK242 could be used as potential interventional therapeutics in the treatment of myocardial infarctions.

9.7: Acknowledgements

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XVII: Statements of authorships (chapter 10)

Title of Paper	Conclusion
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Summaries of 1,2,3 and 4, limitations of each study, future experiments, clinical relevance and concluding thoughts.

Principal Author

Name of Principal Author	Mr Samuel Man lee		
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Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

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Chapter 10: Conclusion

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10.1: Summary of studies 1-4.

10.11: TLR4 may influence contractile recovery in ischaemic-preconditioned hearts; concluding thoughts on study 1 and 2's findings.

The original aim of this thesis was to examine two related questions; 1) Does ischaemic-preconditioning influence toll-like receptor 4 (TLR4) signalling and, 2) Does the transient stimulation of TLR4 trigger a preconditioning response? As discussed, in chapter 3, sub-lethal stress [(caused by either heat [1–3], lipopolysaccharide (LPS) [4,5] or ischaemia [1,6–9]] triggers a preconditioning response which protects against ischaemic-reperfusion injury. Since Murry's original discovery [8], researchers have discovered multiple molecular mechanisms which trigger classical [10–16] and delayed preconditioning [12,17,18]. Activation of opioid receptors [10,18,19], protein nitrosylation [12,17,20], and opening of mitochondrial potassium adenosine tri-phosphate (ATP) channels [15,16,21], are just several examples of mechanisms which trigger cardioprotection. However, gaps in the field still exist, especially regarding how myocardial inflammation is regulated through preconditioning. TLR4 was selected for this project because, while there is a significant amount of literature suggesting its involvement in ischaemic-reperfusion injury [22–28], its role in ischaemic-preconditioning remains poorly defined. As discussed in chapter 2, studies have reported that pretreating animals with low dosages of LPS [4,5], or danger associated molecular pattern molecules (DAMP) [29–32], can protect against ischaemic-reperfusion injury. Considering these findings, it may be possible that ischaemic-preconditioning may trigger TLR4 desensitization when DAMPs are released during the preconditioning phase itself.

To investigate the involvement of TLR4 in ischaemic-preconditioned hearts, TLR4 antagonists were administered during ischaemic-preconditioning itself. In theory, if TLR4 desensitization (during ischaemic-preconditioning) contributes to cardioprotection, then its blockade would depress contractile recovery in ischaemic-preconditioned hearts. Using the isolated heart technique [33–35] real time measurements of left ventricular contractile function could be performed throughout the study. (+)-Naloxone was the first TLR4 antagonist to be examined in study 1. As discussed in chapter 5, the significant intergroup variability observed when studying contractile recovery may be attributed to the type of isolated heart model used [35]. Hence study 2 used the constant pressure isolated heart model to resolve these issues [33–35]. Aside from (+)-naloxone, (+)-naltrexone (a more potent and longer acting TLR4 antagonist) was also examined in study 2 [36,37]. The second study revealed that (+)-naltrexone, but not (+)-naloxone, depressed contractile recovery in ischaemic-preconditioned hearts. Furthermore, both compounds were shown to reduce cytosolic high mobility group box one (HMGB1) levels, a transcriptional regulator known to act as a DAMP during ischaemic-reperfusion injury [38–40]. Finally, increase loss of cytosolic cardiac fatty acid binding protein (cFABP), a cardiac biomarker of injury [41], was reported in ischaemic-preconditioned (+)-naloxone, but not (+)-naltrexone, treated hearts. While the molecular work was conflicting, study 2's results highlight two important findings; 1) Blockade of TLR4 signalling, with (+)-naltrexone, directly influences contractile recovery in ischaemic-preconditioned hearts; and 2) These changes were observed in an acute reperfusion model where a significant part of inflammatory cascade was removed. Since the immune response is unlikely to have influenced contractile function during early reperfusion, it is likely that (+)-naloxone and (+)-naltrexone influenced cardiac function in a non-inflammatory manner. While limited, evidence suggests that both compounds may have suppressed reactive oxygen species (ROS)

