Regulation of the 25-Hydroxyvitamin D_3 1 Alpha Hydroxylase and 24-Hydroxylase Gene Promoters

A THESIS SUBMITTED TO THE UNIVERSITY OF ADELAIDE
FOR THE DEGREE OF DOCTOR
OF PHILOSOPHY

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March 2003

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THESIS SUMMARY

$1,25(OH)_2D_3$ plays important roles in many process such as calcium homeostasis. The vitamin D hydroxylase enzymes, vitamin D 1-hydroxylase (*1α(OH)ase*) and vitamin D 24-hydroxylase (*CYP24*) are the key rate limiting enzymes responsible for the bioactivation and degradation of $1,25(OH)_2D_3$ respectively. Several physiological factors including PTH, $1,25(OH)_2D_3$ and calcitonin interact to regulate the gene expression of the two enzymes resulting in regulation of serum and tissue levels of $1,25(OH)_2D_3$.

The aim of the study in this thesis was to understand the molecular mechanism by which PTH regulates expression of the human *1α(OH)ase* gene promoter in the kidney and also the molecular mechanisms by which $1,25(OH)_2D_3$ and calcitonin regulate expression of the rat *CYP24* promoter in kidney and osteoblast cells.

The up-regulation of the gene for *1α(OH)ase* by PTH under hypocalcemic conditions is fundamentally important for the maintenance of calcium and phosphate homeostasis. The molecular mechanism that underlies this hormonal response has been investigated in the present study by transfection analysis of the human *1α(OH)ase* promoter in kidney AOK-B50 cells. It has been shown that the first 305 bp of promoter sequence can be induced by PTH in transient transfection assays and also within a chromatin environment when stably integrated. Mutagenesis of possible transcription factor binding sites within this promoter length has shown that three sites clustered within the region from –66 to –135 contribute to basal expression. A likely GC box and a CCAAT box site are particularly important for basal expression although these sites are not likely to functionally cooperate in a major way. Mutagenesis of the CCAAT box site consistently reduced PTH induction although mutagenesis of the GC box, EBS and other possible binding sites in the 305 bp of promoter had no significant effect on the level of PTH induction. Other experiments showed that PTH induction

but not basal expression was sensitive to the protein kinase inhibitor H89. I have therefore identified for the first time the sites in the human *1α(OH)ase* promoter responsible for basal expression and provide evidence for the role of a CCAAT box binding protein in a PTH mechanism of induction that involves an H89 sensitive step.

$1,25(OH)_2D_3$ functions in kidney and osteoblast cells to induce *CYP24* gene expression and enzyme activity, representing an important feedback mechanism to avoid toxicity that may result from a high level of $1,25(OH)_2D_3$. In the rat promoter, a GC box (-114/-101) and a CCAAT box (-62/-51) were found to strongly contribute to basal and $1,25(OH)_2D_3$ mediated induction in kidney HEK-293T cells and osteoblast UMR106 cells. The present study has focused on characterising the two sites and related signalling systems. EMSA using antibodies for Sp1 and NF-YB provided evidence for the binding of Sp1 to the GC box and the binding of NF-Y to the CCAAT box. Over expression of Sp1 and NF-Y in *Drosophila* SL3 cells revealed that Sp1 functions at least partially through the GC box and NF-Y acts through the CCAAT box. Use of the mammalian two-hybrid assay provided evidence for an interaction of Sp1 with Ets-1 and also of Sp1 with RXRα. Over expression of a dominant negative NF-YAm29 resulted in reduced basal expression but did not lower the fold $1,25(OH)_2D_3$ mediated induction, suggesting the NF-Y is important for basal expression but not for induction by $1,25(OH)_2D_3$. A model for induction is proposed that involves contributions of Sp1 and NF-Y to basal expression with the NF-Y protein being replaced with another unknown protein in response to $1,25(OH)_2D_3$.

