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Characterization of insulin-like growth factor I regulation of renal 25-hydroxyvitamin D_3 1-alpha hydroxylase in aging

LAI WAN PING

MASTER OF PHILOSOPHY

The Hong Kong Polytechnic University

2003

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Abstract of thesis entitled

" Characterization of insulin-like growth factor I regulation of renal 25-hydroxyvitamin D_3 1-alpha hydroxylase in aging"

submitted by

Miss Lai Wan Ping

for the degree of Master of Philosophy at

The Hong Kong Polytechnic University in

2003

Phosphate (P) is a known regulator in 1,25-$(OH)_2D_3$ metabolism. IGF-I peptide is also known to regulate 1,25-$(OH)_2D_3$ production. In this study, it was hypothesized that the regulation of IGF-I axis during dietary P restriction was responsible for the regulation of a key enzyme, renal 25-hydroxyvitamin D_3 1-alpha hydroxylase (1-OHase), in vitamin D metabolism. Part of the IGF-I axis was studied, including two receptor proteins, insulin-like growth factor 1 receptor (IGFIR), insulin receptor (IR), and two docking proteins, Src-homology collagen (Shc) and insulin receptor substrate-1 (IRS-1). Male Sprague Dawley rats aged 4 to 8 weeks and 8 to 12 weeks were used in the study as young and adult animal models, respectively. Rats were fed with either a normal (NPD, 0.65 % P, 0.60 % Ca) or a low (LPD; 0.1% P, 0.60% Ca) phosphate diet for 0, 1, 2, 3, 5 and 7 days.

In young rats, the expression of 1-OHase as well as the receptors in IGF-I axis in renal proximal tubules were up-regulated in response to LPD. The up-regulation of 1-OHase protein was caused by an enhancement of *in vivo* protein stability. However, this mechanism does not apply to the regulation of receptor proteins in IGF-I axis in young rats. In adult rats, LPD did not alter the expression of 1-OHase protein in renal proximal tubules, but proteins in the IGF-I axis, including both the receptors and docking proteins, were up-regulated during LPD. In addition, the responsiveness to LPD in the IGF-I axis was found to be greater than in young rats. In the *in vivo* study, LPD did not enhance receptor protein stability. These findings showed that regulations of proteins in IGF-I axis and 1-OHase by LPD were age-dependent and their regulations were not operated by the same mechanism, i.e. an enhancement of *in vivo* protein stability.

In the present study, some serum measurements were performed, including P, Ca, 1,25-$(OH)_2D_3$ and IGF-I. Dietary P restriction decreased the serum P levels in both young and adult rats, but showed no effect in serum Ca content. Serum 1,25-$(OH)_2D_3$ level increased significantly by LPD in both age groups. However, serum IGF-I level was found to be decreased in young rats, but increased in adult rats during 7 days of LPD treatment. Results were correlated between serum P level and different protein expressions in young rats. It was found that serum P level was inversely related with renal 1-OHase, IGFIR and IR protein expressions in young rats. In particular, correlations of 1-OHase and IR proteins with the change in serum P level were very similar. Besides, 1-OHase showed a greater positive relationship with IR

protein expression than with IGFIR, suggesting that IR was preferably involved in renal 1-OHase protein regulation.

Declaration

I declare that it is for the purpose of my study of Master of Philosophy only and not for circulation or further reproduction or any other purpose.

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Date: 17 March 2003

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List of Abbreviations

1-OHase	25-hydroxyvitamin D_3 1-alpha hydroxylase
ALPD5	Adult rats fed with 5 days of LPD
ALS	Acid-labile subunit
ANOVA	One-way analysis of variance
AND	Adult rats fed with NPD
BSA	Bovine serum albumin
Ca	Calcium
$CaCl_2$	Calcium Chloride
c-AMP	Cyclic AMP
cDNA	Complementary DNA
CHX	Cycloheximide
CO_2	Carbon dioxide
ECL	Enhance chemiluminescence
GFR	Glomerular filtrate
GH	Growth hormone
GHRH	Growth hormone releasing hormone
HCl	Hydrogen chloride
HRP	Horseradish perioxidase
IGF-I	Insulin-like growth factor I
IGF-II	Insulin-like growth factor II
IGFIR	Insulin-like growth factor Type I receptor
IGFBPs	Insulin-like growth factor binding proteins
IR	Insulin receptor

IRS-1-4	Insulin receptor substrate 1-4
IR-TK	Insulin receptor-tyrosine kinase
KCl	Potassium Chloride
KH_2PO_4,	Potassium dihydrogen phosphate
KHS	Krebs Henseleit saline
LPD	Low phosphate diet
Max	Maximum
mRNA	messenger RNA
Mol. Wt.	Molecular weight
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MSA	Multiplication-stimulating activity
NaCl	Sodium Chloride
$NaHCO_3$	Sodium hydrogen carbonate
NPD	Normal phosphate diet
NSILA	Non-suppressible insulin-like activity
O_2	Oxygen
$1,25\text{-}(OH)_2D_3$	1,25 dihydroxyvitamin D_3
$24,25\text{-}(OH)_2D_3$	24,25-dihydroxyvitamin D_3
$25\text{-}OHD_3$	25-hydroxyvitamin D_3
PCT	Proximal convoluted tubules
PTH	Parathyroid hormone
P	Phosphate

Pi	Inorganic phosphate
P4501α	Type I (mitochondrial) cytochrome P450 enzyme
PI3'K	Phophoinositide 3'kinase
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethanesulfonyl fluride
PTHrP	PTH related protein
PVDF	Polyvinylidene fluoride
SFA	Sulfation factor activity
Shc	Src-homology collagen
SH2	Src-homology 2
SD	Sprague Dawley
SDS-PAGE	Sodium dodecyl sulfate-polyacrlamide gel electrophoresis
TBST	Tris-buffer saline with Tween 20
VDDRI	Vitamin D-dependency rickets Type I
VDR	Vitamin D receptor
v/v	Volume / Volume
w/v	Weight / Volume
YLPD5	Young rats fed with 5 days of LPD
YND	Young rats fed with NPD

Chapter One
Literature Reviews

1.1 Introduction

Hong Kong is one of the regions where osteoporosis is prominent. Osteoporosis is a condition characterized by a microarchitectural deterioration of bony tissue and a consequent increase in fracture risk. In 2001, the incidence of hip fractures in Hong Kong women aged above 65 rose exponentially, and the rate of incidence for elderly women was found to be twice that of elderly men. In the past three decades, the occurrence of osteoporotic hip fractures increased 2 folds. There were 11 patients out of 1000 female individuals and 5 patients out of 1000 male individuals with aged above 70 (Lau *et al.*, 2001). In addition, the prevalence of osteoporosis of the spine increased dramatically from 10 % in the age group between 50 and 59 to 45 % of the age group between 60 and 69, with a male to female ratio of 1 : 2 (Ho *et al.*, 1999). About 30 % of the women and 17 % of the men with osteoporosis suffered one or more spinal fractures (Lau and Woo, 1998). The cost for hip fracture treatment exceeds 1 % of the hospital budget, and it is estimated that the cost will rise exponentially as the population ages (Lau, 2002). A much larger population will suffer from this epidemic bone disease, and much more money will be spent on this issue. Therefore, more public attention is needed on the health of the elderly population, including the prevention and the treatment of osteoporosis. The underlying mechanisms of this disease need to be characterized.

1.1.1 Osteoporosis

Osteoporosis is a global problem. The disease is characterized by low bone mass and structural deterioration of bone tissue, leading to bone fragility and an increase in susceptibility to fractures of the hip, spine and wrist. The bones become extremely porous (Figure 1). Patients suffer from severe pain and long-term physical disability. Both the patients and their families suffer physically and mentally from the problems associated with the disease.

1.1.2 Bone Remodeling

Osteoporosis is, in part, a disorder of bone remodeling. It is caused by a negative calcium (Ca) balance, in which more Ca escapes from the skeleton than returns. The concept of bone remodeling was introduced by Manolagas & Jilka in 1975 (Manolagas & Jilka, 1975). Bone remodeling is the balance between bone resorption and its accretion; it can reflect whether there is a negative, neutral or positive Ca balance in the bone environment at both the local level and the entire skeletal system. When an imbalance occurs, either an increase in relative bone resorption rate or a decrease in relative bone formation rate will result. The remodeling process is affected by various regulators, such as 1,25 dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), hormones and cytokines.

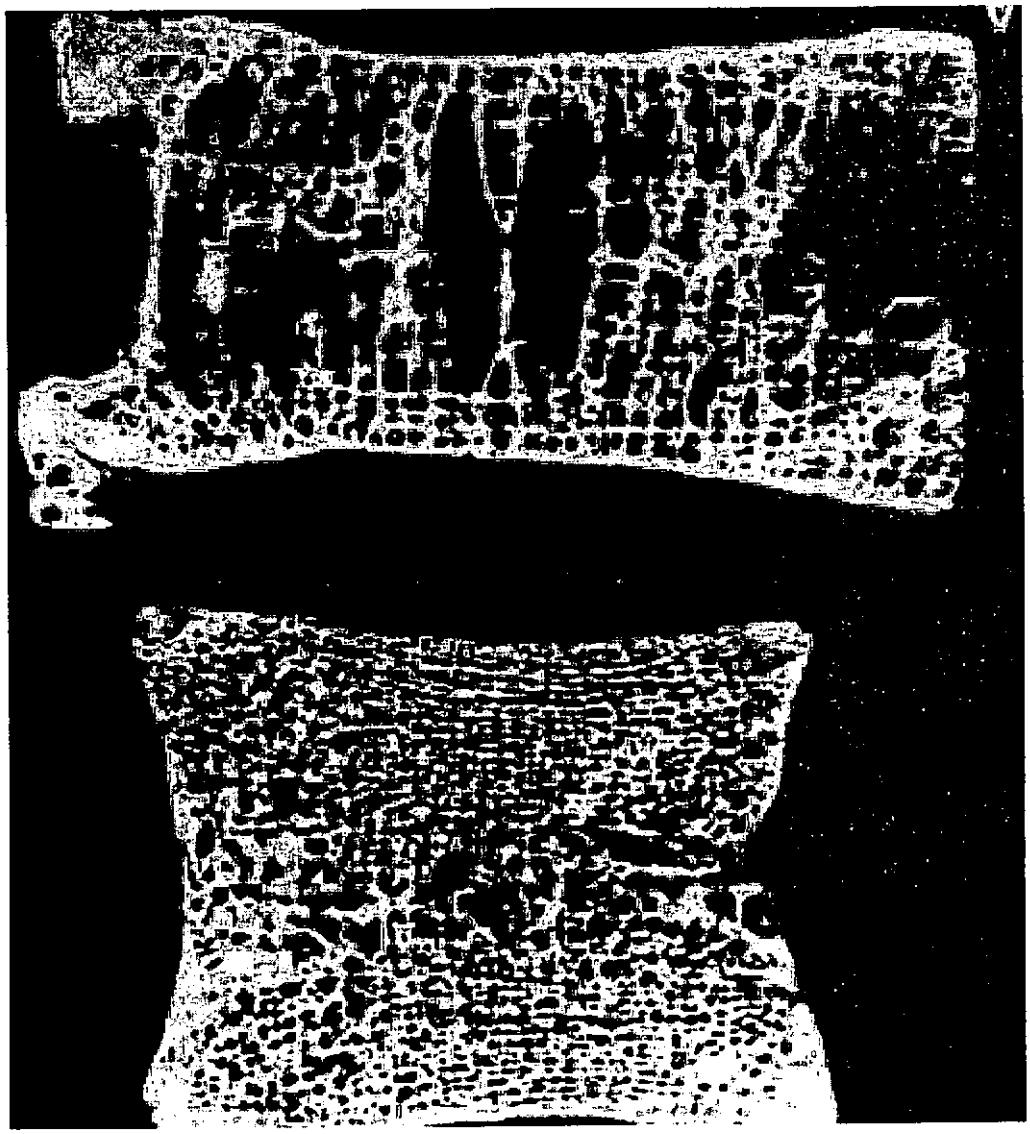

Figure 1: Microradiograph showing a porous lumbar vertebra with long-standing osteoporosis in an elderly subject (upper panel). A healthy lumbar vertera from a young (lower panel) female is shown below (Adapted from Avioli & Krane, 1997).

1.1.3 Pathogenesis of Osteoporosis

1.1.3.1 Type I Osteoporosis

Two types of osteoporosis have been classified (Riggs, 1993). Type I osteoporosis is postmenopausal osteoporosis and occurs in women with estrogen deficiency who are under the age of 75. It is believed that a deficiency in estrogen reduces renal 1,25-$(OH)_2D_3$ production by suppression of parathyroid hormone (PTH) secretion. The suppression is induced when Ca is mobilized from the bone to the circulation. In 1976, Gallagher et al. demonstrated a 30 % decrease in plasma 1,25-$(OH)_2D_3$ level in postmenopausal females having osteoporosis than in their age and sex-matched controls. This correlates with a lower Ca absorption: the postmenopausal group showed a parallel decrease in 1,25-$(OH)_2D_3$ level and Ca absorption (Gallagher *et al.*, 1979). The decrease in both Ca absorption and 1,25-$(OH)_2D_3$ level indicates that the primary defect is likely to be an increase in net bone resorption, resulting in eventual bone loss. Most of the Type I osteoporotic patients suffer from fractures in forearm and vertebra (Riggs, 1993).

1.1.3.2 Type II Osteoporosis

Type II osteoporosis is an age-related bone disease that occurs in both men and women. One of the proposed models of this senile bone disease is the primary decrease in renal 1,25-$(OH)_2D_3$ production with age. The age related reduction of 1,25-$(OH)_2D_3$ production, in turn, mediates a decrease in Ca absorption, resulting in

negative Ca balance. The negative Ca balance leads to the development of secondary hyperparathyroidism which accelerates bone resorption in both cortical and trabecular bone and decelerates bone formation. Patients usually suffer from hip and vertebral fractures in type II osteoporosis.

As mentioned in Section 1.1.2.2, Type II osteoporosis is mainly caused by an age-related alteration of vitamin D_3 metabolism. The age-related decrease in renal 25-hydroxyvitamin D_3 1-alpha hydroxylase (1-OHase) activity, which is an important enzyme involved in synthesizing of bioactive vitamin D_3, is thought to be one of the contributing factors to the age-related change in bone metabolism. This study attempts to review the regulation of this essential vitamin D_3 enzyme, 1-OHase, by using an animal model.

1.2 Vitamin D_3

1.2.1 Sources of vitamin D_3

Vitamin D_3 is classified as a steroid (secosteroid) hormone. It may be obtained from nutritional origin (vitamin D_3 from natural source or vitamin D_2 mostly from pharmaceutical source) or produced photochemically by sunlight (short-wave ultraviolet (UV) – 290-320nm) from a precursor, 7-dehydrocholesterol, which is present in the skin. The vitamin D_3 content is low in most of the food products, except some fatty fish (Table 1). Skin synthesis is a common source of

vitamin D_3 in man and most vertebrates. However, excess UV exposure causes a degradation of previtamin D_3 into other sterol products, such as luminsterol or tachysterol (Figure 2), which dramatically decrease the availability and activity of vitamin D_3. This phenomenon is known as 'natural' vitamin D_3 intoxication (Bouillon *et al.,* 1998) and is one of he reasons why excess sunlight should be avoided.

1.2.2 Transportation of vitamin D_3

The transportation of the two sources of vitamin D_3 is different. The dietary source of vitamin D_3 is transported to the liver through lymph veins after being absorbed from the intestinal chylomicrons, whereas, the skin source is mainly transported by a serum albumin-like plasma carrier protein (DBP). In 1997, Cooke *et al.* demonstrated that less circulating vitamin D_3 is found and its metabolites have a high turnover rate in the DBP-KO mice. These mice are less sensitive to vitamin D_3 intoxication, but more sensitive to vitamin D_3 deficiency (Cooke *et al.,* 1997).

	Dutch Food Composition Compendium	McCance & Widdowson's 'The composition of food' 1991
Milk		0.03
Cheese (Brie 60+)	0.8	0.2
Eggs (chicken, scrambled)	1.4	1.6
Liver		
Pig	0.9	1.1
Cow	0.2	0.3
Meat		
Average, <5 g fat	0.1	Trace
Average, >5 g fat	0.4	Trace
Herring, salted	9.6	22.5
Mackerel, canned in oil	5.8	8.0
Tuna, canned in oil	5.8	5.8

Table 1: Estimated vitamin D_3 content of some food products (100 ug / 100 g) (Bouillon *et al.*, 1998).

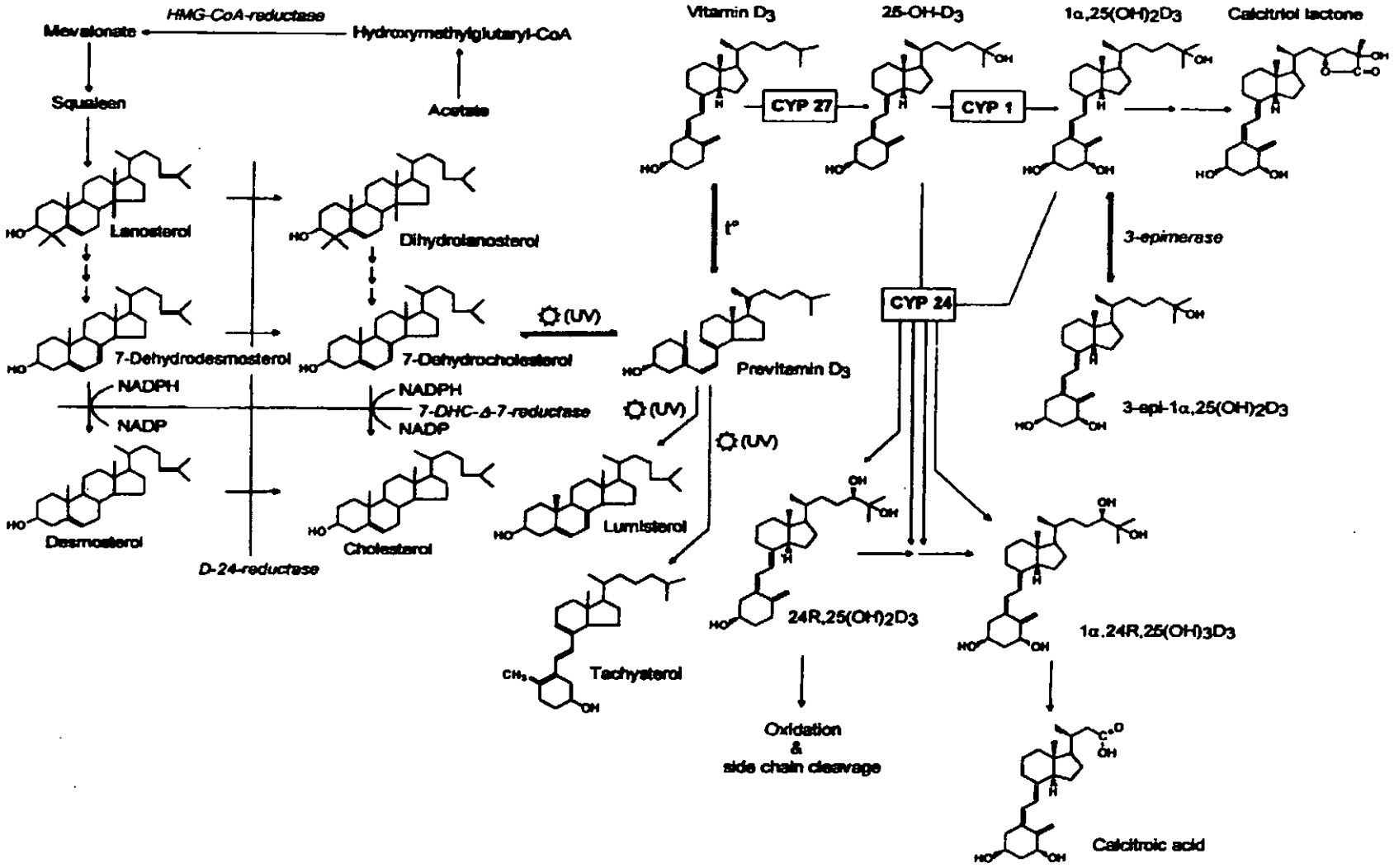

Figure 2: Photosynthesis and the metabolites of vitamin D_3 (Bouillon *et al.*, 1998).

1.2.3 Activation of vitamin D_3

The pro-hormone, 7-dehydrocholesterol is not biologically active. It has to undergo two hydroxylations to become active. The first hydroxylation takes place in the liver and is performed by a hepatic enzyme, 25-hydroxylase (25-OHase), which is not under tight physiological regulation (Portale *et al.*, 2000). It transforms the 7-dehydrocholesterol to yield 25-hydroxyvitamin D_3 (25-OHD_3) as a single process. The circulating 25-OHD_3, about 10^{-7} M, has a longer half-life than vitamin D_3 itself. It is a good marker of the vitamin D_3 intake and the amount of sunlight exposure. It undergoes another hydroxylation at 1-alpha position to form 1,25-$(OH)_2D_3$ by the 1-OHase. 1,25-$(OH)_2D_3$ is the hormonally active form of vitamin D_3 which binds to the vitamin D receptor (VDR) and triggers a series of biological responses. This conversion takes place in the mitochondria of the renal proximal tubules. Besides the 25-OHD_3 and 1,25-$(OH)_2D_3$, other vitamin D_3 metabolites can be formed by different hydroxylations at different hydrocarbon backbone positions, such as 24,25-dihydroxyvitaminD_3 (24,25-$(OH)_2D_3$), by the action of 24-hydroxylase (24-OHase) on 25-OHD_3. The biosynthesis of the vitamin D_3 metabolites is summarized in Figure 2.