and nitric oxide (NO) production. This theory is based on Wang *et al* [42] findings who reported that both compounds limited ROS and NO production in LPS stimulated BV-2 cells. During early reperfusion high levels of ROS [43] and peroxynitrite (produced from NO) [44,45] are produced and cause additional tissue injury. However, the regulated production of ROS and NO during ischaemic-preconditioning is believed to be triggered during classical preconditioning [16,20,46,47]. It may be possible that by preventing the upregulation of ROS and NO during ischaemic-preconditioning, classical preconditioning is partially blocked leading to the depression in contractile recovery. If this theory is true, then this will change our perception of TLR4 and how it is involved in myocardial ischemic-reperfusion injury. To date, most cardiac literature surrounding TLR4 has only considered the immunosurveillance receptor as a mediator of inflammatory injury. However, the fact that the TLR4 network activates a broad range of adaptor proteins and signalling kinases suggests that it may have some influence in triggering classical preconditioning [48]. For example, studies have suggested that TLR4 influences PI3k/Akt signalling in immune cells [49,50] and myocardial ischaemic-reperfusion injury [11,51,52]. The PI3k/Akt pathway, which is part of the "Reperfusion injury salvage kinase" (RISK) [53] network has been reported to suppress pro-apoptotic pathways when stimulated in an acute manner. Whether the desensitization of the TLR4 network triggers acute PI3k/Akt signalling, or through a TLR4 dependent negative feedback response is uncertain. It is also important to consider whether these findings can be replicated in the in-vivo setting. If study 2's findings are mediated through the suppression of TLR4 signalling, it would be assumed that a more potent effect would be observed in an in-vivo infarct model. If TLR4 is indeed desensitized through ischaemic-preconditioning, innate and adaptive immunity may be influenced. Considering the ubiquitous nature of TLR4, and its significant influence of multiple signalling kinases, additional research it required.

On the other hand, from the literature which has already examined this receptor, blocking TLR4 signalling itself may be more effective than triggering TLR4 desensitization. From the clinician's point of view, minimizing inflammatory signalling by blocking an immunosurveillance receptor is far more clinically relevant in regard to reducing ischaemic-reperfusion injury. It is for this reason that the project was revised accordingly and resulted in the designs of study 3 and 4. Before discussing these findings, study 2 results should not be disregarded. Understanding how TLR4 regulates MyD88 signalling in ischaemic-preconditioning asks a bigger question; how malleable is TLR4 signalling? Though evidence suggests that TLR4 expression can be influenced by varying factors (i.e. age, genetics and chronic pathological diseases [54]) the ability of TLR4 to respond to these changes remains poorly defined. Aside from understanding its role in cardiovascular disease, deciphering how the receptor adapts to these changes remains an important question regardless of the field of research.

10.12: The rapid administration of (+)-naloxone or TAK242 limits infarct size in an in-vivo myocardial infarction model.

Recently, the completion of (Canakinumab Anti-inflammatory Thrombosis Outcomes Study) CANTOS [55] revealed that the suppression of specific pro-inflammatory cytokines may be beneficial in the cardiovascular setting. While the study's findings were promising, the treatment's high costs and inability to reduce mortality rates, suggest that the use of Canakinumab as a potential therapeutic against cardiovascular disease is unlikely. It is also uncertain as to whether the suppression of multiple pro-inflammatory cytokines at once is more beneficial. This may be achieved by blocking a more upstream effector such as TLR4.

Hence study 3 and 4 investigated whether (+)-naloxone and (+)-naltrexone can suppress myocardial inflammation and protect against cell death / tissue damage. Before beginning any animal work, in-vitro experiments were conducted to determine whether (+)-naloxone / (+)-naltrexone could suppress inflammation and cell death in a cardiac cell-line, H9C2 [56,57]. Study 3's findings showed that there was a robust suppression of NF-κB translocation into the nucleus when LPS treated H9C2s were co-treated with either compound. Although these results suggest that both compounds suppressed the MyD88 dependent pathway, the RT-PCR data collected was conflicting. When gene expression was studied, it was revealed that co-treating cells with LPS and (+)-naloxone enhanced IL-1β and IL-6 gene expression. Although multiple reasons were postulated as to why these findings were observed (see study 3, chapter 7, discussion), I suspect that these results are related to the timepoint in which the cells were collected. Regardless of the RT-PCR data, the rapid suppression of NF-κB translocation suggests that both compounds act on MyD88 signalling. While study 3's results do not undermine Wang *et al* [42] findings, it does suggest that these TLR4 antagonists may act in a time-dependent manner. Future experiments should examine whether TRIF dependent mediators such as interleukin 10 (IL-10), interferon regulatory factor-3 (IRF-3) and interferon gamma (IFN-γ) are influenced. The 2nd part of study 3 also revealed that neither compound could improve cell viability when H9C2s were exposed to simulated ischaemic-reperfusion conditions. This suggests that neither (+)-naloxone or (+)-naltrexone directly influence cardiomyocyte survival and may instead act on TLR4s expressed on other cell types. This is explained in further detail in the "limitations" section of this chapter.

Once (+)-naloxone was discovered to limit inflammation, the in-vivo infarct study (study 4) was conducted. Because I was interested in studying whether TLR4 antagonists could be used as an interventional therapeutic, (+)-naloxone was administered during the ischaemic period just before reperfusion was restored. Because (+)-naltrexone was no longer available, TAK-242 [58–60], a TLR4 antagonist which attenuates both MyD88 and TRIF signalling, was also tested. Study 4 showed that infarct size could be reduced when either compound was administered before reperfusion was restored. To our knowledge, this is the first study to show that (+)-naloxone and TAK242 limit infarct size and could be used as a rapid interventional therapeutic. These findings are supported by the suppression of molecular biomarkers associated with tissue injury. Caspase-3 (a pro-apoptotic mediator [61–64]) and MMP-9 [65–68] (a collagenase) activity were both shown to be attenuated in infarct hearts treated with either compound. When inflammatory mediators were studied, (+)-naloxone, but not TAK-242, was shown to suppress myocardial inflammation. NF-κB nuclear protein translocation and IL-1β / TNF-α gene expression was downregulated in (+)-naloxone treated hearts. Because hearts were collected 48 hours after ischaemic insult, it is difficult to conclude whether this partial suppression of inflammation is attributed to the fact that innate immunity begins to resolve around this time. While this can easily be tested (see “Future experiments: study 4) the findings from this study do suggest that the anti-inflammatory effects of (+)-naloxone are prolonged.