The results in HEK-293T cells, using pharmacological inhibitors (H89, calphostin C, PD98059 and SB203580) demonstrated that PKA, PKC and ERK1/2/5 MAPK pathways are critical for $1,25(OH)_2D_3$ mediated induction of the *CYP24* promoter. Evidence is presented that the CCAAT and GC boxes are likely to be target sites for the cAMP/PKA cascade. In addition,

there is evidence that the PKC isoform, PKCζ is important for induction of the promoter by $1,25(OH)_2D_3$ but not for basal expression and that this action of PKCζ occurs through the GC box. Hence, it can be proposed that basal expression is driven by Sp1 and NF-Y, both of which may be phosphorylated by PKA. In the presence of $1,25(OH)_2D_3$, NF-Y is replaced by another protein perhaps following phosphorylation of NF-Y by PKA. At the GC box, Sp1 in the presence of $1,25(OH)_2D_3$ could be phosphorylated by PKCζ.

Studies were undertaken to determine whether calcitonin could lower circulating $1,25(OH)_2D_3$ levels, through induction of *CYP24* in kidney cells. It was found that the promoter was stimulated by calcitonin in transiently transfected HEK-293T cells that stably expressed calcitonin receptor. A GC box at -114/-101 and a CCAAT box at -62/-51 in the transiently transfected –298bp promoter predominantly accounted for calcitonin induction. Mutagenesis of either the GC or CCAAT box resulted in the almost totally loss of induction in stably transfected in these cells. Over expression of NF-YAm29 in these cells strongly inhibited basal expression of the *CYP24* promoter and moderately inhibited calcitonin mediated fold induction. ERK1/2 signalling pathways were not involved in the calcitonin-mediated induction as shown by studies with the pharmacological inhibitor PD98059 and a dominant negative of ERK1/2 (ERK1K71R). However, both PKA and PKC pathways are involved in the calcitonin induction mechanism as determined by the inhibitory action of the PKA and PKC inhibitors. PKCζ contributes 50% to calcitonin induction, but not basal expression of *CYP24* promoter expression as shown by over expression of a dominant negative PKCζK281M. Cotransfection of a dominant negative form of Ras (Ras 17N) resulted in calcitonin mediated induction being reduced by about 50%. Therefore a Ras-PKCζ signalling pathway was proposed which acts through the GC box.

Calcitonin and $1,25(OH)_2D_3$ synergistically induced *CYP24* gene promoter activity in both transient transfected and stably transfected cells. This synergy was almost abolished when the two VDREs were mutated, but synergy was still evident when the GC and CCAAT boxes were mutated. The findings have been extrapolated to the *in vivo* situation where it is suggested that induction of renal *CYP24* by calcitonin under hypercalcemic conditions could contribute to the lowering of $1,25(OH)_2D_3$ levels.

DECLARATION

This thesis contains no material that has been accepted for the award of any other degree or diploma by any university. To the best of my knowledge it contains no material that has been previously published by any other person, except where due reference has been made in the text.

I consent to this thesis, when deposited in the university library, being available for photocopying and loan.

Signature:... Date:.....12/03/2003

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ACKNOWLEDGMENTS

I would like to express my sincere thanks to:

- Dr. Brian May, my supervisor, for his permission to undertake the Ph.D. study in the lab, and for his continuous encouragement, guidance and innovative supervision in the field.

- Dr. Prem Dwivedi, with whom I worked closely and happily, for his great supervision, valuable discussion and big patience.

- Josef, the Honest Aussie Boy, for his generous help and discussion (Small arc can make a big picture!).
- Tania, with whom the whole lab is cheering up, for her warm friendship and help.
- Dr. Howard Morris, for his careful review of some chapters.
- Other past and present BKM members, Tim, Satish, Ingrid, Ben, Senali, Andy, Paul, Diseree, Babara, Ana and Oly, for their great chatting and friendship.

- All the departmental staff and postgraduates, especially the support staff in TC, for the nice working environment they are making and maintaining.

Finally I would like to thank dear XJ and Cheryl for their understanding, love and support, and also to my parents and brother for their endless love and encouragement.