1.2.4 25-hydroxyvitaminD_3 1-alpha hydroxylase (1-OHase)

The crucial step of the active vitamin D_3 biosynthesis is by the action of renal 1-OHase. It is mainly synthesized in proximal tubules in the kidney. In 1997, Fu *et al.* highlighted the importance of the 1-OHase gene in vitamin D_3 function by demonstrating the mutation of 1-OHase genes in genetic disorder vitamin D-dependency rickets Type I (VDDRI). VDDRI patients have severe rickets despite normal vitamin D intake and sufficient UV light exposure (Fu *et al.*, 1997). Therefore, understanding of the regulation of 1-OHase is very important.

The renal 1-OHase is a type I cytochrome P450 (P4501α) enzyme, which is present in the inner mitochondrial membrane (Paulson & DeLuca, 1985 and Henry, 1992). It acts as a mixed-function oxidase and uses electrons from NADPH and molecular oxygen (Black & Coon, 1987 and Nebert & Gonzalez, 1987). Significant activity is reported exclusively in the kidney, but activity is also detected in other extra-renal tissues (Bikle *et al.*, 1986; Delvin & Arabian, 1987 and Glorieux *et al.*, 1995).

1.2.4.1 Cloning of various vitaminD_3 hydroxylases

The study of the 1-OHase regulation is limited under normal physiological condition because 1,25-$(OH)_2D_3$ production (or 1-OHase activity) can only be detected under vitamin D-deficient conditions. Therefore, the recent success in cloning of the human, rat, mouse and pig cDNA for 1-OHase facilitates the mechanistic studies of 1,25-

$(OH)_2D_3$ regulation. The 25-OHase and 24-OHase were successfully cloned in 1990 and 1991 respectively; however, 1-OHase was not cloned until late 1997. It was done by four independent research groups by employing different approaches in the cloning of the mouse, rat and human vitamin D_3 1-OHase (Fu *et al.*, 1997; Monkawa *et al.*, 1997; Shinki *et al.*, 1997; St-Arnaud *et al.*, 1997 and Takayama *et al.*, 1997). The successful cloning of 1-OHase benefits the study of its regulation and action at the molecular level.

1.2.4.2 Regulation of vitamin D_3 1-OHase

The vitamin D_3 1-OHase is tightly regulated by numerous factors. Its activity is stimulated by PTH, insulin-like growth factor I (IGF-I), hypocalcemia and hypophosphatemia, and suppressed by hypercalcemia, hyperphosphatemia and its product, $1,25\text{-}(OH)_2D_3$. However, the underlying mechanisms in modulating 1-OHase activity were not fully understood (Feldman *et al.*, 1996; Shinki *et al.*, 1997 and St-Arnaud *et al.*, 1997).

1.2.4.2.1 Parathyroid Hormone (PTH)

It is believed that the regulation of 1-OHase by PTH is mediated, at least in part, through both cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC) signaling pathways (Rost *et al.*, 1981 and Henry, 1985). The stimulatory effect of PTH on 1-OHase transcript levels is cAMP dependent and is successfully blocked by a transcriptional RNA synthesis inhibitor, actinomycin D, in both rats and MCT cells (Murayama *et al.*, 1999). In 2000, Brenza and DeLuca revealed the blocking of 1-OHase activity by actinomycin D treatment in a porcine proximal tubule cell line (AOK-B50), with stably transfected opossum PTH/PTH-related protein (PTHrP) Type I receptor (Bringhurst *et al.*, 1993) and in a human kidney (HKC-8) cell line, which was SV40 transformed (Racusen *et al.*, 1997). They also showed that the effect of PTH is cAMP-dependent at transcriptional level by using forskolin, an inducer of cAMP (Seamon & Daly, 1981) and 8Br-cAMP, a cAMP analog. In addition, other data from Brenza and DeLuca suggest that the effect of PTH on 1-OHase transcription does not require *de novo* protein synthesis, and it is thought that inhibition of protein synthesis by cycloheximide may stabilize 1-OHase transcription.

1.2.4.2.2 Hypocalcemia

Hypocalcemia increases PTH secretion, which in turn, increases 1,25-$(OH)_2D_3$ production (Boyle *et al.*, 1971 and Fraser & Kodicek, 1973).

1.2.4.2.3 $1,25\text{-}(OH)_2D_3$

It is believed that the inhibitory effect of $1,25\text{-}(OH)_2D_3$ on 1-OHase is transcriptional (Feldman *et al.*, 1996). Its suppressive action is well described (Henry, 1979 and Booth *et al.*, 1985). Brenza and DeLuca demonstrated the inhibitory effect of $1,25\text{-}(OH)_2D_3$ on its transcription *in vivo* in AOKB50 cells (Brenza and DeLuca, 2000).

1.2.4.2.4 Hypophosphatemia

It is clear that phosphate (P) is an important regulator of renal $1,25\text{-}(OH)_2D_3$ production (Feldman *et al.*, 1996). Restriction of dietary P increases the conversion of the $25\text{-}OHD_3$ to $1,25\text{-}(OH)_2D_3$, and hence circulating $1,25\text{-}(OH)_2D_3$ (Tanaka & DeLuca, 1973; Hughs *et al.*, 1975; Gray & Napoli, 1983; Maierhofer *et al.,* 1984 and Portale *et al.*, 1986). However, the molecular mechanism of how hypophosphatemia influences $1,25\text{-}(OH)_2D_3$ production is still unknown.

1.2.4.2.4.1 Phosphate (P) Metabolism

A balanced P level is crucial in the body. It plays an important role in maintaining the basic processes of cellular metabolism, growth, and especially in mineralization of the skeleton. Bone mineralization depends on the regulation and availabilities of both inorganic P and Ca, which form the major mineral part of bone,

namely hydroxyapatite. A higher extracellular level of P is maintained in youth than in adulthood. The inorganic P is mainly retained by reabsorption in renal tubules. The P reabsorption is assessed by a ratio of the maximal rate of P reabsorption per unit volume of glomerular filtrate (max TRP / ml GFR or Tm P / GFR). This is termed as Tubular P Transport Capacity. This capacity and plasma P remain remarkedly high during active growing period (Albright & Reifenstein, 1948) and varies according to the growth rate of the individual (Corvilain & Abramow, 1972; Caverzasio *et al.*, 1982; Bonjour & Caverzasio, 1984 and Kiebzak & Sacktor, 1986).

The mechanism involved in regulating P reaborption is complex and known to be regulated by various factors. Previous studies (Albright & Reifernstein, 1948 and Caverzasio *et al.*, 1982) show that a higher level of max TRP / ml GF and plasma 1,25-$(OH)_2D_3$ is found in young growing rats than their adult counterparts. Popvtzer *et al.* and Pushchett *et al.* reported the increase of P reabsorption by 1,25-$(OH)_2D_3$ required the presence of PTH. It is found that chronic administration of growth hormone (GH) can increase renal P transport; however, acute effect (within hour) fails in rats (Westby *et al.*, 1977). It was suggested that GH exerts its indirect effect on renal P transport through the action of IGF-I. In 1990, Caverzasio *et al.* performed another similar experiment by administrating IGF-I to hypophysectomized rats, tubular P reabsorption was stimulated and plasma 1,25-$(OH)_2D_3$ level was found to be increased significantly. This further supports the assumption that IGF-I is an important factor in P metabolism and plays a crucial role in mediating the effect of GH on renal P handling and 1,25-$(OH)_2D_3$ production.

1.2.4.2.5 Growth hormone / insulin-like growth factor axis

Hypophysectomy abolishes the increase in renal 1,25-$(OH)_2D_3$ production and its serum level (Spencer & Tobiassen, 1977 and Gray, 1987) that are induced by the restriction of dietary P (Gray, 1981; Gray *et al.*, 1983; Gray & Garthwaite, 1985 and Gray, 1987). GH administration to the hypophysectomized rats can restore, at least in part, the responsiveness of serum 1,25-$(OH)_2D_3$ level (Gray *et al.*, 1983; Gray & Garthwaite, 1985 and Gray, 1987). These data provide evidence that GH can mediate the response of serum 1,25-$(OH)_2D_3$ level to dietary P change. However, it is not known whether the function of GH is direct or indirect. Previous data showed that IGF-I administration in hypophysectomized rats can restore serum 1,25-$(OH)_2D_3$ level to the same extent as GH, which was increased by dietary P restriction (Bernard *et al.*, 1988). It is known that IGF-I production is stimulated by GH and is believed to be the mediator of the growth-promoting action of GH. Therefore, the data from Bernard and Spencer in 1988, strongly indicated that the effect of GH is mediated by IGF-I to restore the serum 1,25-$(OH)_2D_3$ level in the hypophysectomized rats under dietary P restriction. However, the mechanism by which IGF-I influences 1,25-$(OH)_2D_3$ metabolism is not known.

Besides the various factors mentioned in Section 1.2.4.2, it is known that the serum 1,25-$(OH)_2D_3$ level vary with age. Many reports have shown the effect of aging on vitamin D_3 metabolism.

1.2.5 Aging and vitamin D_3

1.2.5.1 Definition of Aging

In 1995, De Boer and Rudman characterized aging as changes in structure, functions, metabolism and body composition. It closely resembles to those changes caused by adult GH-deficiency. As implied from this definition, aging is closely related to GH availability, and so to IGF-I synthesis, release and activity (Hammerman, 1987 and Toogood *et al.*, 1996).

1.2.5.2 Age-related change in vitamin D metabolism

Age-related changes include many physiological variations throughout the body. The body may or may not overcome or adapt to the changes in appearance, psychological changes, thus diseases may result. In 1997, Wong et al. reported that 1-OHase activity in aging rats failed to increase after five days of LPD. Another experiment from Wong et al. in 2000 showed that aging rats failed to increase 1,25-$(OH)_2D_3$ production under hypocalcemia. In 1994, Friedlander *et al.* indicated that young rats increase renal 1,25-$(OH)_2D_3$ production by PTH in a dose-dependent manner; however, aging rats failed to respond to PTH stimulation. These findings reveal a correlation between aging and the loss of renal 1-OHase response to some major stimuli, such as hypophosphatemia, hypocalcemia and PTH.

Vitamin D_3 metabolism is controlled by many factors including parathyroid hormone, IGF-I, the availability and the metabolism of the Ca and P content, the active vitamin D_3 metabolite, 1,25-$(OH)_2D_3$ and age. Previous studies only concentrated on the study of the changes in serum 1,25-$(OH)_2D_3$ level, the enzymatic activities as well as the transcripts level of 1-OHase. Little attention was paid to the regulation of protein expression of renal 1-OHase. Due to the successful cloning of the 1-OHase genes, it is now possible to produce its antibody. Our research group has obtained a polyclonal anti-1-OHase antibody from Birmingham, UK. In this study, by using the anti-1-OHase antibody, it is possible to study its regulation by LPD at the translational level, and correlate to the result from previous studies in terms of 1-OHase activity (Wong *et al.*, 1997 and Wong *et al.*, 2000) and mRNA transcriptional level (Murayama *et al.*, 1999 and Brenza & DeLuca, 2000).

As mentioned in Section 1.2.4.2, regulation of 1-OHase is closely related to various factors. In particular, the regulation by PTH was extensively studied; whereas the mechanism for how IGF-I regulated 1-OHase was still poorly understood. Previous studies have demonstrated aging resulted in blunted response of 1,25-$(OH)_2D_3$ production, suggesting that IGF-I might be involved in 1-OHase regulation and this might be age-related. In the present study, IGF-I axis was studied to determine how their regulation altered with 1-OHase expression and age under dietary P restriction.

1.3 Insulin like growth factor axis (IGF-axis)

The insulin-like growth factor (IGF) axis consists of IGF peptides, IGF binding proteins (IGFBPs), IGFBP proteases and insulin-like growth factor receptor (IGFIR).

1.3.1 Insulin like growth factors (IGFs)

IGFs are members of the insulin-related-peptide family, including insulin, IGF-I, and IGF-II (Table 2). The first two IGFs characterized were IGF-I and IGF-II. They were isolated from adult human plasma by Rinderknecht & Humbel in 1978. They were referred to as somatomedins in 1972 (Daughaday *et al.*, 1972). Somatomedin was used to denote the uncharacterized factor that mediates the action of GH in the stimulation of somatic growth and displays insulin-like activity (Sara & Hall, 1990). Both IGF-I and IGF-II are single-chain polypeptides with three intrachain disulfide bridges consisting of 70 (Mol. Wt. 7,646) and 67 (Mol. Wt. 7,471) amino acids, respectively. The former has a 3-dimensional structure similar to the proinsulin molecule and, thus, to its immunoreactivity and receptor binding properties. The latter is a neutral peptide which has a high structural homology with IGF-1. Their amino acid sequences have identical residues in 45 positions, giving a sequence homology of 62 % (Figure 3).

Characteristic	IGF-I	IGF-II	Insulin
Structure			
Amino acids (mature)	70	67	51
Homology[1] (A & B domains)	I vs. Ins 43%	I vs. II 74%	II vs. Ins 41%
Cystine bonds	3	3	3
C Peptide	Intact	Intact	Removed
D Region	Present	Present	Absent
Histidines (Zn binding)	Absent	Absent	Present
Activity			
Cellular action	↑ Hypertrophy	↑ Hypertrophy	↑ Hypertrophy
Consequence	↑ Size	↑ Size	↑↑↑ Size
	↑↑ Hyperplasia	↑↑ Hyperplasia	↑ Hyperplasia
	↑↑ Differentiation	↑↑ Differentiation	↑ Differentiation
	↑ Survival	↑ Survival	↑ Survival
Receptors			
Name	Type 1 IGF	IGF - Ins hybrid	Insulin
Structure, = size	$\alpha\beta\beta\alpha$, 370 kDa	$\alpha\beta\beta'\alpha'$, 370 kDa	$\alpha'\beta'\beta'\alpha'$, 370 kDa
Ligand preference	I > II >> Ins	I > Ins ? II	Ins >> II > I

[1]The type 1 IGF-insulin hybrid receptor ($\alpha\beta\beta'\alpha'$), for lack of a better location in the table, is listed under IGF-II but is not the type 2 IGF receptor. An IGF-mediated function of the type 2 IGF receptor has not been clearly defined and is not included in the table. I = IGF-I, II = IGF-II, and Ins = Insulin.

Table 2: Characteristics of the members of IGF superfamily (Mccusker, 1998).

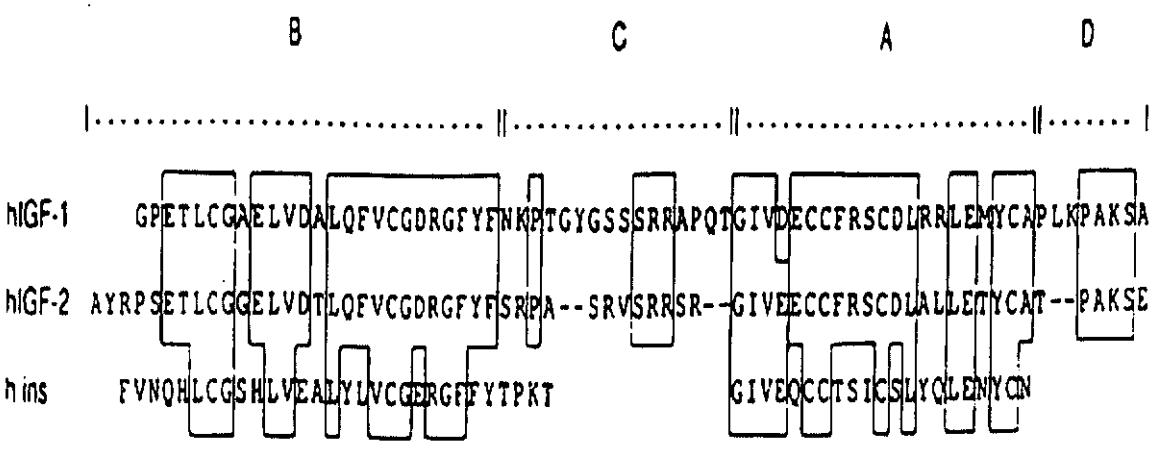

Figure 3: Primary structure of human IGF-I, IGF-II and insulin. Parts of the alignment are shown to give maximal homology. The dash line represents truncation or introduced gaps. Conserved residues between human (h) IGF-I, IGF-II, and insulin are boxed (Sara & Hall, 1990).

1.3.1.1 Discovery of IGFs

IGFs were first discovered as three separate biological activities in serum, namely sulfation factor activity (SFA), nonsuppressible insulin-like activity (NSILA), and multiplication-stimulating activity (MSA) (Sara & Hall, 1990).

Salmon & Daughaday in 1957 suggested that the growth-promoting action of growth hormone was mediated by a substance in which was defined as "sulfation factor activity". It was proposed that the stimulation of sulfate uptake into the cartilage was by the action of serum from normal, but not hypophysectomized rats. The restoration of the serum growth promoting activity was reported in hypophysectomized rats by growth hormone (GH treatment) despite GH itself having no direct effect.

Froesch et al. investigated another line of observation regarding insulin-like substances. By the development of radioimmunoassay for insulin, it is obvious that serum contained far more insulin-like activity than from the content of immunoreactive insulin. This insulin-like activity was expressed as NSILA by the Froesch group in 1963 (Froesch *et al.*, 1963).

In the 1960s, a third team, Dulak & Temin, investigated the serum components needed for the growth of cells in culture. They found that some cell lines depended on the presence of specific factors in serum for their cell proliferation; however, in other cell lines, they did produce their own growth-promoting substances. This activity was

identified in the conditioned medium of rat liver cells and termed " MSA" by Dulak and Temin.

It was clear that the three activities (SFA, NSILA and MSA) represented a similar group of substances with a much wider biological activity than first suspected simply as SFA, NSILA, or MSA. This group of substances was introduced as 'somatomedin' (Daughaday *et al.*, 1972), for which some uncharacterized factors were described that mediated the GH action in the stimulation of somatic growth and displayed insulin-like activity.

1.3.1.2 Function of IGFs

IGFs are potent mitogenic and anti-apoptotic molecules involved in the regulation of cell proliferation in renewing epithelial cell populations of organs including the breast, prostate, colon and lung. They are the anabolic hormones that are active throughout our life. Their action is dependent on the responsiveness of the target cells. When the cells undergo hyperplastic phase, proliferation will result. While the cells are in hypertrophic growth, or are mature, i.e., nondividing cells, such as nerve cells, some anabolic responses result instead, such as protein synthesis (Sara & Hall, 1990). Some studies reported that various tissues synthesized IGF-I during both fetal and adult stages of life. This suggested that IGF-I acts locally (i.e. autocrine or paracrine) along with its endocrine action (D'Ercole *et al.*, 1980 and Adamo *et al.*, 1991). With the ablation of hepatic IGF-I production, it was shown that growth and development was hampered in

mice (Yakar *et al.*, 1999). The circulating IGF-I exerts its endocrine action in somatic cell growth and development. The gene expression level of IGF-I was found to increase from 10 to 100 fold between birth and adulthood (Roberts *et al.*, 1986). Unlike many other regulatory peptides, IGFs have characteristics of both classic 'endocrine' hormones and characteristics of tissue growth factors.

1.3.2 Insulin-like growth factor receptors (IGFIR)

IGFs trigger cellular effects by binding to specific receptors. Type I IGF receptor is structurally and functionally homologous to insulin receptor but binds IGF-I with higher affinity than IGF-II or insulin. However, the monomeric type II IGF receptor is a single-chain polypeptide of molecular weight ~250K. It is structurally similar to the mannose-6-phosphate receptor but not to insulin or type I IGF receptor (Jones & Clemmons, 1995). It has a higher affinity for IGF-II than IGF-I but cannot recognize insulin. The cellular action of IGFs depends on their binding to the kinase receptors. Unlike IGF-I receptor and insulin receptor, the receptor for IGF-II exhibits no intrinsic kinase activity (Figure 4). However, type I IGF receptor and insulin receptor are similar in structure, and share intracellular signaling cascades (Werner & LeRoith, 1995).

IGF-I receptor is a dimeric glycoprotein with a molecular weight between 300K and 350K. It possesses 2 α- and 2 β- subunits in which the α-subunits (~135KD) are exposed extracellularly and β- subunits (~100KD) are transmembranal (Figure 4). The primary structure of human IGF-I receptor was identified from placenta cDNA (Ullrich *et*

al., 1986). It was cloned and sequenced in 1986 by Ullrich *et al.* The mature β-α-α-β receptor was encoded by a precursor protein of about 152KD. The α-subunit, which contains a cysteine-rich domain, is responsible and important for ligand binding (LeRoith *et al.*, 1995), whilst the β-subunit has a cytoplasmic domain with intrinsic tyrosin kinases. In contact cells, IGF-I stimulates tyrosine phosphorylation of the receptor, and consequently, other cellular proteins as well (Jacobs & Cuatrecasas 1986). There is a strong evidence that tyrosine kinase activity of IGF-I receptor is involved in generating cellular signals (Moxham & Jacobs, 1992).