Study 4 also discovered that TAK242 did not suppress myocardial inflammation. These findings are interesting considering that TAK242 is known to be a potent antagonist of TLR4 [58–60]. Again, considering the timepoints in which hearts were harvested, it may be possible

that a more robust anti-inflammatory response would be observed at earlier timepoints. Another possible reason is the concentration used. Hence time and dose-dependent studies are required to determine the optimum dosage required for TAK242 (see future experiments). It is also important to consider whether the reduction of infarct size, caspase-3 and MMP-9, is correlated to the suppression of myocardial inflammation. Studies have reported that caspase-3 and MMP-9 can be activated, or released, through the action of TNF-α [61,69] and NF-κB [70–72]. While this theory is applicable for (+)-naloxone treated hearts, TAK242 did not suppress inflammation. While Matsunaga *et al* [58] has shown that TAK242 does not antagonise other members of the TLR family, it is still unknown as to whether it influences non related TLR pathways.

10.2: Limitations of this project

As discussed throughout this thesis, each study has several limitations which were described in detail. This section will reiterate the limitations of each study as well as additional issues which were not addressed previously.

10.21: Isolated rat and mouse heart technique

The isolated heart technique is a well-established protocol which allows a researcher to monitor direct contractile function real time. However, the technique is highly dependent on the skill set of the researcher, the animal model, and the apparatus which is used [34,35,73]. When examining ischaemic-reperfusion injury, previous users have advised against using the constant flow model. Because flow rate is maintained at a constant state, the heart cannot

adjust coronary flow when required [34]. This can lead to sub-optimal perfusion if the heart requires a higher flow rate after reperfusion. For this reason alone, there was concern as to whether the langendorff model used contributed to the lack of significance reported in study 1. While study 2 resolved this, the short reperfusion protocol limited the usefulness of this data. The reason why post-ischaemic hearts were not perfused for a longer period is related to the variability which can be caused through the model itself. One of the main concerns was that prolonged perfusion could lead to the development of tissue oedema [33]. Another issue which has been discussed repeatedly is that isolated hearts are not perfused with blood. In the context of studying TLR4, the lack of exposure to various inflammatory mediators circulating throughout the bloodstream may have reduced the impact of (+)-naloxone and (+)-naltrexone in ischaemic-reperfusion injury. Furthermore, because each heart was perfused with fresh buffer, any DAMPs released from the myocardium may have been unable to bind onto TLR4. Hence it may be possible that the immunosurveillance receptor was not properly activated during ischaemic-reperfusion injury. Allowing the perfusate to recirculate using a closed system would allow hearts to be re-exposed to buffer containing DAMPs previously released. This is discussed in further detail in the “future experiments section”. Finally, the duration of each experiment limited the ability to find molecular markers associated with inflammation or changes in contractile function. Although multiple attempts were made in study 2 to quantify lactate dehydrogenase (LDH) within the perfusate, the biomarker could not be detected. Another issue was selecting molecular markers associated with inflammation. Because hearts were only perfused for 1 hour, measuring protein level changes in pro-inflammatory markers was unlikely. Considering study 3’s findings, if TLR4 was properly activated, it would have been interesting to see whether changes in NF-κB nuclear translocation could be reported. Finally, while study 2 examined the cytosolic levels of

HMGB1, the nuclear HMGB1 levels were not examined. Comparing the ratio between cytosolic: nuclear HMGB1 levels may provide different results.

10.22: The use of H9C2s and design limits

One of the biggest issues of using H9C2s [56,74] is that they are not cardiomyocytes but instead cardiomyoblasts with cardiomyocyte properties. Because of issues such as timing and costs, primary cardiomyocytes were not tested in study 3. The cell-line was selected because of evidence suggesting that H9C2s display cardiomyocyte like properties when placed in hypoxic-normoxic conditions [57]. To my knowledge, no study has compared the inflammatory profile between primary cardiomyocytes and H9C2s. While inflammatory signalling and TLR4 mRNA was detected in study 3, it is difficult conclude whether these findings are truly reflective of what would be observed in the myocardium. Another thing to consider is the proportion of TLR4 signalling mediated through cardiomyocytes when compared to endothelial cells [28] or white blood cells [49,75]. The second major issue with study 3 was the inability to collect and detect sufficient levels of proteins in H9C2s. While a significant amount of time was spent in finding a method to isolate sufficient protein yields, these attempts proved to be unsuccessful. Due to the large of number of consumables that would have been used, I could not justify spending additional time on optimizing this technique. This decision was made after communicating with another lab who revealed that H9C2s have notoriously low protein yields. This reason may explain why TNF-α was also not detected in the supernatant of LPS stimulated H9C2s when ELISAs were performed. While I was able to isolate RNA from H9C2s, the fact that protein analytical data could not be provided weakens the strength of study 3's findings.