1.3.3 Insulin like growth factor binding proteins (IGFBPs)

At least six IGFBPs have been identified. They are soluble proteins that are ubiquitously present in extracellular fluids, and all of which bind both IGF-I and IGF-II. Most of the IGF peptides circulating in the blood form a complex with IGFBPs and a glycoprotein known as acid-labile subunit (ALS). This ternary complex is stable and with a high molecular weight (~150KD) (Baxter, 1994). Liver produces three components of the ternary complex (Cohen & Rosenfel, 1995), in which the hepatic endothelia and Kupffer cells synthesize IGFBP3, while hepatocytes produce IGF-I and ALS (Arany *et al.*, 1994; Chin *et al.*, 1994 and Villafuerte *et al.*, 1994). The hepatic production of these three components are all stimulated by GH (Underwood *et al.*, 1994). Less than 5% of IGF-I is in free form in the blood circulation (Clemmons and Van, 1984). Complexed IGF-I (15 hours) has a prolonged half- life compared with the free IGF-I peptide (10 to 12 minutes) (Juul *et al.*, 1995 and Rosen & Conover, 1997). In adult

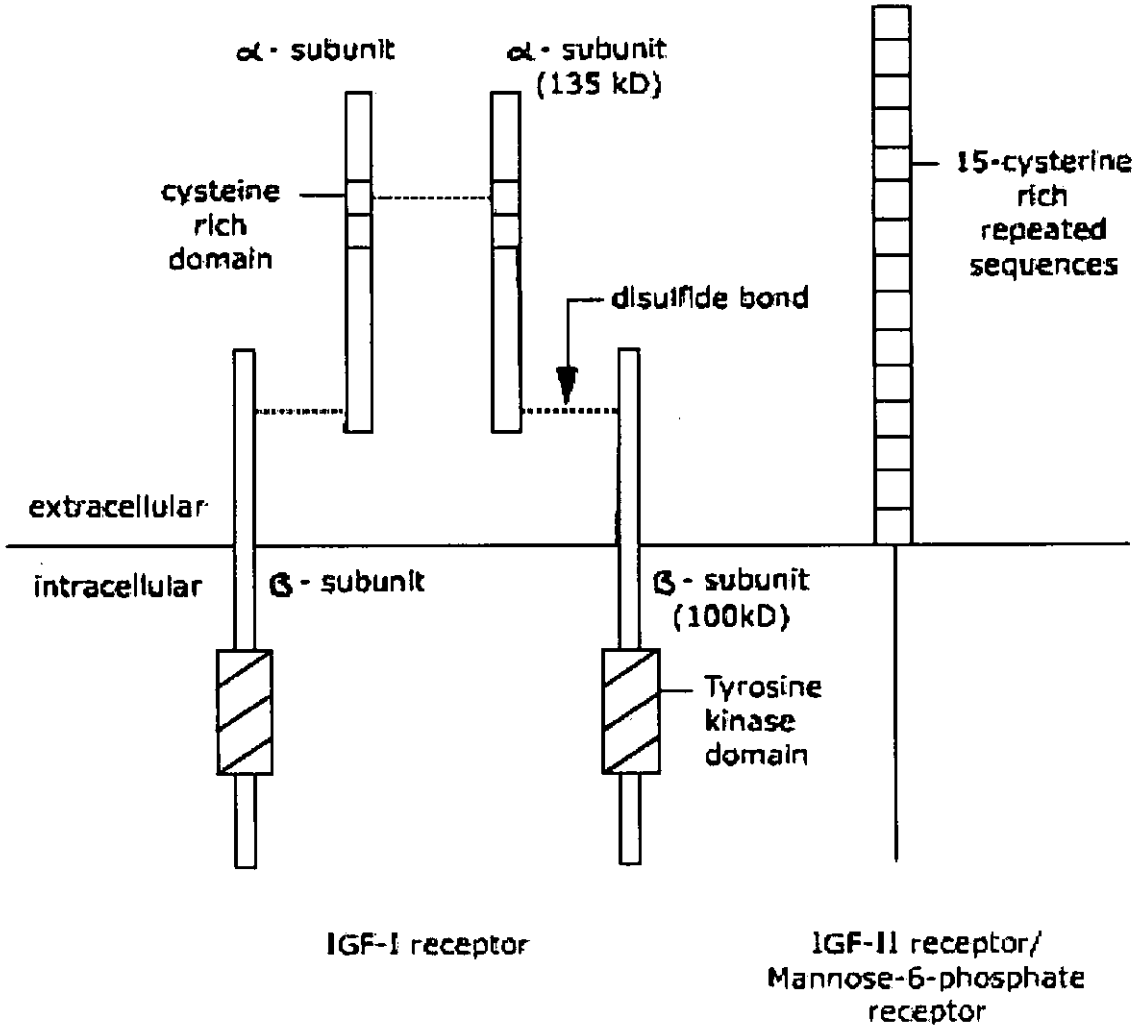

Figure 4: Schematic structures of IGF-I and IGF-II receptors.

circulation, IGFBP3 is the most abundant among six known binding proteins, which have mean levels of 3-5 mg / L (Baxter & Martin, 1986 and Blum *et al.*, 1990). The IGFBP3 level is tenfold higher than the other BP species. However, it is less important in the early childhood circulation (1 mg / L).

When the complex reaches the receptor, IGF-I is released by an action of a protease (Figure 5). The released IGF-I binds to the extracellular α-subunit of the IGF-I receptor and triggers a series of activations. The binding is facilitated and regulated by IGFBPs. The IGFBPs modulate the availability of the circulating IGF-I, and hence the response of cells (Sara & Hall, 1990). In 1987, Elgin et al. demonstrated that IGFBP enhances stimulation of DNA synthesis in fibroblasts (Elgin *et al.*, 1987) by IGF-I as it facilitates the IGF-I binding to the cell surface (Clemmons *et al.*, 1987). The binding of IGFBPs to IGF-I is thought to limit the metabolic effects of the IGF-I pool in circulation. This, in turn, alters IGF-I signal transduction through this transmembrane signaling unit.

1.3.4 Signal Transduction along the IGF-I axis

Insulin-like growth factor I receptor belongs to the large family of growth factor receptors with intrinsic tyrosine kinase activity. Following IGF-I binding to the extracellular α-subunit, conformational change of the receptor results. It allows substrate and Mg^{2+}-ATP bind to the active site, thereby inducing autophosphorylation on multiple tyrosine residues within the β-subunit cytoplasmically (Hubbard *et al.*, 1994).

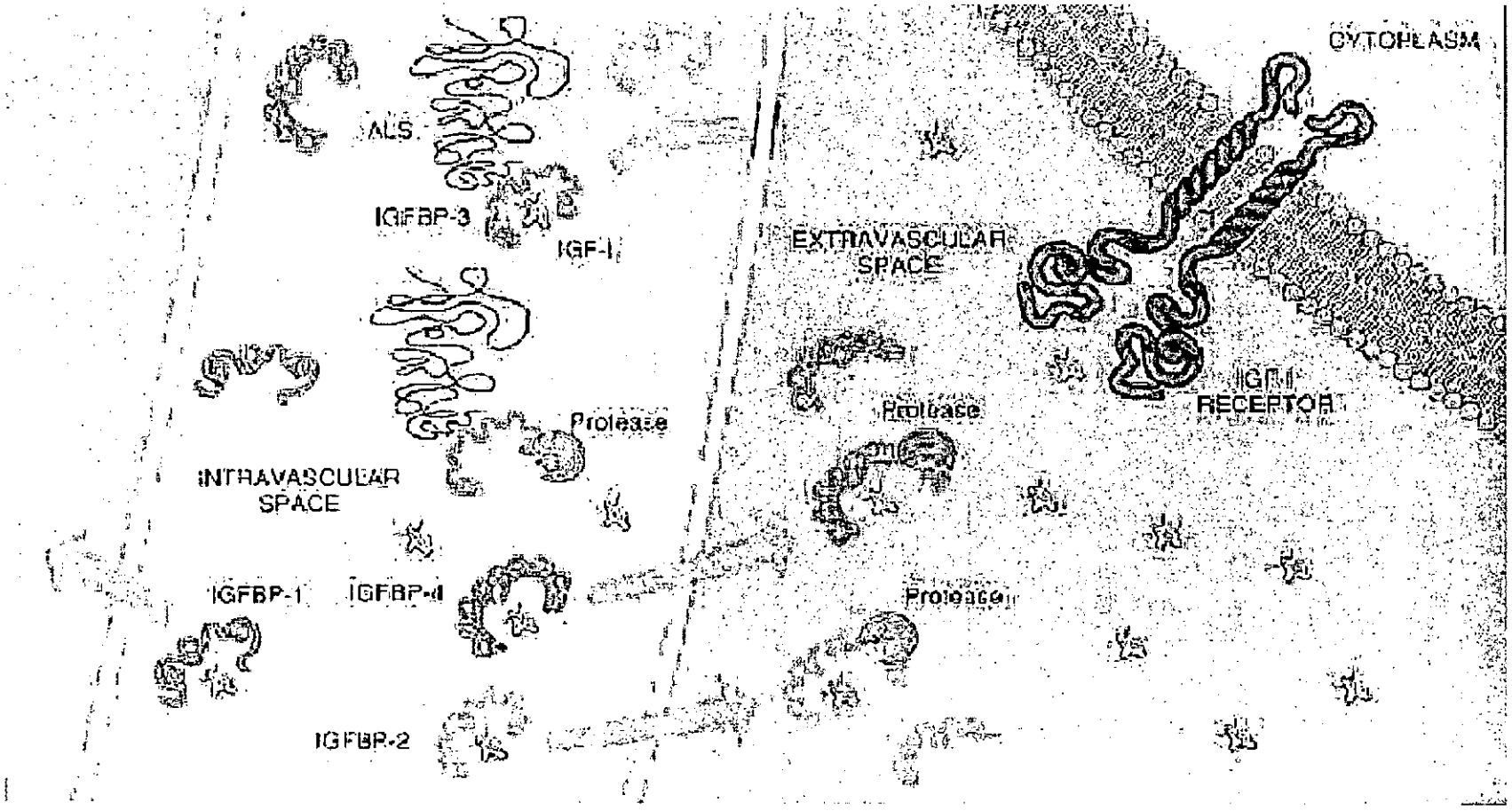

Figure 5: Schematic diagram of IGF-I delivery. IGF-I is delivered by IGFBP-acid-labile subunit (ALS) complex to the IGF-I receptor through the bloodstream. IGF-I is released by a protease and exposed to the extracellular IGF-I receptor alpha subunit.

The major sites for phosphorylation are clustered tyrosines at amino-acid position 950, 1131, 1135, 1136, 1250, 1251 and 1316. The autophosphorylation activates the receptor tyrosine kinase and leads to phosphorylation of other protein substrates (Werner & LeRoith, 2000). The major difference between the IGF-I receptor and other tyrosine kinase receptors is the utilization of a family of docking proteins called insulin receptor substrate (IRS1-4) proteins by IGF-I receptor (Sun *et al.*, 1991; Myers *et al.*, 1993; Sun *et al.*, 1993 and Cheatham & Kahn, 1995). Other docking proteins, such as Src-homology collagen (Shc), are also known to be involved (Dey *et al.*, 1996). These docking proteins then bind to some Src-homology 2 (SH2)-containing proteins, including Grb-2, the p85 regulatory subunit of phophoinositide 3'kinase (PI3'K) and tyrosine phosphatease, SyP (Lavan *et al.*, 1992; Lee *et al.*, 1993; Skolnik *et al.*, 1993 and Sun *et al.*, 1993). They are adaptor proteins, which do not have any intrinsic enzymatic activity. The adaptor proteins continue the signal by additional protein-protein interaction until the signal is transmitted to its end destination to trigger a response, e.g. protein synthesis for growth, differentiation, proliferation or apoptosis. A typical signaling pathway for IGF-I receptor is summarized in Figure 6.

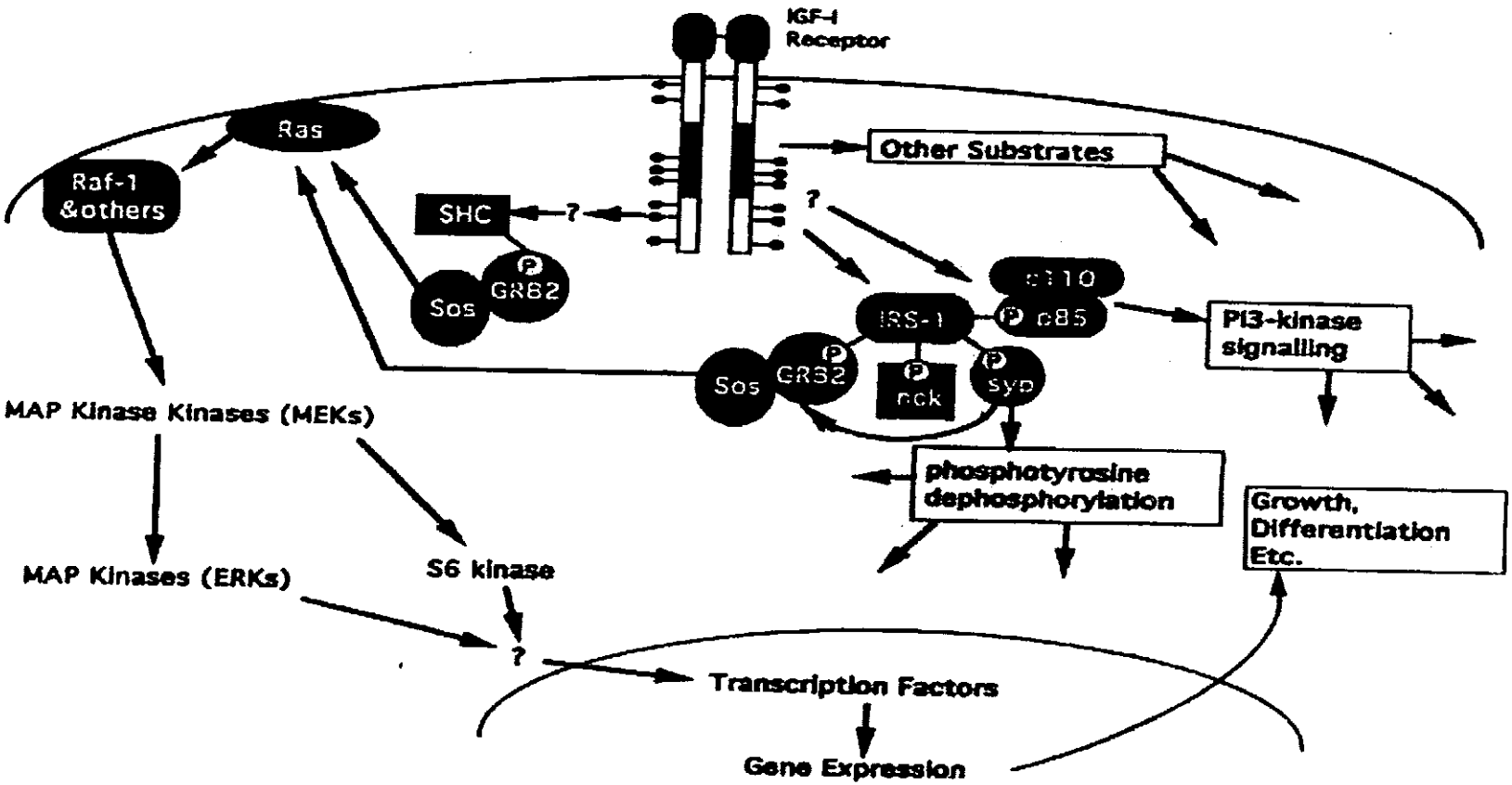

Figure 6: Schematic representation of intracellular signaling pathways of the IGF-I receptor. The activated IGF-I receptor phosphorylates a number of adaptor proteins, such as IRS-I, Shc. These phosphorylated proteins interact with other downstream molecules, e.g. Grb2, p85, Syp and Nck through their SH2 domains. These interactions then activate a cascade of protein kinases and other signaling pathways (LeRoith *et al.*, 1995).

1.3.5 Regulation of IGF-I

IGF-I is mainly produced in the liver, but local production is also found in other tissues. Its biosynthesis is highly GH-dependent, but the local synthesis is, at least partially, GH-dependent in some IGF-I responsive tissues, such as the heart, lung and pancreas. Growth hormone is synthesized and released from the anterior pituitary gland by the GH-releasing hormone (GHRH), whereas its secretion and production are inhibited by somatostatin. GH inhibits its own secretion by stimulating the somatostatin secretion and inhibiting the GHRH secretion. When GH binds to the growth hormone receptors on hepatic cell membrane, the receptor is activated, and eventually leads to gene expression and translation of IGF-I and its binding proteins. The newly synthesized IGF-1 and binding proteins are either released to the blood stream to complete their endocrine action or to perform local paracrine or autocrine actions through the peripheral fluid. The circulating IGF-1 has a negative feedback to the hypothalamus, in order to control the release of growth hormone. In 1987, Daughaday and Trivedi showed that patients with Laron dwarfism, who lack GH receptors, have the lowest IGF-I level, and that patients who have complete GH deficiency have a decreased IGF-1 level. Besides GH dependency, it is suggested that nutrients also regulate IGF-I production (Hall & Sara, 1983). Emler and Schalch have demonstrated the fact that refeeding restores hepatic mRNA IGF-I level of a fasting rat (Emler & Schalch, 1987). A schematic diagram of IGF-I and Growth Hormone regulation is shown in Figure 7.

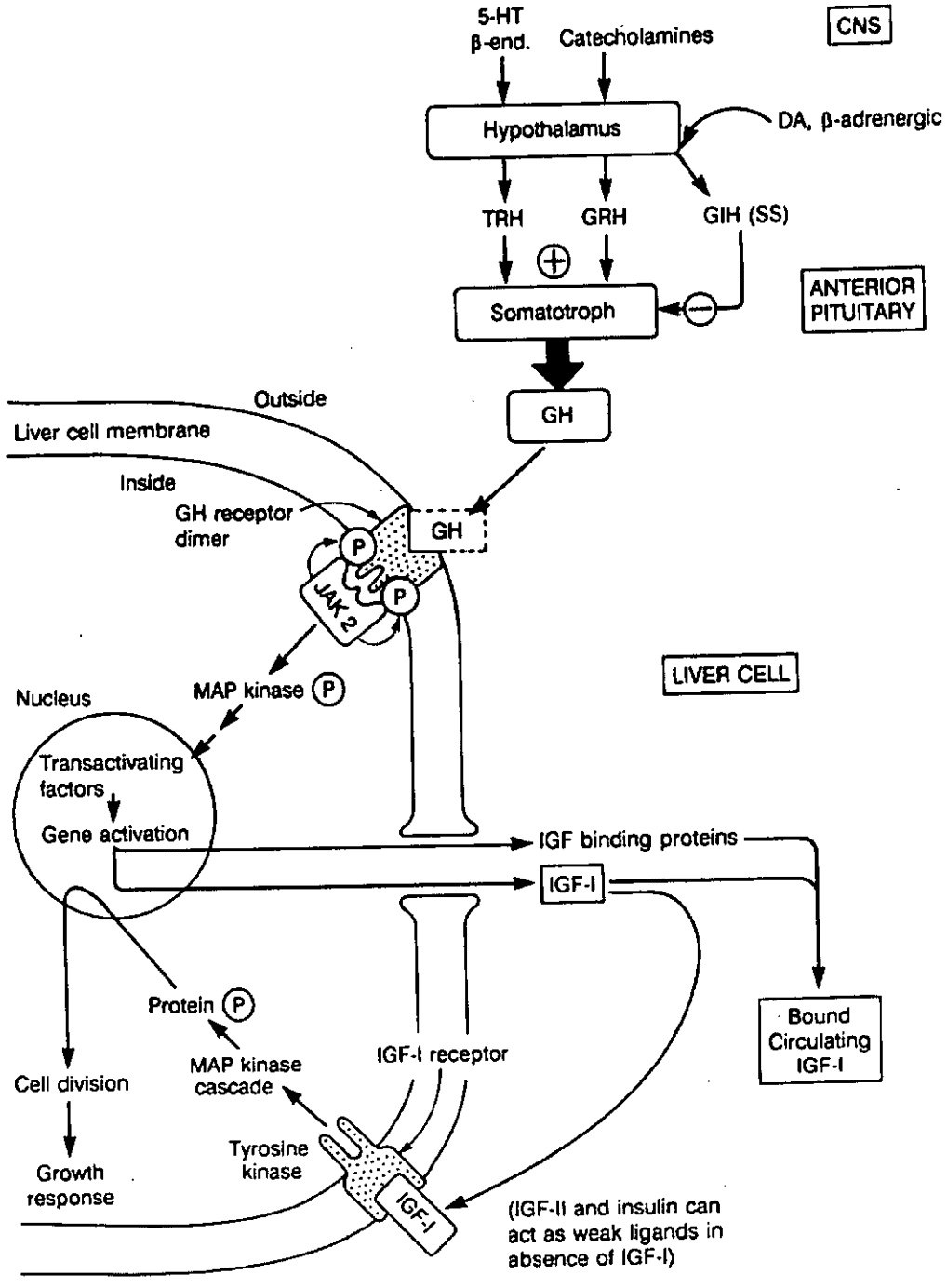

Figure 7: Schematic diagram of the regulation of IGF-I by GH. GH is released from the anterior pituitary gland. It then binds to the hepatic GH receptor to initiate the production of both the IGF-I and IGF-BP. Both products are then released to the bloodstream in order to act on other target tissues, or IGF-I acts locally to promote somatic growth (Norman & Litwack, 1997).

Besides the GH, the availability of IGF-I was also varied with age. It is found that the mean IGF-I level was lower in the elderly which is similar to that detected in GH-deficiency patients (Hammerman, 1987 and Toogood *et al.*, 1996).

1.3.6 Aging and IGF-I

1.3.6.1 Age-related variation in IGF-I level

As mentioned in Section 1.2.5.1, aging is defined (Hammerman, 1987 and Toogood *et al.*, 1996) and is related to the GH availability and hence the IGF-I. Age-related variation in circulating IGF-I level increases in puberty and decreases in the elderly. The decrease in IGF-I level in elderly can be attributed to various factors, such as the change in gonadal sex steroid hormones, impaired somatotroph secretion, nutritional status and age-related variation in IGF binding proteins (IGFBPs) (Arvat *et al.*, 2000).

1.3.6.1.1 Gonadal hormones

Clemmons and Vans proposed that age-related decrease in gonadal hormones may account for majority of the decrease in IGF-I activity in the elderly. Estradiol stimulates IGF-I synthesis and secretion from non-hepatic tissues (Hernandez, 1995 and Attie *et al.*, 1990) and up-regulates the hepatic GH-receptors (Gabrielsson *et al.*,

1995). However, estrogen replacement does not increase IGF-I levels in the menopausal women because it increases the pulsatile GH secretion (Dawson-Hughes *et al.*, 1986; Wiessberger *et al.*, 1991; Kelly *et al.*, 1993; Bellantoni *et al.*, 1996 and Friend *et al.*, 1996). This implies that estradiol impairs the post-GH mechanism and its peripheral sensitivity (Wiessberger *et al.*, 1991 and Mannor *et al.*, 1991). Although decrease in IGF-I level is gender-independent, pulsatile GH secretion in women is more obvious (Clemmons & Van, 1984).

Despite the association between total IGF-I level and gonadal steroid levels, no relationship between total IGF-I level and free androgen and estrogen levels has been found. It is suggested that there is no direct interaction between the biological activity of IGF-I level and gonadal hormones (Janssen *et al.*, 1998). However, it is a fact that both the IGF-I level and gonadal hormones change with age.

1.3.6.1.2 Somatotroph secretion

The age-related reduction of IGF-I levels is GH-dependent. It is reported that GH production rate reduces progressively after puberty at a rate of 14% per decade (Clemmons & Van, 1984). Previous studies showed that GHRH also decreases in the elderly (Corpas *et al.*, 1993 and Ghigo *et al.*, 1996). Because of the relationship between aging and GH deficiency, there is some rationale to treat elderly with somatropin (recombinant human GH) and / or IGF-I, GHRH or its synthetic GH secretagogues to reverse the age-related changes in body functions and

structures (Rudman, 1985 and Corpas *et al.*, 1993). However, in terms of its benefits and adverse effects, use of GH on elderly is not encouraged (Ghigo *et al.*, 1997; Smith *et al.*, 1997 and Ghigo *et al.*, 1998).

1.3.6.1.3 Nutritional status

Starvation and chronic malnutrition reduce IGF-I synthesis. It is reported that long-term nutritional impairment may lead to GH resistance (Thissen *et al.*, 1994; Underwood *et al.*, 1994 and Ross & Chew, 1995). Sufficient energy and proteins are needed in order to maintain their effectiveness in GH receptor and postreceptor mechanisms, and on IGF-I post-translational mechanisms and peripheral degradation of IGF-I (Thissen *et al.*, 1994; Underwood *et al.*, 1994 and Ross & Chew, 1995). Deficiency of some micronutrients may impair GH and IGF-I activities. Lack of zinc leads to peripheral GH resistance and a decrease in hepatic IGF-I mRNA expression (Martin *et al.*, 1997). Depletion of proteins and micronutrients always occurs in the elderly (Rosen & Conover, 1997), and may, at least in part, account for the reduced IGF-I synthesis in the elderly population.

1.3.6.1.4 Insulin-like growth factor binding proteins (IGFBPs)

The level of IGFBPs exerts a short-term modulation of IGF-I bioavailability (Lee *et al.*, 1993 and Rajaram *et al.*, 1997). Circulating IGFBP-3 level increases in puberty, as does IGF-I. Its level declines progressively. However, the availability

of IGFBP-1, which inhibits the IGF-I availability, increases with age (Rutanen *et al.*, 1993; Benbassat *et al.*, 1997 and Rajaram *et al.*, 1997), so it has become a significant determinant of aging.

1.4 Correlation of dietary P restriction, IGF-I regulation, vitamin D_3 metabolism and aging

IGF-I is tightly regulated by GH. IGF-I is believed to mediate the action of GH. Many previous studies reported that administration of IGF-I (Bernard *et al.*, 1988) or GH itself (Gray *et al.*, 1983; Gray & Garthwaite, 1985 and Gray, 1987) restored the increase in serum 1,25-$(OH)_2D_3$ level and renal 1-OHase activity in the hypophysectomized rats under LPD treatment. However, under low P condition, renal 1-OHase activity did not increase in aging rats (3 months and 24 months of age), but IGF-I infusion can restore the LPD-induced increase in renal 1-OHase activity and serum 1,25-$(OH)_2D_3$ level in these rats (Wong *et al.*, 1997). In 1996, Wu and his co-worker demonstrated the decreases in the mRNA expression of renal 24-OHase under LPD (Wu *et al.*, 1996). As anticipated, the mRNA and protein of renal 1-OHase expressions were found to increase under dietary P deprivation (Yoshida *et al.*, 2001). These data strongly suggested that LPD regulates 1,25-$(OH)_2D_3$ in which it involves the action of 1-OHase and is IGF-I dependent. However, the underlying cellular and molecular mechanisms by which P regulates the renal production of 1,25-$(OH)_2D_3$ and how the IGF-I involved in different age models are not known. A schematic diagram of the correlation between vitamin D metabolism and the IGF-I regulation is summarized in Figure 8.

As argued in last section, a tight correlation was found among vitamin D metabolism, LPD, IGF-I and aging. In the present study, it was proposed that the change in 1-OHase response during LPD is due to change in responsiveness to IGF-I (protein expression in IGF-I axis) in rats and the change is age-dependent. Owing to the successful cloning of cDNA for 1-OHase, it is possible to study its regulation at the transcriptional and translational levels. This study was mainly divided into two parts. Part 1 (Chapter 3) involved the regulation of renal 1-OHase protein by low P condition. Part 2 (Chapter 4) examined the effect of LPD on the regulation of several proteins in the IGF-I axis. The following diagram shows a proposed model of IGF-I signaling pathway for the vitamin D metabolism under low P condition in a rat model (Figure 9).

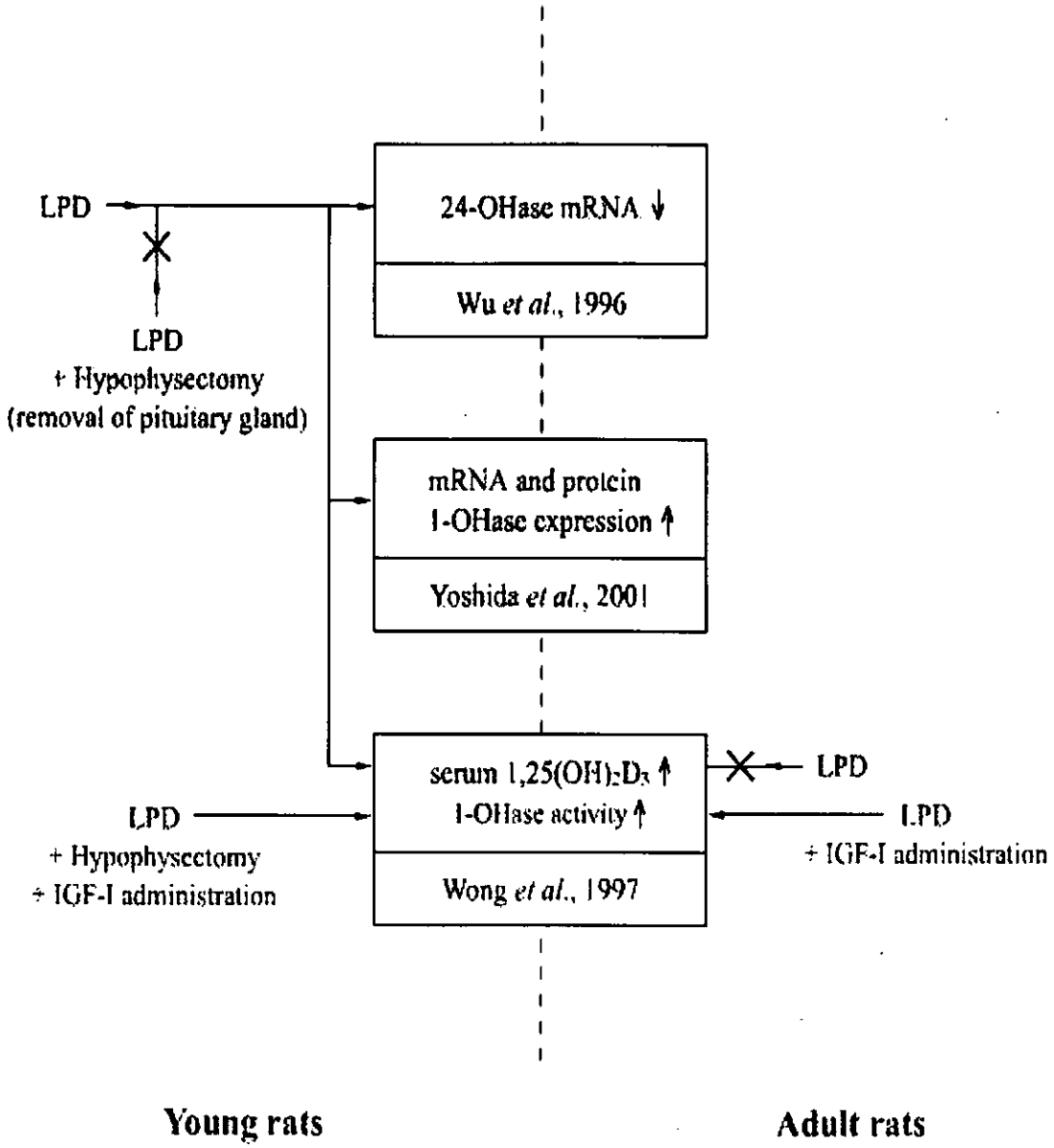

Figure 8: Schematic diagram of the correlation between vitamin D metabolism and the IGF-I regulation under low phosphate condition in rats.

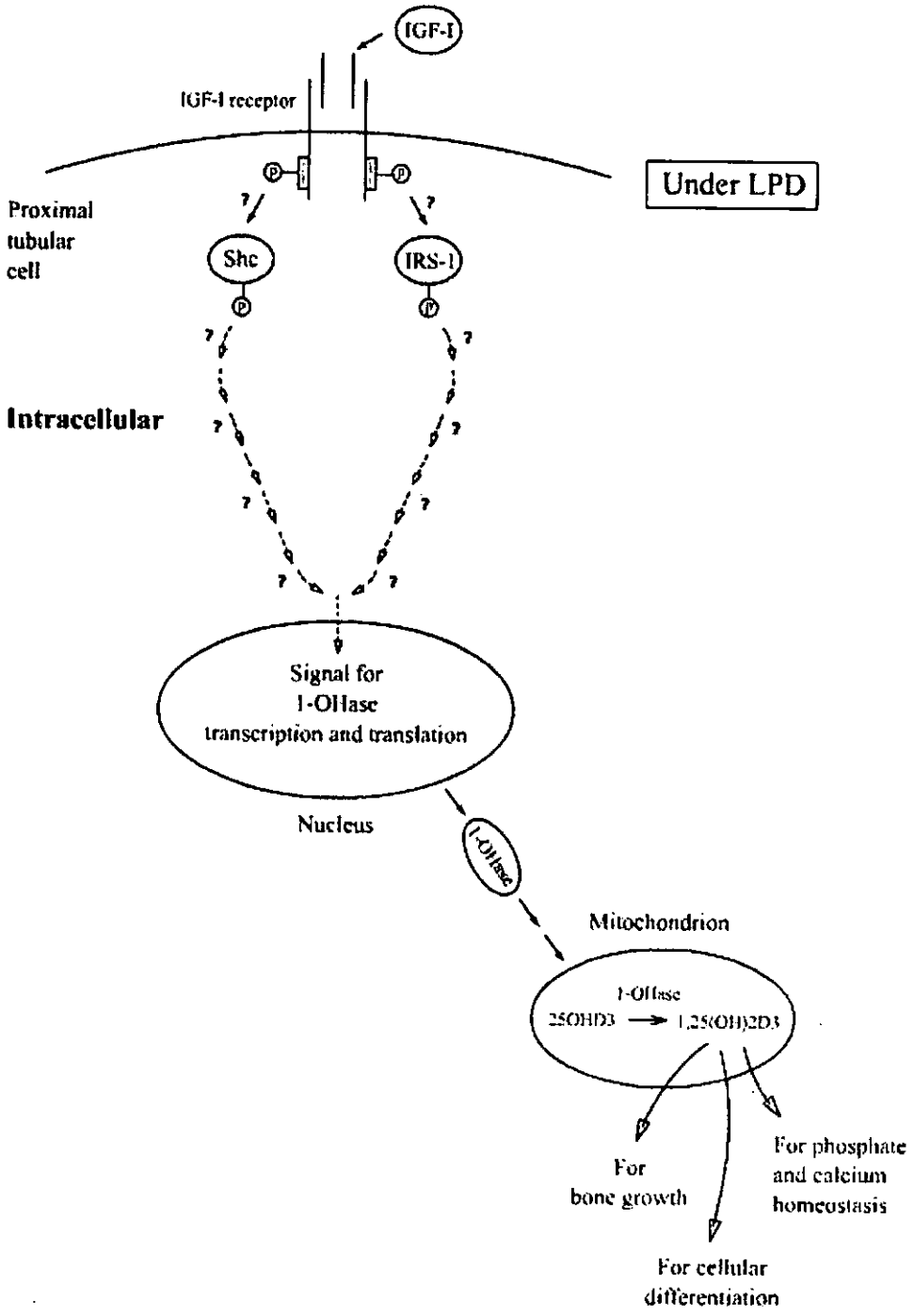

Figure 9: Proposed model of IGF-I axis and its signaling pathway on the regulation of 1-OHase in a renal proximal tubular cell in rat fed LPD.

Chapter Two

Hypothesis, Objectives and Significance

2.1 Hypothesis and Objectives

Phosphate (P) is a well-known determinant of 1,25-$(OH)_2D_3$ production. Dietary P restriction increases the synthesis and serum concentration of 1,25$(OH)_2D_3$. The rate of its production inversely varies with serum P concentration. It is also known that regulation of 1,25-$(OH)_2D_3$ is IGF-I- and age-dependent upon hypophosphatemia. However, the underlying mechanisms are still not known.

2.1.1 Hypothesis

The hypothesis of the present study are (1) the regulation of renal 1-alpha hydroxylase by dietary P deprivation is age-dependent; (2) low phosphate diet (LPD) can modify the responsiveness of renal proximal tubules to IGF-I by altering the expression of proteins in IGF-I axis in rats and this modification is age-dependent; and finally (3) the change in protein expression in IGF-I axis upon LPD is responsible for the change in renal 1-OHase protein expression in rat model.

2.1.2 Objectives

The study was designed to characterize the regulation of renal 1-OHase in rat during LPD feeding and to determine its effect on the expression of signaling proteins in IGF-I axis in rat renal proximal tubules. By using rats as animal model, it was (1) first to determine if the regulation of 1-OHase protein changes

with age; (2) to determine if the stability of 1-OHase protein altered; (3) to study the expression of insulin-like growth factor (IGFIR), insulin receptor (IR), insulin receptor substrate-1 (IRS-1) and src-homology collagen (Shc) and (4) to study if the stability of these proteins change in response to low P diet in both young and adult rats.

2.2 Significance of the study

Osteoporosis is one of the major public health problems of the elderly population worldwide. Its pathogenesis is closely related to diet and to the physiological changes in bone and mineral metabolism with age. The age-related alteration in vitamin D metabolism is a contributing factor for the development of Type II osteoporosis. Understanding the complex interplay of hormonal and dietary factors in the regulation of renal 1-alpha hydroxylase at the molecular and cellular level is therefore important. It is hoped that novel therapeutic targets along the IGF-I axis can be identified for treatment of age-related osteoporosis.

Chapter Three

Expression of Renal 25-Hydroxyvitamin D_3-1 Alpha Hydroxylase protein in rats: regulation by dietary phosphate restriction and age

3.1 Introduction

The active 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) plays an essential role in phosphate (P) and calcium (Ca) homeostasis, bone growth, and cellular differentiation. Its synthesis is by the activation of vitamin D_3, which is either synthesized in the skin or ingested from food. The activation undergoes sequential 25-hydroxylation in the liver and 1-hydroxylation in the kidney (Jones *et al.*, 1998). The second hydroxylation is carried out by 25-hydroxyvitamin D_3 1-alpha hydroxylase (1-OHase). This enzyme belongs to the cytochrome P-450 superfamily and is predominantly localized in renal proximal tubular cells (Kawashima *et al.*, 1981). The activity of this enzyme is tightly regulated by PTH, Ca, P and its product, 1,25-$(OH)_2D_3$, itself. The successful cloning of 1-OHase cDNA (Fu *et. al.*, 1997; Monkawa *et al.*, 1997; Shinki *et al.*, 1997; St-Arnaud *et al.*, 1997 and Takayama *et al.*, 1997) facilitates studying of the mechanism in which low phosphate diet (LPD) treatment increases serum 1,25-$(OH)_2D_3$ concentration at the molecular level.

Extensive studies have demonstrated the effect of LPD on renal 1-OHase regulation. Serum P level has been shown to vary inversely with the renal 1-OHase protein expression (Yoshida *et al.*, 2001), activity (Wong *et al.*, 1997) and serum 1,25-$(OH)_2D_3$ level (Wong *et al.*, 1997) in rats with age ranged from 4 to 8 weeks. The decrease in serum P level by LPD could be restored, in part, by the adaptive increase in 1,25-$(OH)_2D_3$ production. Previous studies showed that the regulation was at the transcriptional level, with an increase in 1-OHase

mRNA (Yoshida *et al.*, 2001) and decrease in 24-OHase mRNA (Wu *et al.*, 1996) level. However, the mediating molecular mechanisms are unknown. Due to physiological change, adult rats aged between 12 and 16 weeks, failed to increase renal 1-OHase activity and serum 1,25-$(OH)_2D_3$ level during LPD (Wong *et al.*, 1997). This finding suggests that renal 1-OHase regulation by LPD is age-dependent. However, the regulation of renal 1-OHase protein in adult rats under LPD has not been determined. In addition, it is still not known if the increases in renal 1-OHase activity (Wong *et al.*, 1997), protein and mRNA expressions (Yoshida *et al.*, 2001) under LPD in young rats (4 to 8 week-old) require *de novo* protein synthesis. In 2000, Brenza and DeLuca showed that up-regulation of 1-OHase mRNA expression by PTH did not require *de novo* protein synthesis, and that the inhibition of protein synthesis might stabilize the 1-OHase transcript. However, their findings were only drawn from the mechanistic studies regarding the 1-OHase mRNA, but not at protein level, under the PTH regulation.

In this part of my study, protein expression of 1-OHase was investigated upon dietary P restriction in both young (4 to 8 weeks) and adult rats (12 to 16 weeks). In addition, protein stability of 1-OHase in rats fed normal P and low P diet is compared in order to give a further understanding of 1-OHase regulation at the translational level.

3.2 Materials and Methods

3.2.1 Animals and Diets

An animal model was employed to study the effect of low phosphate diet (LPD) on 1-OHase regulation. LPD was induced by a designated diet in order to provide a platform to study the mechanism of vitamin D regulation. Male Sprague Dawley (SD) rats were employed as our animal model as the purpose of this study was to explore the age-related effect on 1-OHase regulation, the hormonal change in female rats would vary during the experimental period and interfere the results detected. Studies by Wong showed that 5-day LPD treatment increased both serum 1,25-$(OH)_2D_3$ and 1-OHase activity in 4 to 8-week-old young rats, but not in 12 to 16-week-old adult rats (Wong *et al.*, 1997). In the present study, rats aged 4 to 8-week-old (as young rat model) and 12 to 16-week-old (as adult rat model) were also used. Expression of 1-OHase protein was measured to see if the change in vitamin D metabolism (reported by others) found in adult rats was due to the inability of the up-regulation of 1-OHase protein.