The other major part of study 3 was to determine whether (+)-naloxone and (+)-naltrexone could limit cell death when H9C2 cells were exposed to simulated ischaemic-reperfusion injury. The protocol was designed after reading Gáspár *et al* [76] study. Although significant cell death was reported in study 3, the administration of either (+)-naloxone or (+)-naltrexone failed to protect against simulated ischaemic-reperfusion injury. While this negative result suggests that (+)-naloxone or (+)-naltrexone does not directly influence cell viability, the cell-line used, and the lack of circulating inflammatory mediators, may explain for the lack of a response. Finally, considering study 3's results in the scope of this project, it is important to consider whether the results of LPS stimulated H9C2s are applicable in terms of studying TLR4 activity in ischaemic-reperfusion injury. While studies [40] have reported that DAMPs released from necrotic cells trigger TLR4 signalling, the differences between LPS and DAMP induced activity remains poorly defined.

10.23: In-vivo infarct model

The aim of study 4 was to study whether TLR4 antagonists could reduce infarct size when reperfused for 48 hours. The main reason for this was to ensure that each heart could be exposed to the complete innate immune response. While the results collected are interesting, harvesting blood and heart samples after 48 hours may have been sub-optimal in terms of studying innate immunity. This reason alone may explain why TAK-242 did not suppress myocardial inflammation or why TNF-α could not be detected in plasma samples when ELISAs were run. Another issue with study 4 is that TRIF dependent markers were not examined in the tissue homogenate. Considering when the hearts were harvested, and the evidence suggesting (+)-naloxone / TAK242 suppressive effects on the TRIF pathway [42],[58], I should

have looked at these markers using western blots or RT-PCR. Study 4 was also designed to measure cardiac biomarkers such as LDH, creatine kinase (CK) and cardiac troponin (cTnT) in blood samples collected. Unfortunately, because blood was collected through cardiac puncture [77] this caused the artificial release of cardiac biomarkers in the blood. Analysis of LDH levels in plasma samples (data not shown) revealed high levels of LDH in all groups (including shams). Another limitation is the model selected for this study. When considering patients who suffer from cardiovascular disease it is important to remember the cohort which is studied. Old age, poor diet, and co-morbidities (such as atherosclerosis or diabetes) are not often considered in the animal models used. Hence the relevance of study 4, where young, healthy rats were used, may not be applicable in the clinical setting. Considering that immunosenescence is correlated with old age [78], and that TLR4 is involved in the progression of atherosclerosis [79] and diabetes [80], it is uncertain whether the infarct limiting effects of (+)-naloxone and TAK242 would be influenced under these conditions.

10.24: Data analyses

Understanding the relationship between changes in contractile function and inflammatory mediators requires advanced statistics. By using Rstudio to construct multiway-ANOVAs (i.e. three or four-way ANOVAs) and regression models I could have examined whether these relationships could be explained through statistical modelling. However, because of issues related to timing and available resources, the additional groups or sample numbers required to conduct these analyses was not possible. For example, including groups such as sham + drug treatments would have allowed me to determine whether the drugs themselves were responsible for causing physiological or molecular changes. On the other hand, by increasing

the number of groups for each study the sample size required per group is increased to maintain statistical power.

10.3: Future experiments

10.31: isolated hearts

One of the biggest issues with study 1 and 2 is the intergroup variability reported. While the sample sizes used for both studies were sufficient, increasing the sample size of 10 per group is recommended. This would allow me to perform more complex statistical analyses while accounting for intergroup variability. With study 2,3, and 4, reporting that (+)-naloxone and (+)-naltrexone influence myocardial function and inflammation, the next logical step is to conduct dose and time dependent studies in ischaemic-preconditioned hearts. Furthermore, after study 4's findings, it would be interesting to see whether administering (+)-naloxone / (+)-naltrexone directly after ischaemia in isolated hearts, would show different results. It is also important to check that the positive stereoisomers themselves did not influence contractile function; hence each compound should be perfused into sham hearts in future studies. Finally, TAK242 should also be investigated to determine whether a more specific TLR4 antagonist would show different results in study 2.