The two rat groups were fed with either a normal P diet (Harlan Teklad, USA; NPD; 0.65 % P, 0.60 % Ca; TD#98005) or a low P diet (LPD; 0.1 % P, 0.60 % Ca; TD#98004). Rats were first fed with NPD for two days to normalize the physiological level of serum P as the P content of their regular diet was lower than that of NPD. After two days of normalization, rats were fed either a NPD for two day (Day 0), or a LPD for one day (Day 1), two days (Day 2), three days

(Day 3), five days (Day 5) or seven days (Day 7). The Day 0 represented the control group for receiving zero day of LPD. The basal expression of the 1-OHase protein in the 2-day NPD control group, which served as Day 0 group in this study, was found no difference with that in a 5-day NPD fed group (Results not shown). A time-course of LPD was studied in order to show both the acute and chronic effects on the protein expression. The detailed composition of the diets was summarized in Table 3. The rats were given deionized distilled water *ad libitum*. The kidneys were then harvested and renal proximal tubular cells were isolated.

3.2.2 *In vivo* protein stability analysis

In order to determine whether the responses on 1-OHase to LPD in rats (Yoshida *et al.*, 2001 and Wong *et al.*, 1997) was due to a change in protein stability, an *in vivo* mechanistic study was performed by using a protein synthesis inhibitor, cycloheximide (CHX). Rats were fed with either NPD or LPD as described in Section 3.2.1. Two rat groups, Day 0 and Day 5, were used to receive CHX administration, as our data showed that a maximal up-regulation of renal 1-OHase protein expression takes place on Day 5 (data shown in the Results Section 3.3.2). A single administration of CHX was injected (n = 4) intravenously through the tail, with the dosage of 0.3mg / 100g body weight. This dosage has been found to inhibit over 95 % of protein synthesis in rat liver (Alessenko *et al.*, 1997) at 1 hour after injection. The rats were sacrificed at 0, 1, 2, 4 and 6 hours after CHX injection. Rats injected with 0.9 % NaCl alone served as control. Kidneys were collected, and renal proximal tubular cells were

isolated for measuring renal expression of proteins. Besides the expression level of proteins, percentage of protein degradation was also calculated in each time point by the equation, [(Expression level at 0 hour – Expression level at each individual time point) ÷ Expression level at 0 hour x 100%].

3.2.3 Preparation of Renal Proximal Tubules

The renal proximal tubules were used in the present study. It is known that renal 1-OHase was abundant in mitochondria, especially in renal proximal convoluted tubules (PCT). It was the principal site of 1,25-$(OH)_2D_3$ production (Brunette *et al.*, 1978 and Kawashima *et al.*, 1981), and was also the region responsible for Pi reabsorption. In addition, renal proximal tubules showed a high level of IGF-I receptor expression, thus allowing a parallel study of the regulation of IGF-I axis under LPD condition.

3.2.3.1 Preparation of kidney slices

Rats were deeply anesthetized with ether, and dissected along the abdomen. Blood was collected and placed on ice. The kidneys were removed and placed immediately in ice-cold Krebs Henseleit saline (KHS), 18 % NaCl (w / v), 1.15 % KCl (w / v), 0.11M $CaCl_2$, 0.2M KH_2PO_4, 3.8 % $MgSO_4$ (w / v), 1.8 % $NaHCO_3$ (w / v), pH 7.4, which was previously gassed with 95 % O_2 and 5 % CO_2 (Gas A) for 30 minutes. The proximal tubules were isolated from the detached kidneys following the protocol introduced by Vinay *et al.* in 1981. The

kidneys were decapsulated and were cut into half longitudinally. The medulla parts were removed and the resultant cortex portions were placed in ice KHS buffer. Renal cortex was then sliced into very thin layer using a microtome and the slices were pooled together.

	HarlanTeklad	
	Normal Diet (NPD) (0.6 % Ca, 0.65 % P)	Low Phosphate Diet (LPD) (0.6 % Ca, 0.1 % P)
	Cat. No.: 98005	Cat. No.: 98004
Composition	**G / Kg**	**G / Kg**
Casein	110.0	110.0
Egg Whit Solid, spray-dried	97.9	97.9
DL-Methionine	3.0	3.0
Sucrose	551.1	575.7
Corn Starch	100.0	100.0
Corn Oil	50.0	50.0
Cellulose	20.0	20.0
Vitamin Mix, Teklad (40060)	10.0	10.0
Potassium Phosphate, monobasic	**24.6**	--
Calcium Carbonate	14.7	14.7
Potassium Chloride	5.6	5.6
Sodium Bicarbonate	4.6	4.6
Magnesium Oxide	3.8	3.8
Sodium Chloride	3.7	3.7
Sodium Selenite	0.50	0.5
Ferric Citrate	0.21	0.21
Manganous Carbonate	0.123	0.123
Zinc Carbonate	0.056	0.056
Chromium Potassium Sulfate	0.0193	0.0193
Cupric Carbonate	0.011	0.011
Potassium Iodate	0.0004	0.0004

Table 3: Composition of the Normal Diet (NPD) and the Low Phosphate Diet (LPD)

3.2.3.2 Digestion of kidney slices

The pooled slices were washed with KHS buffer for three times and were digested with Type I collagenase (Worthington, USA), supplemented with 500 ul 10 % BSA. The mixture was transferred to a 50-ml Erlenmeyer flask and gassed (Gas A) for 30 seconds. The flask was capped and incubated in a shaking water bath for digestion at 37 °C for 45 minutes. The undigested tissue or collagen fibers were removed using a tea-strainer. The tissue suspension was then washed twice with 30 ml of ice-cold KHS and cells were collected after centrifuged for 1 minute at 600 rpm.

3.2.3.3 Isolation of proximal tubular cells

After washing, cell suspension was ready for separation by Percoll gradient. Percoll (Pharmacia, USA) was freshly diluted to a final concentration of 50 % with deionized water and salt solutions. Thus the final concentration of each salt was the same as that in KHS buffer. The diluted Percoll was gassed (Gas A) for 30 minutes. The tissue pellet was resuspended in ice-cold Percoll solution and the whole resuspension was allowed to spin at 9,000 rpm for 30 minutes at 4 °C. The lowest layer, identified to be proximal tubule cell, was collected by a 10-ml glass pipette. They were washed twice with ice-cold KHS buffer to remove any trace of Percoll solution.

3.2.3.4 Lysis of proximal tubular cells

After washing, the cells were lysed by Nonidet P-40 buffer, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 % glycerol, 1 % Nonidet P-40, 1 mM Sodium Vanadate, which was supplemented freshly with various protease inhibitors, 2 ug / ml leupeptin, 2 ug / ml aprotinin and 1mM PMSF and with additional protease inhibitor cocktail from Pharmacia. The cell lysate was resuspended in 2X Laemmli buffer. The resuspension was boiled for 5 minutes and chilled on ice after boiling. The sample was aliquoted and was kept at -20°C before further analysis. The protein content of the cell lysate was quantified by Bradford assay (BioRad, USA). All chemicals were purchased from Sigma, USA, unless otherwise specified.

3.2.4 Serum Collection

Blood was collected using a syringe with a 19 ½" needle through the abdominal aorta and was allowed to clot at 4 °C. The blood was centrifuged at 4,000 rpm for 20 minutes at 4 °C. The clear serum portion was collected and was ready for Ca, P and 1,25-$(OH)_2D_3$ measurements, where serum Ca and P levels were the result from two separate experiments with n = 7, while the result for 1,25-$(OH)_2D_3$ was from one experiment with n = 3.

3.2.4.1 Phosphate Chemistry

Serum inorganic P was detected by a commercial kit from Sigma Diagnostics ® (Cat No. 670-C). Blood cells should be removed from serum as quickly as possible in order to avoid hemolysis or diffusion. As hemolysis caused a release of organic P from the red cells, this process could disturb the actual amount of inorganic P in serum detected (Henry, 1964).

3.2.4.1.1 Basic Principles and Method

The underlying mechanism was based on the formation of a complex between the inorganic P in serum and the acidified ammonium molybdate. The complex was reduced by Fiske & Subba Row solution. A blue phosphomolybdenum complex was formed and could be detected at wavelength of 660nm.

The detection method of inorganic P could be referred to the Sigma Diagnostics ® procedure NO. 670. The detection method employed the method of Fiske and SubbaRow, which was introduced in 1925 (Kessler & Wolfman, 1964). The serum collected was diluted with deionized water and treated with 20 % (w / v) Trichloroacetic Acid (TCA) in the following volume ratio, serum : water : 20 % TCA, 1 : 5 : 4. The mixture was allowed to stand for 5 minutes to allow protein and lipid P precipitation. The precipitant was removed by centrifugation at 4,000 rpm for 10 minutes. Two parts of supernatant were

diluted with three parts of deionized water. The diluted sample was then reacted with one part of acidified ammonium molybdate (Sigma Diagnostics ®, Cat No. 661-11). The whole mixture was mixed and shaked gently. One-fourth part of Fiske & Subba Row Solution (Sigma Diagnostics ®, Cat No. 661-8) was then added and allowed to stand 10 minutes for colour development. The colour mixture was transferred to cuvettes. Then absorbance at wavelength of 660nm was recorded. The absorbance reading was completed within 10 minutes after colour development. The absorbance from the sample recorded was referred to a calibration curve. The calibration curve was created by using a standard P solution (Sigma Diagnostics ®, Cat No. 661-9). The preparation was the same as that of the sample, except that P standard solution was used.

3.2.4.2 Calcium Chemistry

3.2.4.2.1 Basic Principles and Method

The serum Ca interacted with o-Cresolphthalein Complexone, pH 10-12 to form a Ca-Cresolphthalein Complexone Red Complex. This red complex can be detected at wavelength 575nm.

Serum Ca was detected by a commercial kit from Sigma Diagnostics ® (Cat No. 587-A). The detection method can be referred to as Sigma Diagnostics ® procedure NO. 587. Ten micro-liters of serum sample was added with one ml of Ca Reagent Working Solution, which was prepared by combining one part of

Ca Binding Reagent (Sigma Diagnostics ®, Cat No. 587-2) with one part of Ca Buffer (Sigma Diagnostics ®, Cat No. 587-3). The mixture was allowed to stand for 3 minutes for colour development. The colour was detected at a wavelength of 575nm within 30 minutes. The absorbance recorded was referred to a calcium calibration curve by using the 15 mg / ml $CaCl_2$ stock solution instead of the serum sample.

3.2.4.3 1,25-$(OH)_2D_3$ Chemistry

3.2.4.3.1 Basic Principles and Method

Serum 1,25-$(OH)_2D_3$ was detected by a commercial kit from Immundiagnostik (Cat No.: K2112). This is a competitive enzymatic immuno-assay for 1,25-$(OH)_2D_3$ measurement. It is based on the competition of 1,25-$(OH)_2D_3$ presented in the sample with the labeled 1,25-$(OH)_2D_3$ for the binding site of the antibody specific for this metabolite.

1,25-$(OH)_2D_3$ was first isolated from the serum sample by 2 separate extraction columns (Immundiagnostik, Cat No.: SE2112 and SB2221). This step is necessary to isolate the desired metabolite from the other vitamin D_3 metabolites, 25-$(OH)D_3$ and 24,25-$(OH)_2D_3$. After separation, antibody solution (450 ul) was added to 20 ul of the samples, calibrators and controls (the last two were supplied by the kit) for 1-hour incubation at room temperature. Two hundred microliters of these pre-incubated samples were added into a 96-well

micro-plate accordingly. The test was carried out in duplicates. The plate was covered with plastic film and incubated for 16 hours at 4°C. After incubation, the contents were discarded and the wells were washed with 300 ul washing buffer for 5 times. An incubation of 200 ul conjugate was followed for 1 hour at room temperature. The wells were then washed for 5 times with the washing buffer. They were added with 200 ul substrate and left for 30 minutes at room temperature in the dark. Fifty microliters of stop solution was added and the colourimetric signal was detected at 450 nm against 620 nm as reference. A calibration curve is constructed from the calibrators and assisted by the GraphPad software Prism 3.0. Results of the samples were read from the calibration curve.

3.2.5 Sodium dodecyl sulfate-polyacrlamide gel electrophoresis and Western blotting

The expression and degradation of renal 1-OHase protein were detected by Western blotting analysis. Fifty micrograms of the proximal tubular cell lysates was subjected to 10 % Sodium dodecyl sulfate-polyacrylamide reducing gel electrophoresis. After proteins were resolved, they were electro-transferred onto an Immobilon P PVDF membrane (Millipore, USA). The membrane was first activated in 100 % methanol. The electroblotting was either performed under 4 °C overnight at 22 volts or 90 minutes at 100 volts by an iced-transblotting buffer, 192 mM glycine, 25 mM Tris and 10 % (v / v) methanol.

3.2.5.1 Antibody Incubation and Immunodetection

After blotting, the membranes were dried and blocked by 5 % (w / v) milk powder in Tris-buffered saline with Tween 20 (TBST), 500 mM NaCl, 20 mM Tris, pH 7.4, 0.05 % Tween 20 for 2 hours in room temperature. It was followed by a 5-minute wash by TBST solution. The filter was then incubated with sheep anti-1-OHase antibody solution, 1 : 500 (v / v), at 4°C overnight (The Binding Site, Birmingham, United Kingdom). The antibody solution was prepared from 1 % Bovine Serum Albumin (BSA) in TBST. After the overnight incubation, the blot was washed three times with TBST for 5 minutes each. It was followed by a 2-hour incubation of secondary anti-sheep IgG antibody, 1 : 5000 (v / v) (The Binding Site, Birmingham, United Kingdom). The antibody was conjugated with horseradish perioxidase (HRP). Again, three washes followed. After the washings, the blot was detected by an enhanced chemiluminescence (ECL) kit (Pierce, USA). The signal was amplified and analysed by the software Lumi-Analyst 3.1 from Lumi-Imager (Boehringer Manngeim).

3.2.6 Data analysis and normalization

The chemiluminescent signals captured by the Lumi-Imager were expressed as BLU. It was an arbituary unit which can reflect the expression level of the proteins under the study. The signals from the same protein under the same diet treatment and age group were averaged first. The mean signal from each group was then divided by the one from NP fed group so that the NP group

became one. The resulting ratio was compared with each other in order to show the protein expression difference between different treatments. Since the comparison was generated from many blots, blot to blot variation might introduce an inaccuracy during comparison. Hence, a control sample was added in equal amount (10 ug) to each blot, which was the cell lysate from human proximal tubules cell line, HKC-8. The chemiluminescent signal from this control was first taken a ratio to each sample before any direct comparison. Therefore, a more comparable and accurate result was generated.

3.2.7 Statistics

Data were reported as the mean ± standard error of mean. Significance of differences between group means was determined by one-way analysis of variance (one-way ANOVA). Significance of differences among means was determined by posttest, using Tukey's method. A p value < 0.05 was considered statistically significant. Linear regression analysis was generated by the software GraphPad Prism 3.0.

3.3 Results

As discussed in Section 3.1, LPD increased the 1-OHase activity and serum 1,25-$(OH)_2D_3$ of rats aged 4-8 weeks (Wong *et al.*, 1997). The present study was to determine the change in 1-OHase regulation at the translational level by LPD in both young (4-8 weeks) and adult rats (12-16 weeks), and to determine if the protein level change was the result of the alteration of protein stability.

3.3.1 Serum chemistries of Ca, P and 1,25-$(OH)_2D_3$ under the LPD treatment

Serum Ca, P and 1,25-$(OH)_2D_3$ chemistries of both rat groups were analyzed and summarized in Figure 10-12, respectively.

3.3.1.1 Serum Chemistries of young rats

In young rats, serum Ca level ranged from (9.18 ± 0.27) mg / dl to (10.27±0.42) mg / dl (Figure 10). It remained steady throughout the LPD treatment. Serum P rapidly declined from a normal level of (12.82 ± 0.63) mg / dl (Day 0) to (7.68 ± 1.11) mg / dl ($p<0.005$) during the first day of LP (Day 1) (Figure 11). By the second day (Day 2), serum P level remained at the low level until Day 7 of LPD ($p < 0.005$). This indicated that LPD decreased the serum P level significantly but not the serum Ca level, suggesting that regulation of serum P was not as tight as that of serum Ca. For the serum 1,25-$(OH)_2D_3$ content (Figure 12), no significant change was observed on the first day of treatment

(Day 1), however, a sharp increase of 1,25-$(OH)_2D_3$ content was followed by Day 2 (169.55 ± 5.54) pg / ml ($p<0.05$) from the normal level (43.47 ± 5.40) pg / ml (Day 0). This high level was prolonged till Day 7 (198.89 ± 30.79) pg / ml ($p < 0.005$).

3.3.1.2 Serum Chemistries of adult rats

Similar to young rats, no significant change was found in serum Ca level throughout the 7 days of LPD treatment in adult rat (Figure 10). The normal serum Ca level (Day 0) was (8.55 ± 0.62) mg / dl. There was no difference from the normal Ca level (Day 0) in young rats. As in young rats, the serum P level of the adult rats had a sharp decrease from the normal level (9.60 ± 0.61) mg / dl (Day 0) to (5.55 ± 0.87) mg / dl on Day 1 ($p < 0.001$) (Figure 11). It rose to (5.99 ± 0.28) mg / dl during Day 2 ($p < 0.05$) and remained at this level till Day 7 ($p < 0.05$). The normal P level (Day 0) in adult rats was significantly lower than that found in young rats ($p<0.05$). Unlike the young rats, serum 1,25-$(OH)_2D_3$ content showed a delayed increase on Day 5 (110.76 ± 17.52) pg / ml ($p<0.05$) from the normal level (31.59 ± 5.52) pg / ml (Day 0) in the adult counterparts upon LPD treatment (Figure 12).

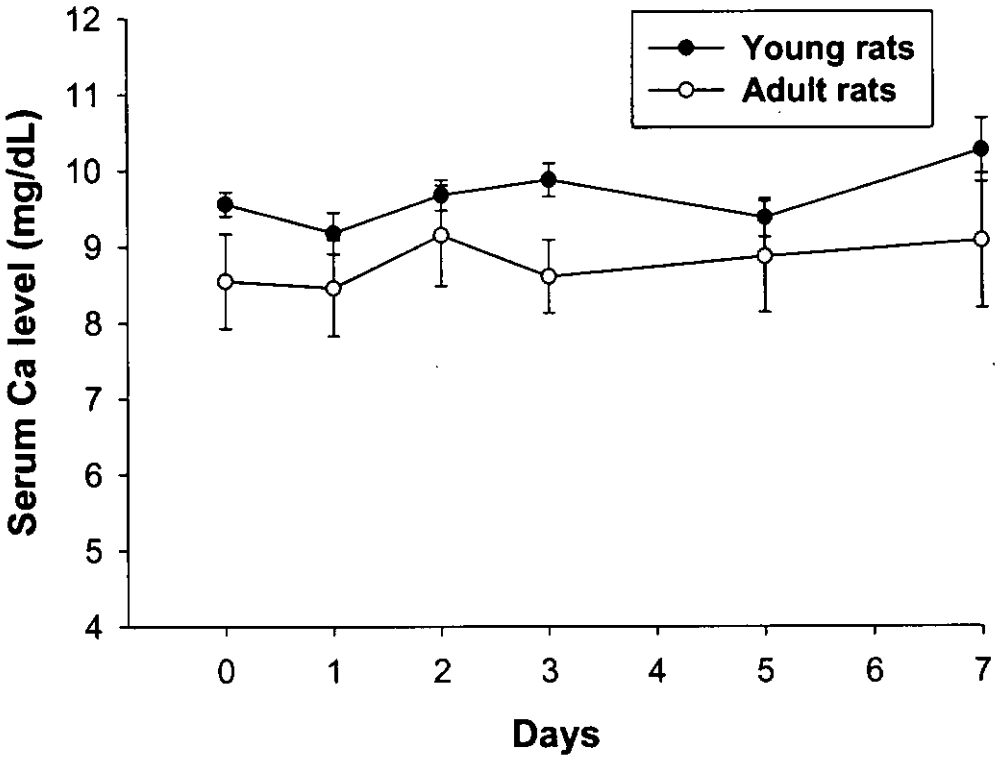

Figure 10: Serum Ca level of young and adult rats under LPD treatment. Rats were fed either normal phosphate diet (NPD) containing 0.6 % Ca, 0.65 % P as controls (Day 0) or low phosphate diet (LPD) containing 0.6 % Ca, 0.1 % P for 1, 2, 3, 5 and 7 days (n = 7 for individual time point). No significant change in serum Ca level was found upon the treatment. Results were demonstrated as mean ± SEM.

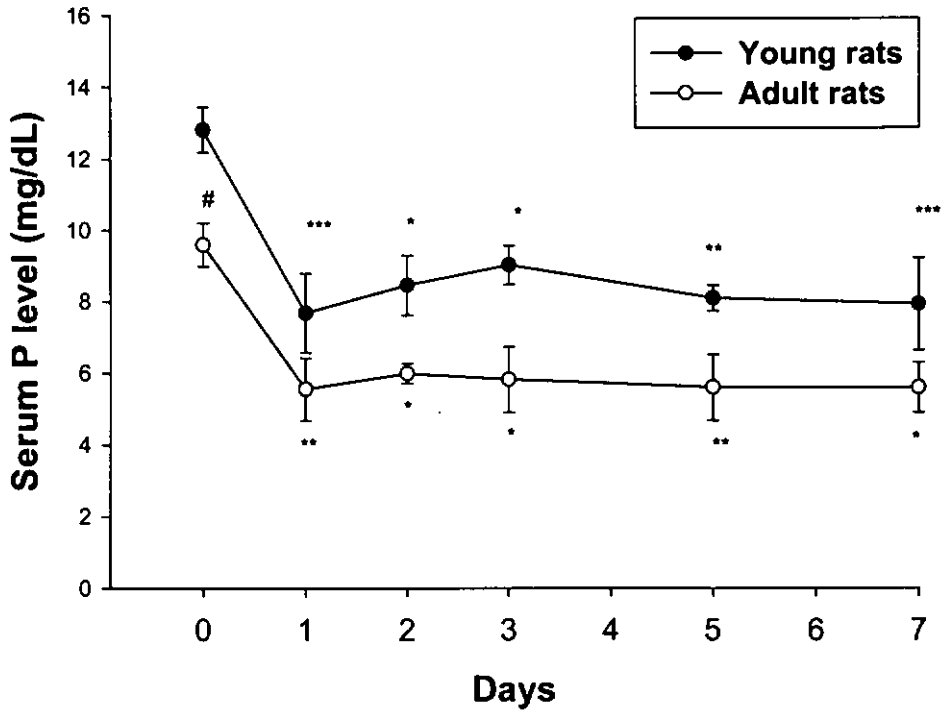

Figure 11: Serum P level of young and adult rats under LPD treatment. Rats were fed either normal phosphate diet (NPD) containing 0.6 % Ca, 0.65 % P as controls (Day 0) or low phosphate diet (LPD) containing 0.6 % Ca, 0.1 % P for 1, 2, 3, 5 and 7 days (n = 7 for individual time point). Both young and adult rats showed a sharp decrease in serum P level upon the first day of treatment. The normal serum P content (Day 0) in adult rats was significantly lower than that in young rats. Results were demonstrated as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.005$, vs Day 0 within the same age group; $^{\#}p < 0.05$, vs Day 0 in young group.

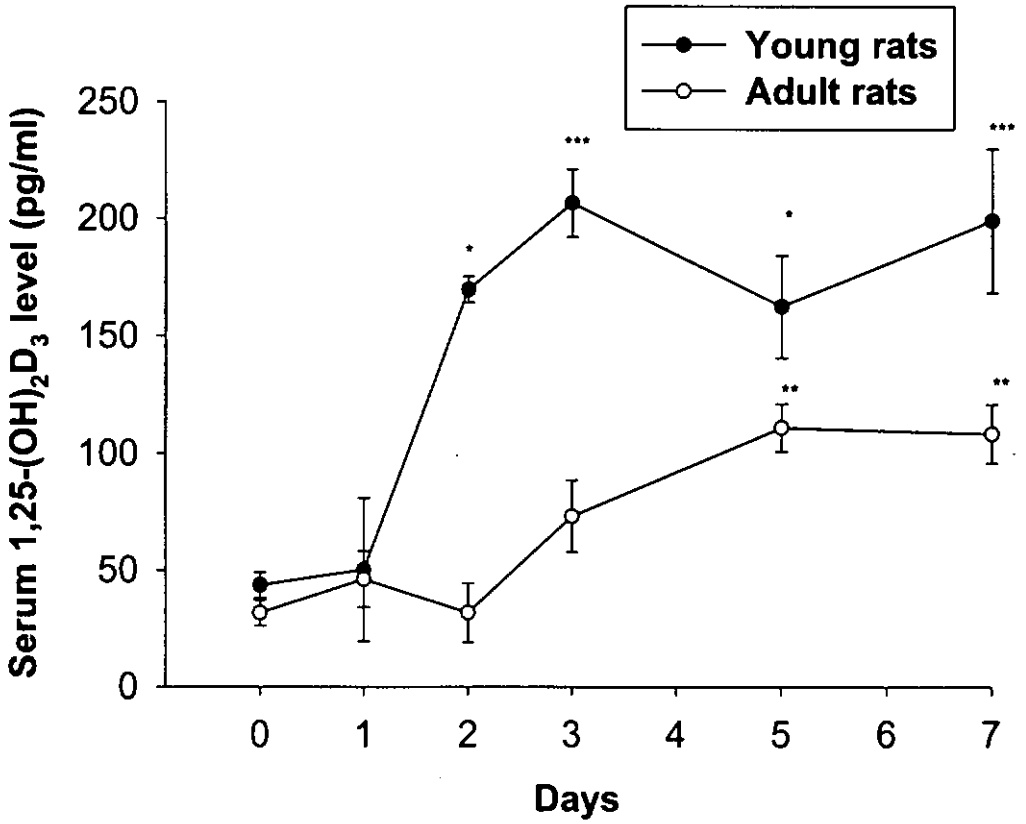

Figure 12: Serum 1,25-$(OH)_2D_3$ level of young and adult rats under LPD treatment. Rats were fed either normal phosphate diet (NPD) containing 0.6 % Ca, 0.65 % P as controls (Day 0) or low phosphate diet (LPD) containing 0.6 % Ca, 0.1 % P for 1, 2, 3, 5 and 7 days (n = 3 for individual time point). Serum 1,25-$(OH)_2D_3$ level was increased in both age groups. The observed increase was significant on the second day (Day 2) of treatment in young rats, while it was delayed on the fifth day (Day 5) in the adult counterparts. No difference was found in the basal serum level between the two age groups. Results were demonstrated as mean ± SEM. $^{*}p < 0.05$ and $^{**}p < 0.005$, vs Day 0 within the same age group.

3.3.2 Protein expression of renal 1-OHase in young and adult rats under the LPD treatment

The protein expression patterns of renal 1-OHase in both young and adult rats under LP condition were shown in Figure 13 and Figure 14. In young rats, Western blot analysis showed that LPD increased renal 1-OHase protein content gradually from Day 0 to Day 7, with a significant maximal increase of 2.5 folds on Day 5 ($p < 0.001$) (Figure 14, dark bar). On the contrary, no significant increase was found throughout 7 days of LPD treatment in the adult counterparts. However, a significant decrease was reported in 1-OHase protein expression on Day 2 (0.5 fold, $p < 0.001$) (Figure 14, gray bar). The basal level (Day 0) of renal 1-OHase protein expression in adult rats was found to be 2-fold higher than that in young rats ($p < 0.005$).

3.3.3 *In vivo* stability analysis of renal 1-OHase protein expression

In the last section, 1-OHase protein expression was found to increase on Day 5 in young rats, but not in adult rats. It is of our interest to determine whether the increase in 1-OHase protein in young rats was due to an increase in protein stability or involved any other mechanism, e.g. an increase in rate of protein synthesis. In this experiment, the susceptibilty of 1-OHase protein under different diet conditions was revealed. As an increase in expression level of a specified protein could be the result of either an increase in rate of protein synthesis or a decrease in rate of protein degradation, we asked if the increase in 1-OHase protein expression on Day 5 of LPD was caused by an alteration of 1-OHase protein stability. Young and adult rats fed with either 5 days of NPD or

LPD were injected intravenously with CHX, a protein synthesis inhibitor to determine the *in vivo* stability of 1-OHase protein. In the absence of de novo protein synthesis, monitoring of the expression level of a specified protein over a period of time will allow us to compare the stability of the protein under different conditions, i.e. the expression level of a more stable protein (lower degradation rate) will be decreased slower than that of a less stable protein (high degradation rate). The *in vivo* stability of 1-OHase protein in rats was found to be age and P dependent. Under normal P condition, renal 1-OHase protein expression decreased gradually in young rats within 6 hours of CHX injection (Figure 15 and 16). The degradation was found to be significant after 4 hours with 54 % ($p < 0.05$), and 6 hours with 59 % ($p < 0.05$) protein degraded (Figure 16). However, the expression in adult rats remained unchanged. The results suggested that renal 1-OHase protein was more stable in adult rats fed with NPD (Figure 16). Upon LPD treatment, renal 1-OHase protein expression in young rats remained unchanged within 6 hours of CHX injection, suggesting that LPD increased the *in vivo* stability of renal 1-OHase protein in young rats. In contrast, the expression of renal 1-OHase protein upon CHX treatment was not different between adult rats fed either NPD or LPD. (Figure 15 and 16). This indicates that LPD does not alter the *in vivo* stability of 1-OHase protein in adult rats.

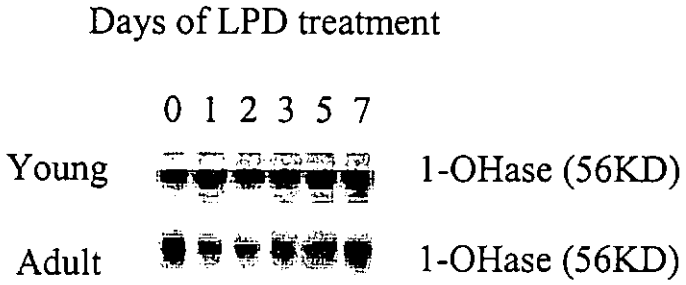

Figure 13: Western blotting analysis of renal 1-OHase protein expression in young and adult rats under dietary P restriction. Rats were either fed with normal phosphate diet (NPD) (0.60 % Ca, 0.65 % P) or low phosphate diet (LPD) (0.60 % Ca, 0.10 % P) for 0, 1, 2, 3, 5 and 7 days. Day 0 served as a control. Fifty micrograms of renal proximal tubules cell lysates were resolved in 10 % SDS-PAGE analysis and electro-transferred to PVDF membrane for Western Blotting. Upper panel represented young rats, while lower panel represented adult rats.

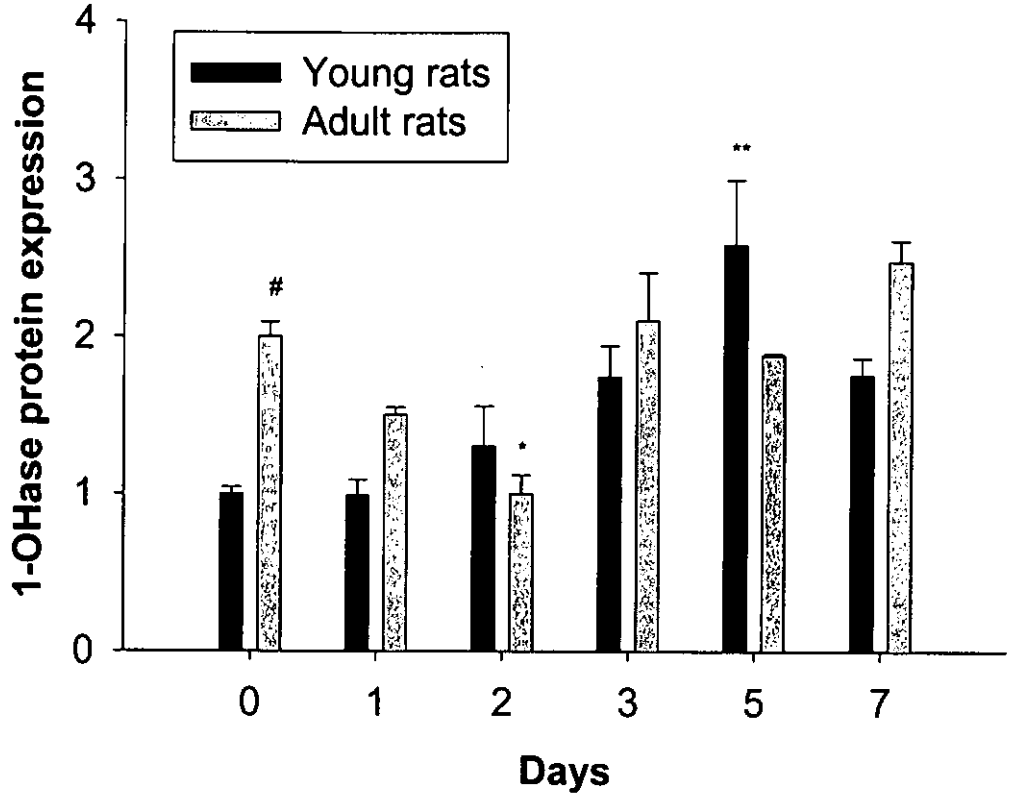

Figure 14: Time course of renal 1-OHase protein expression in response to low phosphate diet (LPD) in young (dark bar) and adult rats (gray bar). A gradual increase in 1-OHase protein expression was observed in young rats. The up-regulation was found to be significant on Day 5 (2.5 folds) vs Day 0. For adult rats, no significant increase was found, but a decrease was observed on Day 2 (0.5 fold) vs Day 0. The basal expression level (Day 0) of 1-OHase was found to be significantly higher (2 folds) in adult rats than that in young rats. $^{*}p < 0.005$, $^{**}p<0.001$ vs Day 0 within the same age group; $^{\#}p < 0.005$ vs Day 0 in young group.

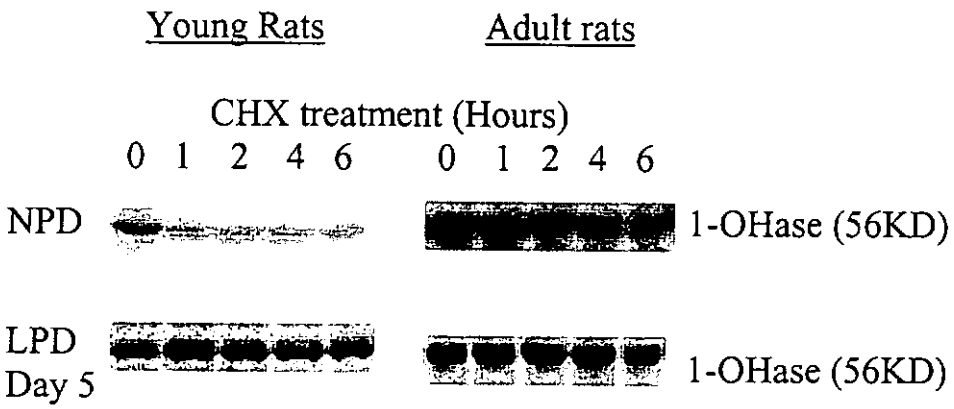

Figure 15: Western blotting analysis of the effect of cycloheximide (CHX) injection on renal 1-OHase protein expression in young (Left) and adult (Right) rats in response to 5 days of LPD feeding. A dose of 0.3 mg / 100g body weight of CHX was injected into the rats fed with normal phosphate diet (NPD) (0.60 % Ca, 0.65 % P) or low phosphate diet (LPD) (0.60 % Ca, 0.10 % P). Fifty micrograms of renal proximal tubules cell lysates were resolved in 10 % SDS-PAGE and electro-transferred to PVDF membrane for Western Blotting. Upper panel represented NPD group while lower panel represented LPD Day 5 group.

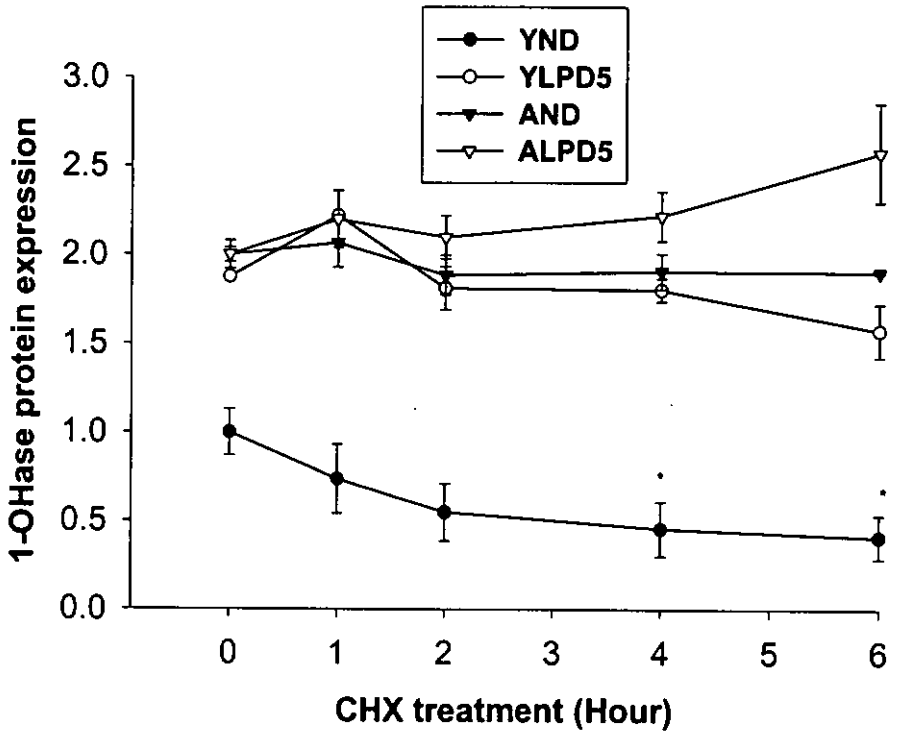

Figure 16: Effect of cycloheximide on renal 1-OHase protein expression in young and adult rats fed 5 days of LPD. The change in 1-OHase protein expression level in response to CHX was shown. There was a gradual decrease in protein expression in YND group only. The decrease was found to be significant at 4 hours (54 %) and 6 hours (59 %) after CHX injection. No significant change in 1-OHase protein expression was found in YLPD5, AND and ALPD5 groups. $*p < 0.05$ vs 0 hour in YND group.
YND represented young rats fed with NPD,
YLPD5 represented young rats fed with 5 days of LPD,
AND represented adult rats fed with NPD,
ALPD5 represented adult rats fed with 5 days of LPD.

3.3.4 Regression analysis

As anticipated, serum P level was inversely varied with renal 1-OHase protein expression (Figure 17) in young rats [1-OHase = (-0.18 ± 0.037) x serum P level + (3.38 ± 0.39), $p < 0.0001$, r^2=0.54], but such a reciprocal correlation was not observed in that of adult rats (data not shown). The serum 1,25-$(OH)_2D_3$ level showed a negative correlation with serum P level [Serum P level = (-0.021 ± 0.0061) x serum 1,25-$(OH)_2D_3$ level + (13.97 ± 1.01), $p < 0.005$, r^2=0.45] (Figure 18), whilst it showed a positive correlation with the expression of renal 1-OHase protein in young rats (Figure 18). These correlations indicated that the increase in serum 1,25-$(OH)_2D_3$ level parallel the up-regulation of 1-OHase protein expression in renal proximal tubules isolated from young rats fed with LPD.

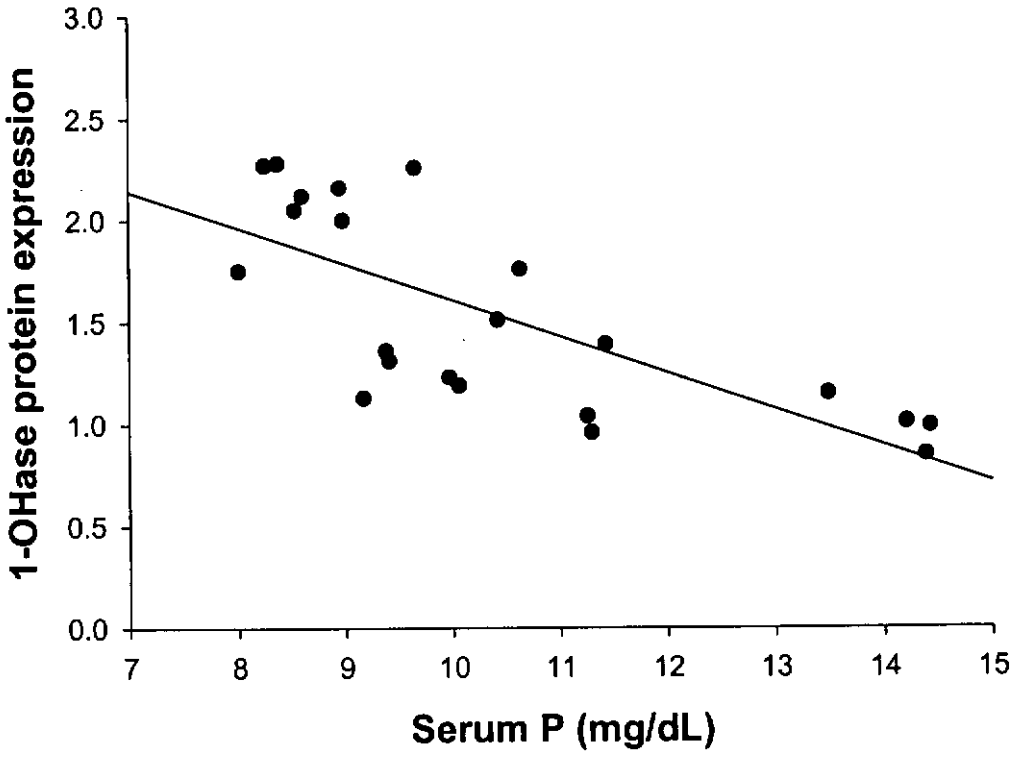

Figure 17: Measurement of 1-OHase protein expression of individual rats as a function of serum P level for rats fed LPD for 0-7 days. A negative relationship between renal 1-OHase protein expression and serum P level was found in young rats [1-OHase = (-0.18 ± 0.037) x serum P level + (3.38 ± 0.39), r^2=0.54]. The slope is significantly different from zero ($p < 0.0001$).