Another potential experiment which was mentioned in the "limitations" section was perfusing hearts in a "closed system" where the buffer is recirculated. By using a recirculating system, the hearts are more likely exposed to DAMPs released during ischaemia or early reperfusion. However, it is important to consider the potential issues with this system. It

should be assumed that without fresh perfusate, glucose and salts levels in the recirculating buffer would gradually decline and thus influence contractile function. Depending on the duration of the experiment, and the number of times the buffer is recirculated, this could contribute to the possible decline in contractile function and be misinterpreted as a treatment effect. On the other hand, by increasing the volume of buffer used per heart, any DAMPs released would be diluted in the buffer solution. It would also be interesting to see whether it would be possible to track ROS and NO levels throughout the experiment. Recently, Andrienko *et al* [13] tested whether ROS levels could be measured real-time in ischaemic-reperfused isolated rat hearts. By administrating ROS binding dyes into the heart prior ischaemia, ROS levels could be detected real time using a custom built fluorometer. This technique could be used to measure ROS levels during ischaemic-preconditioning and early reperfusion.

Selecting biomarkers of interest for the isolated heart experiments was difficult. After study 1's LDH results, quantification of alternative biomarkers [i.e. creatine kinase (CK) or cardiac troponin (cTnT)] in the perfusate could be tested to confirm whether the TLR4 antagonists suppressed tissue injury. Other potential target(s) of interest is to measure kinase phosphorylation in the left ventricle. For example, the mitogen activated protein kinase (MAPK) family is known to be regulated through TLR4 signalling [48]. P38, c-Jun N-terminal kinases (JNK), and extracellular signal–regulated kinases 1/2 (ERK 1/2) have all been reported to undergo phosphorylation during the 1 hour of reperfusion [14,81,82]. Another potential area of interest is examining the protein translocation between the cytosolic and nuclear

environment. After study 3's findings NF-κB nuclear translocation could be studied within the left ventricle.

10.32: Follow-up in-vitro experiments for study 3.

Study 3 was the first to report that (+)-naloxone and (+)-naltrexone could suppress myocardial inflammation in H9C2s stimulated with an acute application of LPS. However, as shown by the RT-PCR results, neither compound could suppress TNF-α, IL-6 and IL-1β gene expression when co-treated with LPS for 6 hours. Because the immunohistochemistry and RT-PCR results were conflicting, it was difficult to conclude whether the blockade of TLR4 with (+)-naloxone or (+)-naltrexone was directed through MyD88 signalling. Harvesting cellular homogenate at earlier timepoints may resolve this. Furthermore, since study 3 did not measure TRIF dependent markers, IRF-3 and IFN-γ activity should also be studied.

Compared to H9C2s, primary cardiomyocytes would have been a more ideal model to test the effects of TLR4 inhibition. The use of primary cells would likely have resolved the issue of harvesting sufficient protein levels for quantitative analyses using western blots or ELISAs. It would have also been wise to test endothelial cells (either cell-line or primary derived) to determine how they respond when given the same experiments. It would be expected that endothelial cells, which have been reported to express higher levels of TLR4 [28], would be more responsive to LPS or DAMP stimulation and trigger a more robust immune response.

10.33: Follow up in-vivo experiments

While study 4 showed that (+)-naloxone and TAK242 are cardioprotective, additional experiments could be performed to validate these findings. To ensure that (+)-naloxone and TAK-242 did not contribute to infarct size or influence the expression of any molecular markers of interest, both drugs should be infused into sham hearts. Furthermore, dose dependent experiments should be performed to determine the optimum concentration required to limit infarct size. This is particularly important as the innate immune response should be suppressed without influencing inflammation's role in the repair phase. Considering that TAK-242 suppressed infarct size but not inflammation, it is also important to determine whether the TLR4 antagonist suppressed TLR4-MyD88 signalling during early reperfusion. By testing heart and blood samples at earlier timepoints a more prominent response, regarding the measurement of innate immune factors, may be observed. Since Shimamoto *et al* [27] reported the suppression of inflammatory genes as early as 2 hours it would be expected that 6-24 hours would be the optimum timepoint to measure innate immunity.

Study 4 was also unable to detect differences in LDH levels (data not shown) because blood samples were collected by cardiac puncture. To avoid this blood sampling could be collected by catheterizing the femoral artery and vein. As shown by Jespersen *et al* [77], this technique would allow for repeated blood samplings while preserving the need to administer the drug of interest directly into the bloodstream. I could also track changes in cardiac biomarkers and cytokines released at different timepoints while reducing animal usage and costs at the same time. Study 4 also attempted to measure TNF-α in the plasma though no differences were

detected. Whether this is attributed to the TNF-α kit used or because of the fact blood samples were collected 48 hours later is unknown. This issue may be resolved by collecting blood samples at earlier timepoints or by using a more sensitive ELISA or multiplex kit.