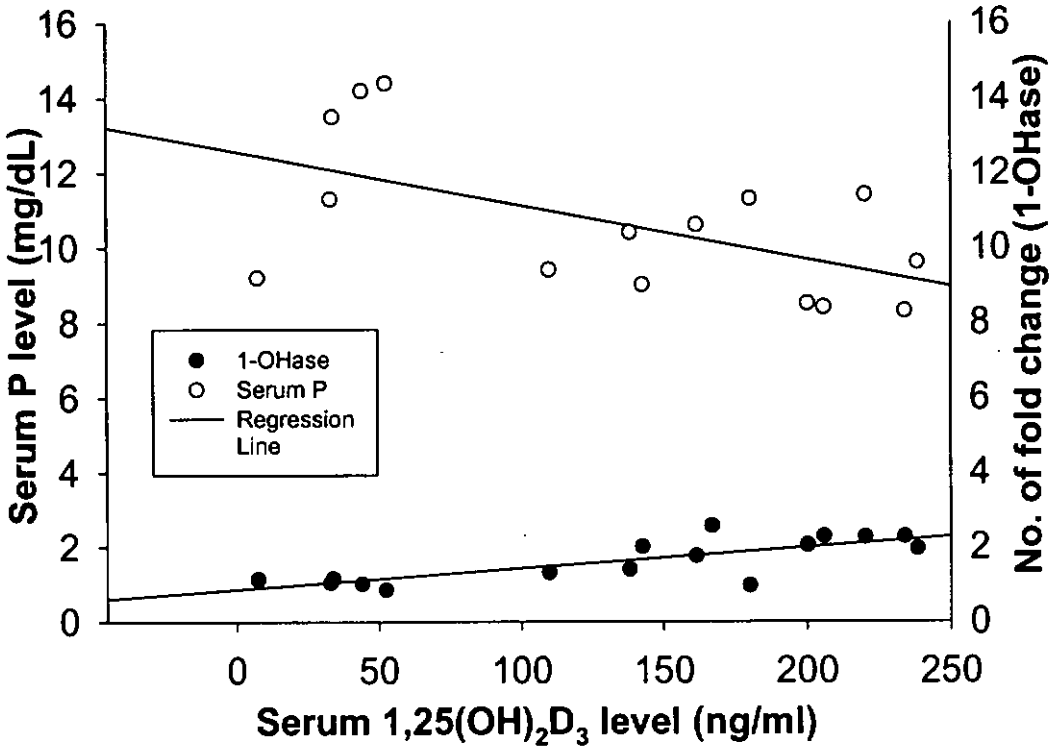

Figure 18: Measurement of serum 1,25-$(OH)_2D_3$ level of individual rats as a function of serum P level and 1-OHase protein expression for young rats fed LPD for 0-7 days. A negative relationship between renal serum P level and serum 1,25-$(OH)_2D_3$ level was found in young rats [Serum P level = (-0.021 ± 0.0061) x serum 1,25-$(OH)_2D_3$ level + (13.97 ± 1.01), r^2=0.45]. The slope is significantly differed from zero ($p < 0.005$). Whilst a positive linear relationship was found between renal 1-OHase protein expression and the serum 1,25-$(OH)_2D_3$ level in young rats [1-OHase = (0.0057 ± 0.0012) x serum level + (0.85 ± 0.19), r^2 = 0.61]. The slope is significantly different from zero ($p < 0.0005$).

3.4 Discussions

Dietary P restriction induced a significant decrease in serum P level in both young and adult rats. This decrease demonstrated that the low P diet that we used was effective to induce hypophosphatemia. However, serum Ca level was found to be constant throughout the duration of LPD treatment in both age groups under investigation. This showed that regulation of serum Ca content was tight and was not altered by the dietary P deficiency for up to 7 days. Besides the two minerals, serum 1,25-$(OH)_2D_3$ level was also measured, the result from this measurement also provide information for the protein expression of its enzyme, renal 1-OHase.

The decline in serum P level showed different effects on renal 1-OHase protein expression in rat of different ages. The results demonstrated that the change for the renal 1-OHase expression was time-dependent upon LPD in young rats. There was a trend for an increase in 1-OHase expression from Day 0 to Day 7. The expression reached a maximum (2.5 folds; $p < 0.001$) on Day 5, and remained high on Day 7 with 1.7-fold. However, no increase in expression was observed in the adult rats throughout the 7 days of LPD treatment. On the contrary, a decrease in renal 1-OHase expression was found in adult rats on Day 2 of LPD treatment (0.5 fold, $p<0.001$). The protein expression patterns were totally different between the two age groups. This implied that the regulation of renal 1-OHase protein by LPD was age-dependent.

The present data clearly show that the increase in 1-OHase protein was lost in adult rats. The result agreed with the study by Wong *et al.* in 1997. They showed a blunted response of 1-OHase activity to LPD when compared with that of their younger counterparts. This suggested that adult rats might be less physiologically adaptable than young rats. The up-regulation of 1-OHase protein might be, part of, the adaptive response for restoring serum P level. However, the failure in up-regulation of renal1-OHase in adult rats might suggest that other mechanisms might be involved in adaptation to restore serum P, or that a longer period of P restriction is needed for adaptive response. Thus, further investigations are needed to delineate the adaptive response in adult rats.

The present study have shown that the loss of adaptive increase in 1-OHase activities during dietary P restriction in adult rats is due to their inability to increase in expression of the 1-OHase protein. In addition, a blunted response on serum 1,25-$(OH)_2D_3$ level (as indicated by a lower serum 1,25-$(OH)_2D_3$ level and a delayed response) was also found in adult rats in this study (Figure 12). Both young and adult groups showed an increase in serum 1,25-$(OH)_2D_3$ level. However, a delayed increase was observed in adult rats on Day 5 (vs Day 2 in young rats). The increase in serum 1,25-$(OH)_2D_3$ level can be explained directly by the increase in renal 1-OHase protein expression and activity (Wong *et al.*, 1997) in young rats, but not in adult rats. It suggested that other components, in addition to renal 1-OHase, might play an important role in regulating the circulating pool of 1,25-$(OH)_2D_3$ level. For example, 24-OHase, which acts as a clearing action for 1,25-$(OH)_2D_3$ production, can also affect the circulating level

of 1,25-$(OH)_2D_3$. The vitamin D enzymes work with each other to regulate the circulating level of vitamin D metabolites.

Our experiment demonstrated an up-regulation of renal 1-OHase by LPD in young rats but not in adult rats. However, the mechanism involved in LPD up-regulation of renal 1-OHase protein as well as the age-related alteration in its expression were not known. Hence, a mechanistic study was performed to determine if the up-regulation was due to a change in protein stability and this can also provide new insights for the understanding of the age-related change in renal 1-OHase regulation. Protein stability changed with both age and dietary P level. It was found that renal 1-OHase proteins were stable in adult rats as CHX treatment did not cause any reduction in 1-OHase protein expression level in adult rats fed either NPD or LPD (figure 15 and 16). This result might explain why adult rats have a higher basal expression level of 1-OHase protein than young rats. However, it is unclear why higher expression and higher stability of 1-OHase proteins are found in adult rats than in young rats fed NPD. Nevertheless, the present study has demonstrated that one of the mechanisms involved in LPD up-regulation of 1-OHase protein expression in young rats, namely, the enhancement of protein stability of 1-OHase were no longer operating in adult rats.

In 2002, Cheung *et al.* have reported a decrease in total number of proteins under LPD in young rats by two-dimensional electrophoresis analysis. They suggested the decrease might be due to the shutting down of those less important proteins; and / or the turning on of those involved in the adaptation to

LPD. The increased stability of 1-OHase protein in young rats upon LP condition shown in the present study might be explained by the removal of some degrading proteins or formation of some protective proteins for 1-OHase under LPD, in order to adapt to imbalance of serum P. Thus, further experiment is needed for the verification of these hypotheses.

In the present study, an inverse relationship was found between serum P and 1-OHase as observed at the protein level (Figure 17). In addition, serum $1,25\text{-}(OH)_2D_3$ showed a negative and positive linear correlation with serum P and 1-OHase protein, respectively (Figure 18) in young rats. These correlations provided strong evidence that mineral P is an important determinant in inducing the expression of 1-OHase at the protein level in young rats.

3.5 Conclusions

In this chapter, it was demonstrated that the regulation of renal 1-OHase protein expression by LPD was both P- and age-dependent. The lost in ability to up-regulate the renal 1-OHase protein production in adult rats in response to LPD suggested that other mechanisms might be involved for adaptation to LPD in adult rats. The *in vivo* mechanistic studies showed that the increase in renal 1-OHase protein expression in young rats was due to the enhancement of protein stability. Further studies will be needed to investigate the mechanism involved in the age-related change in renal 1-OHase regulation and to identify other possible mechanisms that might be involved in maintaining mineral homeostasis in adult animals.

Chapter Four

Regulation of IGF-I axis by dietary P restriction in rats: Effect of age

4.1 Introduction

Insulin-like growth factor I (IGF-I) is characterized as somatomedins, which performs normal fetal and postnatal growth and development. It is involved in the regulation of cell proliferation and serves an endocrine and / or paracrine / autocrine function for promoting somatic growth. IGF-I is believed to be the mediator of the growth-promoting actions of Growth Hormone (GH) (Salmon and Daughaday, 1957). GH can increase the circulating level of IGF-I, probably by inducing its production in the liver (Schalch *et al.*, 1979). The hepatic IGF-I exerts its function either locally or to other target tissues, e.g. the intestines or kidneys, by a release into the circulation. IGF-I elicits its action by binding to its receptors, thus initiating a series of protein-protein interactions for signal transduction and ultimately cell growth.

Hypophysectomy is the surgical removal of pituitary gland, thus the source of GH and IGF-I. Hypophysectomy reduces the production and serum level of 1,25-$(OH)_2D_3$ (Spencer *et al.*, 1977 and Gray, 1987) and abolishes the increase in serum level of 1,25-$(OH)_2D_3$ induced by LPD (Gray, 1981). These findings suggested that GH, IGF-I or both would lead to the increase in serum 1,25-$(OH)_2D_3$ level induced by LPD. In 1998, Bernard and Spencer demonstrated that the IGF-I restored the LPD induced increase in serum 1,25-$(OH)_2D_3$ level, the degree of restoration was to the same level as stored by GH in the hypophysectomized rats. In addition, hypophosphatemia was not associated with changes in GH secretion (Tenenhouse *et al.*, 1988) or plasma IGF-I concentrations (Gray *et al.*, 1983). These findings strongly suggest that

the GH-dependent increase in serum 1,25-$(OH)_2D_3$ level induced by LPD is mediated through IGF-I (Bernard *et al.*, 1998).

In 1997, Wong *et al.* showed that the increase in 1-OHase activity during LPD was lost in adult rats. The blunted response in adult rats was overcome by administrating recombinant human IGF-I. 1-OHase activity was restored to the same level as that found in young rats. The result indicated that 1-OHase activity was age-dependent and this age-related loss was reversible by IGF-I administration (Wong *et al.*, 1997). In addition, decreases in serum IGF-I and GH levels are associated with aging (Corpas *et al.*, 1993).

There has been extensive evidence indicating the involvement of IGF-I in vitamin D metabolism. However, how it is being involved and the mechanisms for the control of vitamin D metabolism by P and IGF-I remain unclear. It was hypothesized that the responsiveness of renal proximal tubule to IGF-I changed in rats in response to LPD. Such changes might be due to a change in the expression of signaling proteins in the IGF-I axis. This resulted in a change in renal 1-OHase regulation during LPD. Based on the close relationship between the IGF-I peptide and the vitamin D regulation, it was proposed that IGF-I-axis was involved in the regulation of vitamin D metabolism. Some of the components in IGF-I axis were studied by using the same LPD platform as that used in renal 1-OHase protein regulation (in Chapter 3).

In this chapter, part of the IGF-I axis profile was studied. This includes (1) insulin-like growth factor Type I receptor (IGFIR); (2) insulin receptor (IR);

(3) insulin receptor substrate-1 (IRS-1) and (4) src homolgy collagen protein (Shc), where Shc family was comprised of three isoforms of 46, 52 and 66 kDa. The first two proteins were membrane bound receptors belonging to the IGF family: IGF-I receptor was highly specific for the ligand IGF-I, but insulin receptor could also recognize it at a lesser extent. The third and fourth proteins were the common docking proteins in the IGF-I signaling pathway. All the proteins were previously shown to be involved in IGF-I axis (LeRoit *et al.*, 1995). The effect of age on the protein expression level of these proteins under LPD will be assessed to determine if the responsiveness of IGF-I axis to IGF-I change with age, and with vitamin D regulation.

4.2 Materials and Methods

4.2.1 Animal and Diets

An animal model was used to study the expression level of specific proteins in IGF-I axis under LPD. The model used here was the same as that used for studying 1-OHase protein regulation in Chapter 3. Similarly, two age groups were employed and rats were fed with either normal phosphate diet (NPD) or low phosphate diet (LPD) for specified number of days. The rat treatment was performed as described in Section 3.2.1, unless otherwise stated.

4.2.2 *In vivo* protein stability analysis

In order to determine the stability of proteins in IGF-I axis in response to LPD, an *in vivo* mechanistic study using cycloheximide to inhibit *de novo* protein synthesis was performed on both rat groups. The time point Day 5 was selected for the study of CHX effect, because the up-regulation of 1-OHase protein expression was the highest on Day 5. This experiment was carried out in parallel with that of the study of 1-OHase. The experimental preparation was similar to that used in studying 1-OHase as described in Section 3.2.2.

4.2.3 Preparation of Renal Proximal Tubules

The renal proximal tubules were used for studying the expression of specific proteins in IGF-I axis. The experimental procedures for isolation were performed as described in Section 3.2.3.

4.2.4 Serum Collection

Blood was collected for serum analysis. Similarly, serum chemistries for calcium (Ca), phosphate (P) and IGF-I were performed. Referred to Section 3.2.4 for the principles and the procedures.

4.2.4.1 IGF-I chemistry

4.2.4.1.1 Basic Principles and Methods

Serum IGF-I level was detected by a commercial kit from Diagnostic Systems Laboratories, Inc. (Cat. No.: DSL-10-2900). It was a competitive binding enzyme immunoassay. The assay was performed in duplicate and three observations were used for each sample treatment. In the assay, the IGF-I from the sample competed with the biotin-labeled antigens for a limited number of anti-rat IGF-I binding sites.

Serum sample was first incubated with sample buffer I and II as described in the instruction manual. The pre-treated serum sample (50 ul) was added into

the wells in the pre-marked microtitration wells accordingly. Hundred microliters of rat IGF-I-Biotin Conjugate Solution were added to each well and followed by 100 ul of rat IGF-I antiserum. The wells were incubated for an hour at room temperature with shaking on an orbital shaker. After incubation, the wells were aspirated and washed with washing buffer for five times. An addition of 200 ul of the streptavidin-enzyme conjugate solution was followed. The wells were then incubated for a further 30 minutes at room temperature with shaking. They were then aspirated and washed again with washing buffer for five times. Hundred microliters of the tetramethlbenzidine (TMB) chromogen solution was added to each well and the wells were incubated in the dark for 30 minutes at room temperature. After incubation, 100 ul of the stopping solution was added to each well. The colorimetric signal was detected through the wavelength 450 nm with 620 nm as reference. The quantification of serum IGF-I content was detected by referring to a standard curve, which was created by a series of standard samples supplied by the kit.

4.2.5 Sodium dodecyl sulfate-polyacrlamide gel electrophoresis and Western blotting

The expression of specific proteins in IGF-I axis and the protein degradation were evaluated by Western Blotting analysis. The experimental procedures were as described in Section 3.2.5.

4.2.5.1 Antibody incubation and Immunodetection

Western blotting procedures were similar to those described in Section 3.2.5.1. The dilutions for rabbit anti-IGFIR beta subunit antibody, rabbit anti-IR beta subunit antibody, rabbit anti-Shc antibody and rabbit anti-IRS1 antibody were 1 : 2000 (v / v), 1 : 2000 (v / v), 1 : 500 (v / v) and 1 : 500 (v / v), respectively. The working dilution for the secondary anti-rabbit IgG was 1 : 1000 (v / v). All antibodies were purchased from Santa Cruz Biotechnology (Santa Crutz, CA, USA).

4.2.6 Data analysis and Normalization

The method for data analysis and normalization should be referred to Section 3.2.6.

4.2.7 Statistics

Statistical analysis was performed in this part of the study. The statistical methods should be referred to Section 3.2.6.

4.3 Results

IGF-I restored the increase in renal 1-OHase activity that was lost in adult rats (Wong *et al.*, 1997) and hypophysectomized rats (Bernard *at al.*, 1988) under LPD. These findings suggested a close relationship between IGF-I and the regulation of vitamin D under LPD. Such regulation seems to be age-dependent. In the last Chapter, it was found that LPD condition can up-regulate the 1-OHase protein expression and this may be explained by the enhancement of protein stability, although these did not happen in adult rats. In this section, the stability of specific proteins in IGF-I axis was also studied to determine if the change in protein expression was due to the change in protein stability.

4.3.1 Serum chemistries of both young and adult rats under LPD treatment

For both serum Ca and P measurement, the results were described and interpreted in Section 3.3.1. For serum IGF-I level, a decreasing trend was measured in young rats with a significant decrease on Day 7 (288.11 ± 24.38) ng / ml ($p < 0.05$) vs Day 0 (735.49 ± 0.63) ng / ml. On the contrary, an increasing trend was observed in adult rats. The IGF-I content ranged from Day 0 (536.80 ± 58.0) ng/ ml to Day 7 (1046.40 ± 274.04) ng/ ml. No difference of the normal IGF-I level (Day 0) was observed between both age groups.

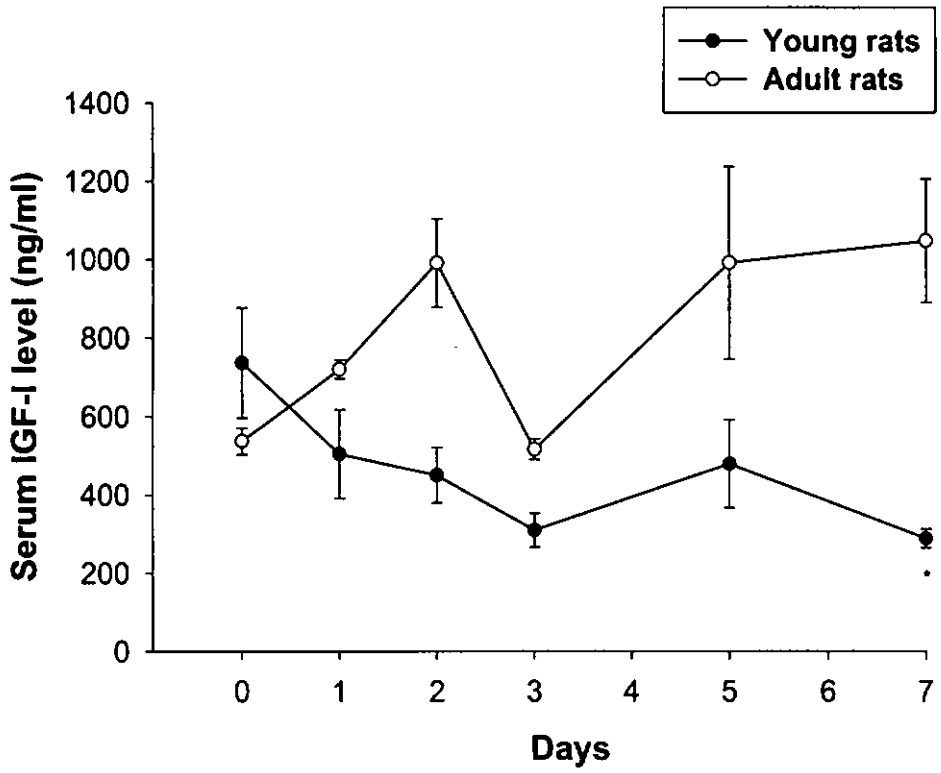

Figure 19: Serum IGF-I level of young and adult rats under dietary P restriction. Rats were fed either normal phosphate diet (NPD) containing 0.6 % Ca, 0.65 % P as controls (Day 0) or low phosphate diet (LPD) containing 0.6 % Ca, 0.1 % P for 1, 2, 3, 5 and 7 days (n = 3 for individual time point). Serum IGF-I level decreased generally throughout 7 days of diet treatment in young rats. A significant decrease was found on Day 7 (288.11 ± 24.38) ng / ml vs Day 0 (735.49 ± 0.63) ng / ml. However, a general increasing trend was observed in the adult counterparts. Results were demonstrated as mean ± SEM. $^{*}p<0.05$ vs Day 0 of young rats.