10.34: Using appropriate animal models for infarct studies

Although this study collected heart samples after 2 days it would be interesting to see whether allowing the animals to recover for a longer period would show different results. Considering the fibrosis and wound healing processes occurs over several days it is uncertain as to whether (+)-naloxone or TAK-242 influenced this window. Since both TLR4 antagonists were reported to suppress MMP-9 activity it would be expected that tissue remodelling and fibrinogen content would change. Examining collagen deposition in the left ventricle may provide clues to how TLR4 suppression influences the remodelling of the extracellular matrix. Finally, to properly test the impact of (+)-naloxone and TAK-242, future studies should use older animal models or ones which suffer from pre-existing diseases (such as atherosclerosis or diabetes). Since these are common co-morbidities associated with cardiovascular disease, using these models would be more clinically relevant. It would also determine the effectiveness of (+)-naloxone and TAK242 under these conditions and how they influence these co-morbidities.

10.4: Clinical relevance / importance of this data

To our knowledge, study 4 was the first study to show that the direct administration of either (+)-naloxone or TAK242 before reperfusion limits infarct size and injury biomarkers. Regarding clinical relevance, the most promising findings is the fact that both TLR4 antagonists could be administered rapidly during the ischaemic phase and still limit infarct size. This suggests that TLR4 antagonists could be used as interventional therapeutics during surgical conditions. As discussed in the beginning of this thesis, ischaemic heart disease remains a significant cause of mortality throughout the developed world. Because of the wide implications of myocardial inflammation in cardiovascular diseases, these findings may have potential relevance in other pathologies. Considering co-morbidities, such as atherosclerosis or diabetes, have been reported to be influenced by TLR4 signalling, (+)-naloxone and TAK242 may provide additional benefits in suppressing inflammation triggered through these diseases. On the other hand, these findings require careful interpretation. High doses of TLR4 antagonists should be avoided to minimise the possibility causing the complete suppression of TLR4 signalling. The reason for this is that treated patients may be at risk of suffering from septic shock if the immune response is dampened. Although the number of patients who suffered from sepsis in CANTOS was low [55], additional care should be taken in suppressing immunosurveillance receptors. For future studies, it may be wise to monitor the bacterial levels in post-op animals to determine whether TLR4 pretreated animals are more susceptible to sepsis.

10.5: Concluding thoughts

The findings from this project have provide several insights into the role of TLR4 in both myocardial ischaemic-preconditioning and ischaemic-reperfusion injury. Like most studies,

the data collected has raised more questions about the role of TLR4 in the cardiovascular setting. The concept of TLR4 biphasic signalling remains an area of interest especially regarding its ability to respond to TLR4 ligands. While it is disappointing that I was unable to examine the theory of DAMP induced preconditioning during this project, the evidence from study 2 has provided some interesting findings. Future experiments discussed in this chapter provide potential ways to explore the role of TLR4 and DAMPs in myocardial preconditioning. Perhaps the most encouraging findings from this project, is study 4. The fact that (+)-naloxone and TAK242 were able to limit size when rapidly administered suggests that TLR4 antagonists could be used as an interventional therapeutic. If TLR4 signalling can be carefully regulated, TLR4 blockers could be used to reduce mortality rates or even alleviate the risks associated with cardiovascular diseases.

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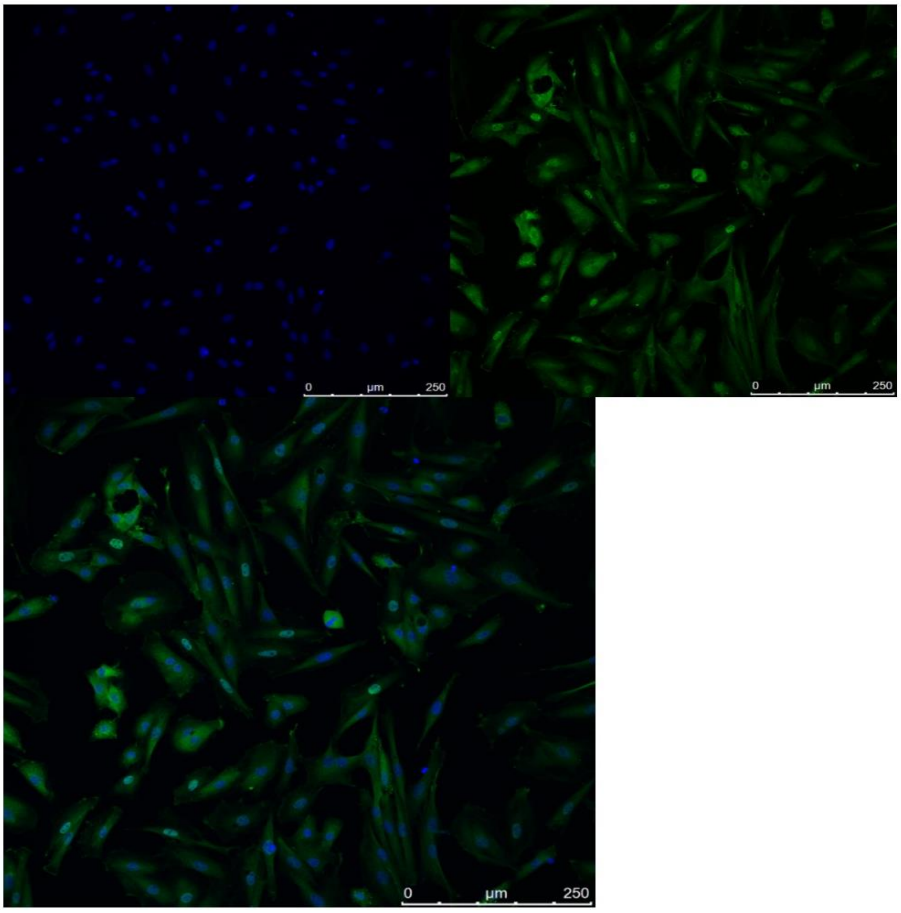
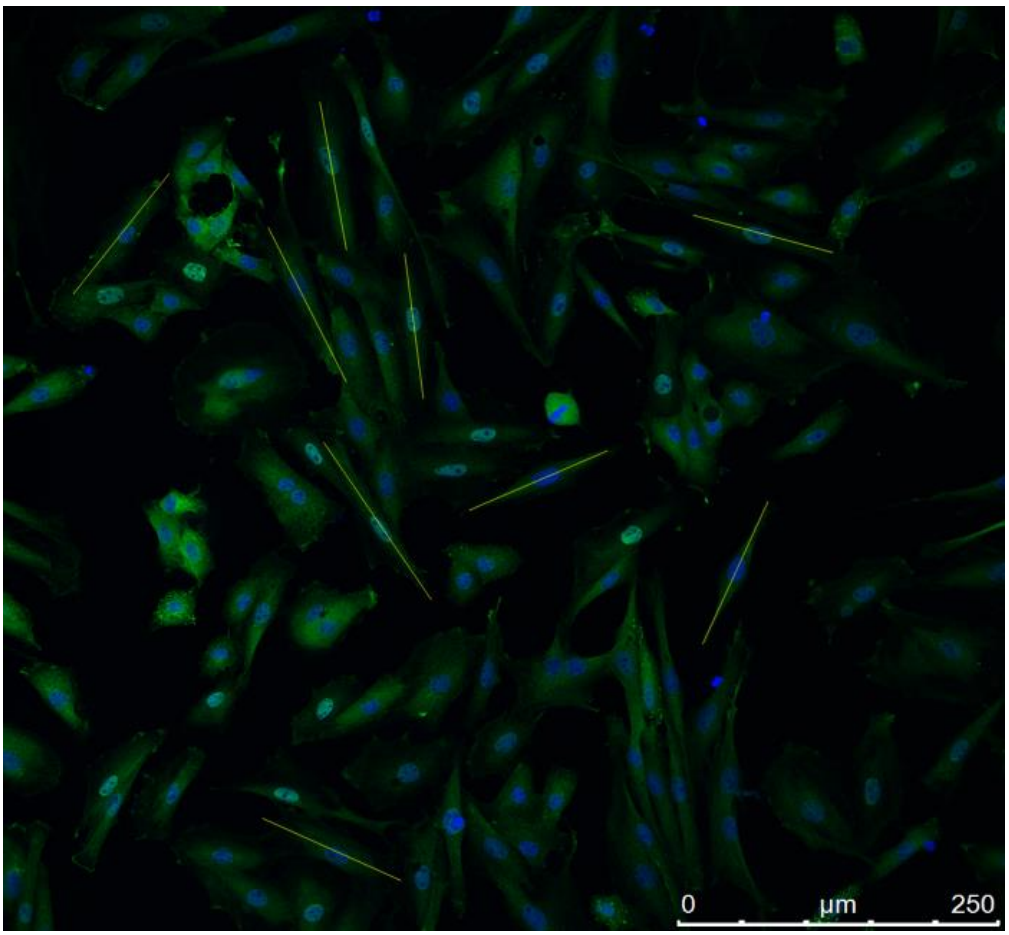
XIX: Appendices

ImageJ analyse: detail description of the analytical method used to determine NF-κB cytosolic – nuclear translocation.

1. All tif images taken on the confocal are loaded into ImageJ with the image type set as RGB.
2. Using RGB allows ImageJ to separate single image channels representing DAPI (nuclear, blue, top left), NF-κB staining (green, top right) and an overlay of both images (bottom right) as shown below.



3. Using the line tool, lines (shown in yellow) are drawn across 30 random cells for each image as shown.



4. Each line represents a measurement of intensity across each cell. Using the custom macro designed for studying NF-κB translocation, a text file containing these measurements are generated. Because the overlay is composed of two images two numerical values are generated per measurement, one for DAPI the other for NF-κB.

5. The macro used for the data ImageJ analyses.

```
// prepare stacks
original_stack = getImageID;
plot_stack = 0;
total_slices = nSlices;

// save to file
home = getDirectory("home"); // Returns the path to users home directory.
if (home == "")
   exit("No desktop directory available");
temp_file = home + "Desktop/" + getInfo("image.filename") + "_profiles.txt";
f = File.open(temp_file);




b = roiManager("count");
for(i = 0; i < b; i++) {
                roiManager("Select", i);
   // cycle through image leaves
   for (slice = 1; slice <= total_slices; slice++) {

      showProgress(slice, total_slices);

      selectImage(original_stack);
      setSlice(slice);

      // get plot data
      value = getProfile();
      positions = lengthOf(value);

      // write slice plot to file

      file_str = toString(value[0]);
      for (pixel = 1; pixel < positions; pixel++)
         file_str += ", " + value[pixel];
      print(f, file_str);

   }
   print(f);
}
setSlice(1);
setBatchMode(false);
restoreSettings();
```

6. The text file is then modified through Microsoft Excel (settings: delimit: ON, tab: OFF, comma: ON) before it is loaded into Rstudio.
7. The text file is then analysed under the following script to generate a histogram plot of DAPI and NF-κB.

```
# import libraries
library(ggplot2)
library(dplR)
library(pracma)
library(scales)
# import dataframe
data<- read.delim('data.txt',sep = '\t' , header = FALSE)
data<- as.data.frame(t(as.matrix(data)), row.names = NULL


GetPlots<- function(df, R, G, B){ #df =  dataframe, order of R/G/B channels, which col ie.(3,1,2)
  for (i in 3: ncol(df)){
    if (i %% 3 == 0){ # if divisible by 3
      ndf = df[,((i-2):i)] # subset df into columns
      # Standardise column names
      colnames(ndf)[R]<- 'Red'
      colnames(ndf)[B]<- 'Blue'
      colnames(ndf)[G]<- 'Green'
      # Plot
      plot<- ggplot(data = ndf, aes(x = seq_along(Red),
                                    y = c(Red,Green,Blue)))+
        geom_line(aes(y = Green), colour = "#66CC99")+
        geom_line(aes(y = Blue), colour = '#00CCCC')+
        geom_line(aes(y = Red), colour = '#CC0000')+
        theme_bw()+
        scale_x_continuous(breaks = seq(0,300,10))+
        labs(y = "Intensity",  x="Distance(Pixels)", title = paste("Cell number", toString(i/3)))
      plotname<- readline(prompt  = "Enter File Name(.png):")
      ggsave (plot, filename = plotname)

GetPlots(data, 1,2,3)
```

```
#intensitydata: dataframe with intensity values
#locationdata: dataframe with pixel locations of nucleus and membrane of cells
#POIChannelNumber: Channel (1st,2nd,3rd...) in which protein of interest is at (integer please)
#TotChannelNumebr: Total number of channels measure

IntegrateIntensity<- function(intensitydata, locationdata, POIChannelNumber, TotChannelNumber){
    # Create empty list/vector
  percentdata<- c()
  totalnucleus<- c()
    #for number of cells on frame
  for (cellID in 1:15){#because we only sample 15 cells / image
      # select cols in intensity data
    intensityVals<- intensitydata[,(TotChannelNumber*cellID-(TotChannelNumber-POIChannelNumber))]
    intensityVals<- data_frame(intensityVals)
      #sequence lower, upper bounds for cell, nucleus
    NucleusLower<-locationdata[cellID,"NuclearLower"]
    NucleusUpper<-locationdata[cellID,"NuclearUpper"]
    CellLower<-locationdata[cellID, "CellLower"]
    CellUpper<-locationdata[cellID,"CellUpper"]
    NucleusPosition<- seq(NucleusLower,NucleusUpper,1)
    CellPosition<- seq(CellLower, CellUpper,1)
      #slice intensity values for nucleus, cell
    NucleusIntensity<- slice(intensityVals, NucleusLower:NucleusUpper+1)
    TotalIntensity<- slice(intensityVals, CellLower:CellUpper+1)
      #integrate values
    NucleusVector<- as.vector(as.matrix(NucleusIntensity[,1]))
    NucleusIntegral<- trapz(NucleusPosition, NucleusVector)
    CellVector<- as.vector(as.matrix(TotalIntensity[,1]))
    TotalIntegral<- trapz(CellPosition, CellVector)
      #Percent nuclear expression of protein
    newpercentage<- NucleusIntegral/TotalIntegral * 100
      #Append percent to list
    percentdata<- c(percentdata, newpercentage)
    totalnucleus<- c(totalnucleus, NucleusIntegral)

  return(percentdata) #returns list of percent expression of POI in Nucleus vs whole cell
```

```
# import locationdata

locationdata<- read.delim("locationdatafile", header = TRUE)

#integrate area under curve under

locationintegrated<-IntegrateIntensity(intensitydata, locationdata, 2, 3)
```

8. This produces a histogram plot of the DAPI and NF-κB histogram plots combined as shown in the example below. Blue represents the nucleus whereas green is NF-κB. Since there is an overlapping between the two histograms plots, cell number 3 would be defined as a cell where significant level of NF-κB was activated translocated into the nucleus.