4.3.2 Expression of specific proteins in IGF-I axis in young and adult rats under LPD treatment

In young rats, the expression of insulin-like growth factor Type I receptor (IGFIR) in renal proximal tubule (Figure 20A and Figure 21) reached a maximum on Day 5. The level of IGFIR expression on Day 5 was 1.86 ± 0.30 fold ($p < 0.05$) higher than of that on Day 0. The trend of IR regulation was similar to that of IGFIR (Figure 20B and Figure 22). Significant increase in IR expression in renal proximal tubule was found on Day 5 (2.23 ± 0.06 fold, $p < 0.005$) and Day 7 (2.22 ± 0.4 fold, $p < 0.01$). For Shc protein expressions (Figure 20C), an increasing trend was observed; however, the signal was too weak for quantification by using software from LumiAnalyst version 3.1. Nevertheless, two isoforms of Shc, p46 and p52 could still be identified. For the other docking protein, IRS-I, its expression was not detectable (Figure 20D) in renal proximal tubule in young rats. The MCF-7 cell lysate served as a positive control for the IRS-I detection (Figure 20D).

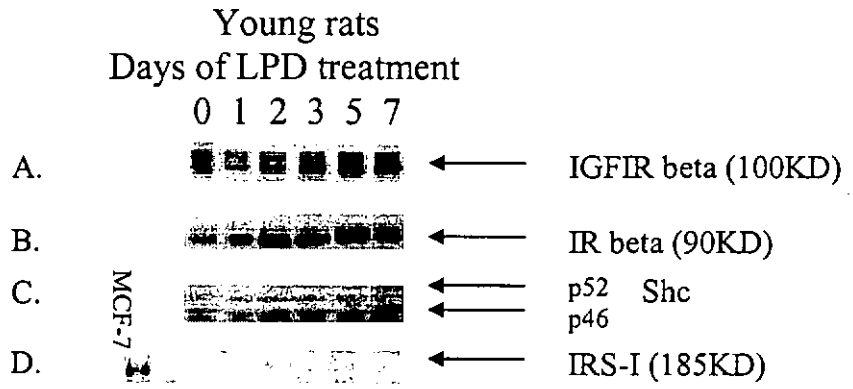

Figure 20: Western blotting analysis of various proteins along the IGF-I axis in young rats under dietary P restriction. Fifty micrograms of renal proximal tubules cell lysates were resolved in 10% SDS-PAGE analysis and electro-transferred to PVDF membrane for Western blotting. (A). IGFIR; (B). IR; (C). Shc and (D). IRS-1. The MCF-7 was the human breast cancer cell line, which served as a positive control for IRS-1.

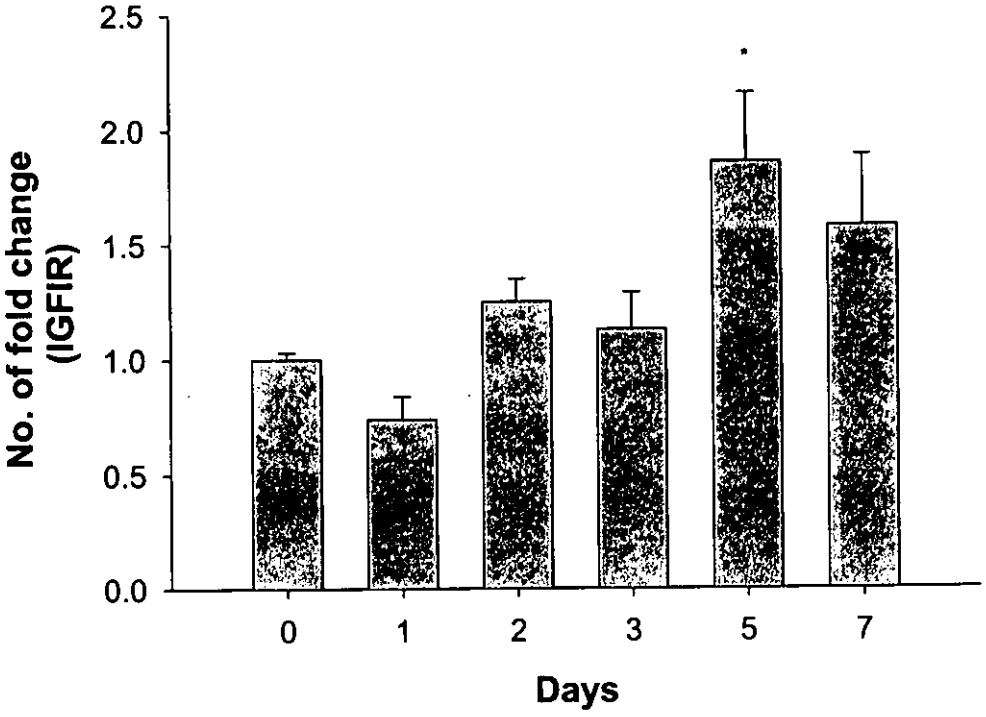

Figure 21: IGFIR protein expression in young rats in response to dietary P restriction. The IGFIR beta protein expression was up-regulated on Day 5 (1.86 ± 0.30 fold), for n = 5 to 6, from 2 separate experiments. Data were analyzed by one-way ANOVA and a posttest, by Tukey's method. A p value of <0.05 was considered statistically significant. $^{*}p < 0.05$ vs Day 0.

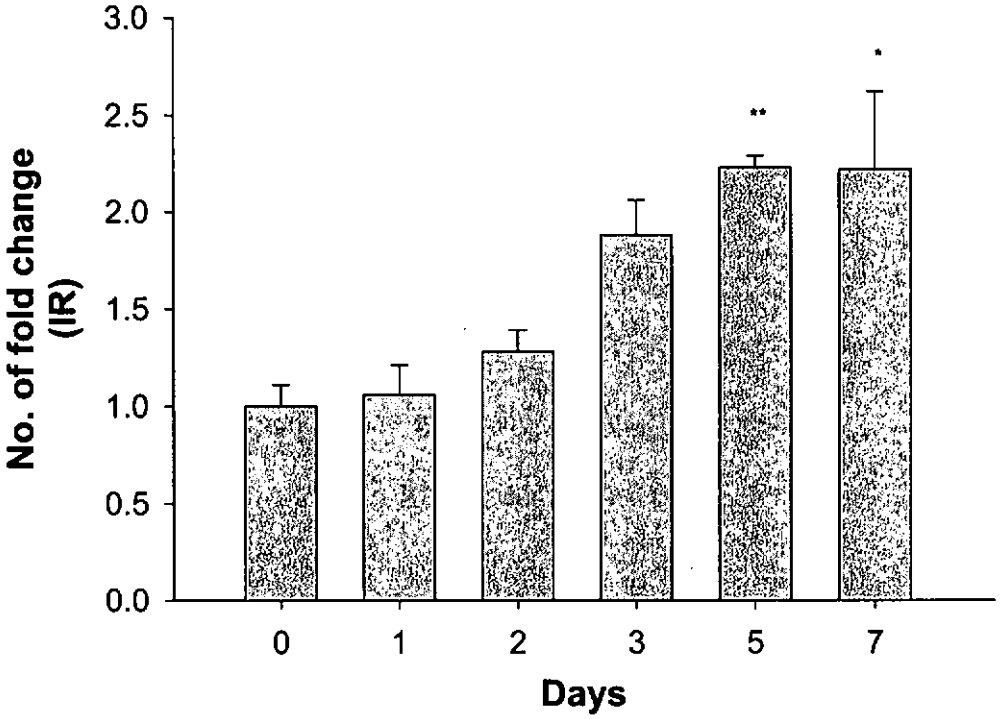

Figure 22: IR protein expression in young rats in response to dietary P restriction. A significant increase in the IR beta protein expression was found on Day 5 (2.23±0.06 folds) and Day 7 (2.22±0.4 folds), for n = 7, from 2 separate experiments. Data were analyzed by one-way ANOVA and a posttest, by Tukey's method. A p value of <0.05 was considered statistically significant. $^{*}p<0.01$, $^{**}p<0.005$ vs Day 0.

In adult rat, the expression of IGFIR protein in rat renal proximal tubule was found to be significantly increased on Day 1 (2.44 ± 0.30 fold, $p < 0.05$). It returned to basal level on Day 2 and rose to level of 5-fold higher on both Day 3 and Day 5 ($p < 0.005$). The expression of IGFIR remained upregulated on Day 7 (3 folds, $p < 0.005$) (Figure 23A and Figure 24). As compared with that in young rats, the up-regulation in adult rats occurred earlier (Day 1 vs Day 5 in young rats) and to a higher extent (5-fold vs 1.86-fold increase in young rats).

For the expression of IR protein in rat renal proximal tubule (Figure 23B and Figure 25), a similar pattern of expression as IGFIR was found. On the first two days of treatment, no significant change was observed. However, a remarkable 4-fold increase in IR was found on Day 3 and Day 5. The level of expression returned to basal level on Day 7. Again, the up-regulation occurred earlier (Day 3 vs Day 5 in young rats) and to a higher extent (4-fold vs 2-fold increase in young rats) in adult rats.

For the expression levels of the docking proteins, Shc, only two isoforms were detected. They were p46 and p52 (Figure 23C and Figure 26). Their expressions were very low during the first two days of LPD conditions. However, a remarkable increase in Shc expression was found on Day 3 (6.37 ± 0.11 folds, $p < 0.001$) and Day 5 (7.79 ± 1.60 folds, $p < 0.001$). The expression decreased dramatically and returned to basal level on Day 7. For the other docking protein, IRS-1, the basal level remained low till Day 2 but was detectable in renal proximal tubule. Its expression level was significantly up-regulated on Day 3 (13.59 ± 4.61 folds, $p < 0.05$) and it remained high on Day 5,

but returned to basal level on Day 7. The regulations of these two docking proteins, Shc and IRS-1, were very similar in both age groups. It was obvious that LPD up-regulated these two docking proteins significantly in adult rats, but not in young rats.

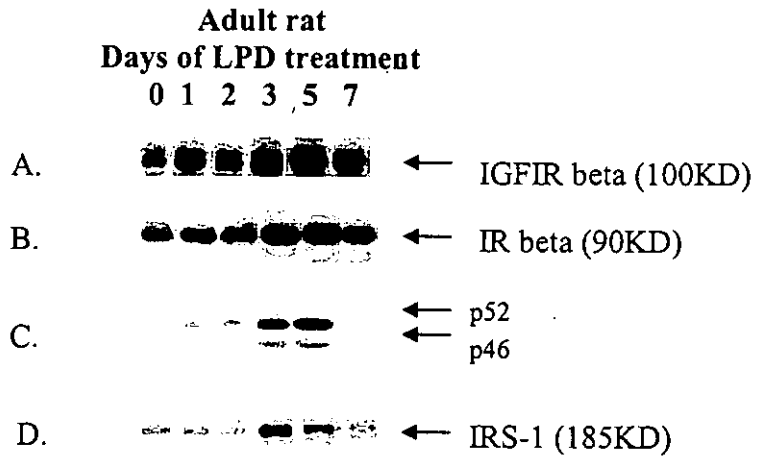

Figure 23: Western blotting analysis of various proteins along the IGF-I axis in adult rats under dietary P restriction. Fifty micrograms of renal proximal tubules cell lysates were resolved in 10 % SDS-PAGE analysis and electro-transferred to PVDF membrane for Western blotting. (A). IGFIR; (B). IR; (C). Shc and (D). IRS-1

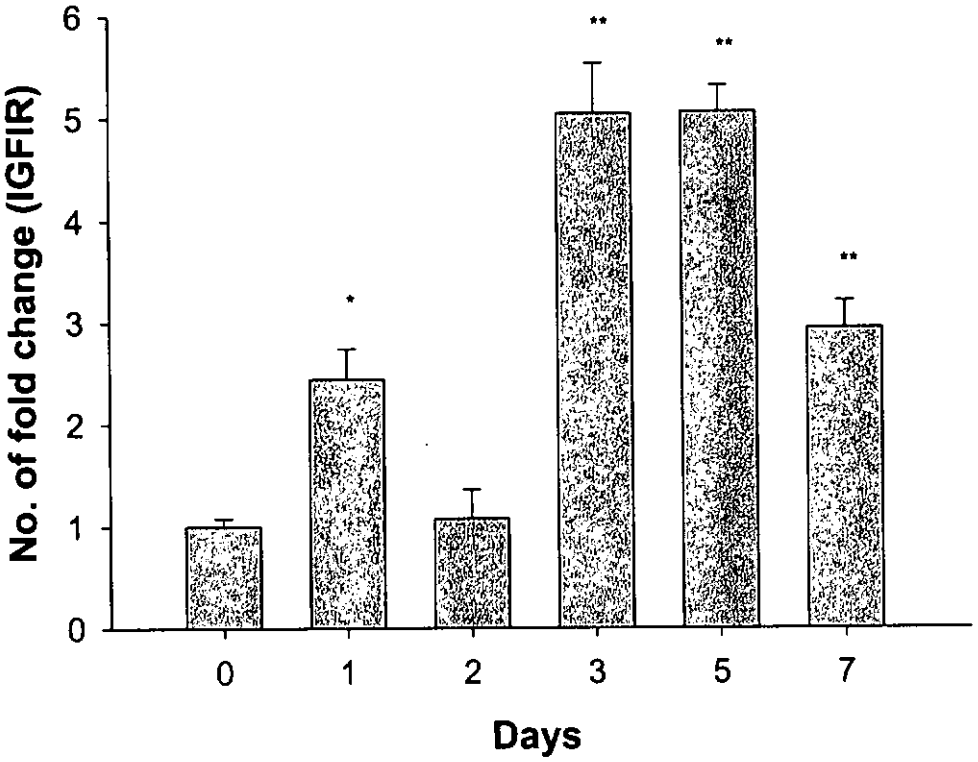

Figure 24: IGFIR protein expression in adult rats in response to dietary P restriction. Fifty micrograms of renal proximal tubules cell lysates were resolved in 10 % SDS-PAGE analysis and electro-transferred to PVDF membrane for Western blotting. The IGFIR protein expression increased in the first day (2.44 ± 0.30 folds) of LP treatment, then returned to normal in Day 2. Its expression reached a maximum on Day 3 (5.05 ± 0.49 folds) and Day 5 (5.067 ± 0.26 folds) and dropped back to (2.95 ± 0.27 folds) on Day 7 vs D0 (1.00 ± 0.08 fold). Data were analyzed by one-way ANOVA and the posttest, by Tukey's method, for n = 7, from 2 separate experiments. A p value of <0.05 was considered statistically significant. $^{*}p < 0.05$ and $^{**}p < 0.005$ vs Day 0.

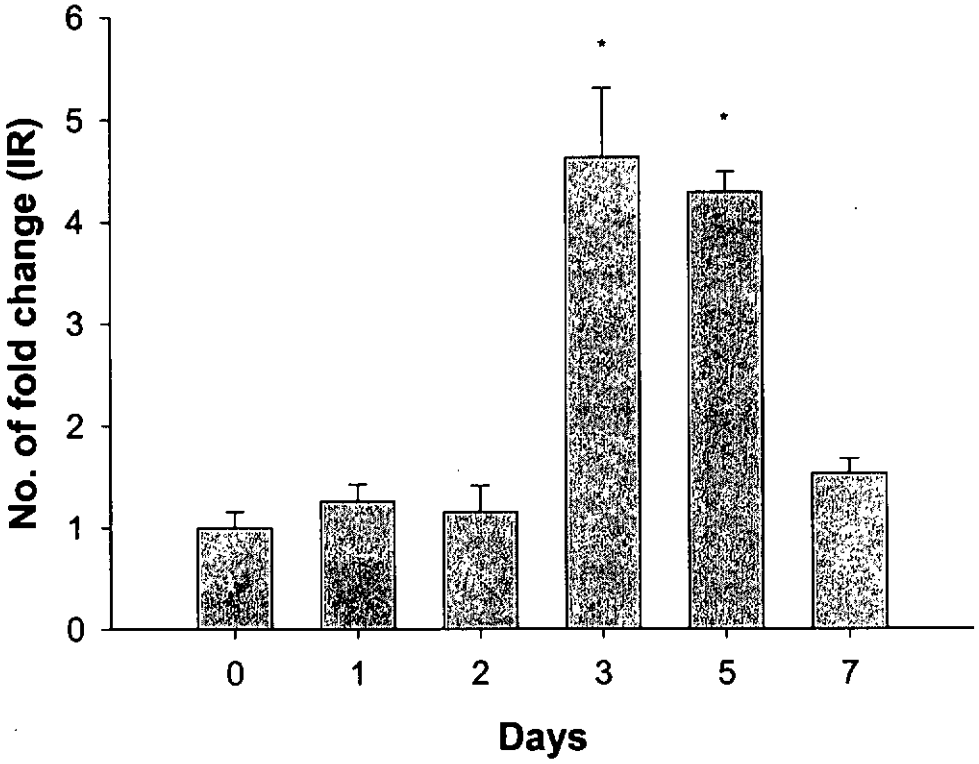

Figure 25: IR protein expression in adult rats in response to dietary P restriction. Fifty micrograms of renal proximal tubules cell lysates were resolved in 10 % SDS-PAGE analysis and electro-transferred to PVDF membrane for Western blotting. The IR protein expression had no change in the first two days and reached a maximum in Day 3 (4.63 ± 0.67 folds) and Day 5 (4.29 ± 0.20 folds). It then returned to normal in Day 7, vs Day 0 (1.00 ± 0.16 fold). Data were analyzed by one-way ANOVA and the posttest, by Tukey's method, for n = 7, from 2 separate experiments. A p value of < 0.05 was considered statistically significant. $^{*}p < 0.001$ vs Day 0.

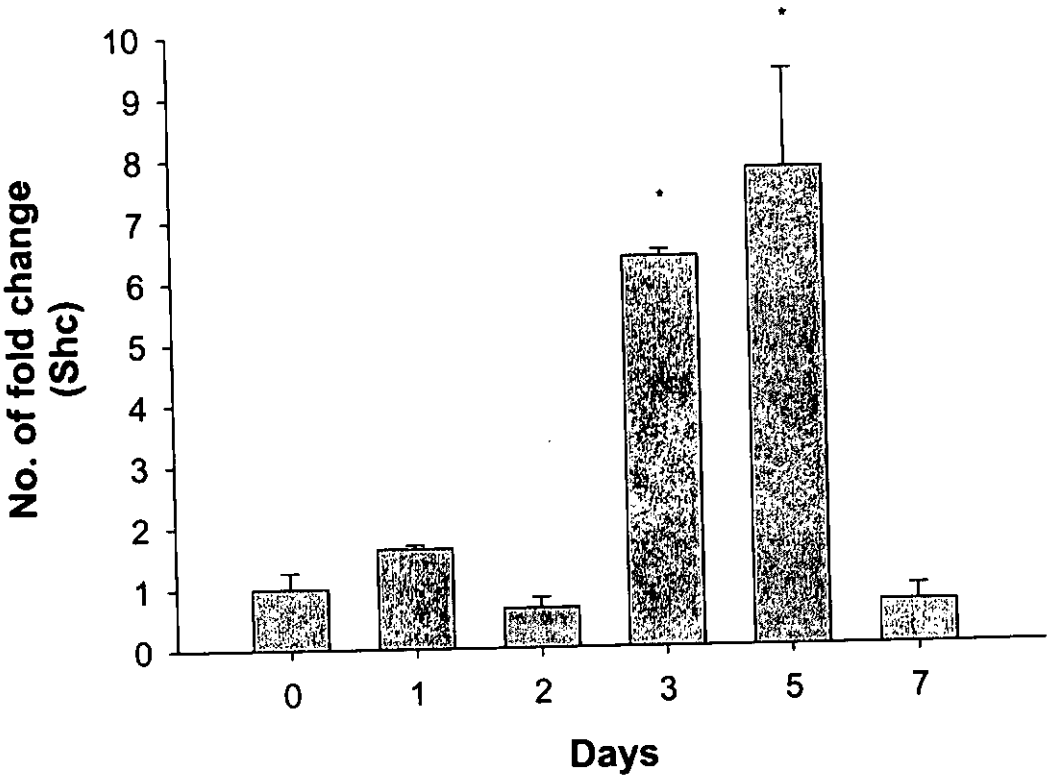

Figure 26: Shc protein expression in adult rats in response to dietary P restriction. Fifty micrograms of renal proximal tubules cell lysates were resolved in 10 % SDS-PAGE analysis and electro-transferred to PVDF membrane for western blotting. There was no change in Shc protein expression during the first two days. On Day 3 and Day 5, there was a sharp increase with a fold change of 6.37 ± 0.11 and 7.79 ± 1.60, respectively. The expression then returned to normal on Day 7, vs Day 0 (1.00 ± 0.26). Data were analyzed by one-way ANOVA and the posttest, by Tukey's method, for n = 3. A p value of < 0.05 was considered statistically significant. $^{*}p < 0.001$ vs Day 0.

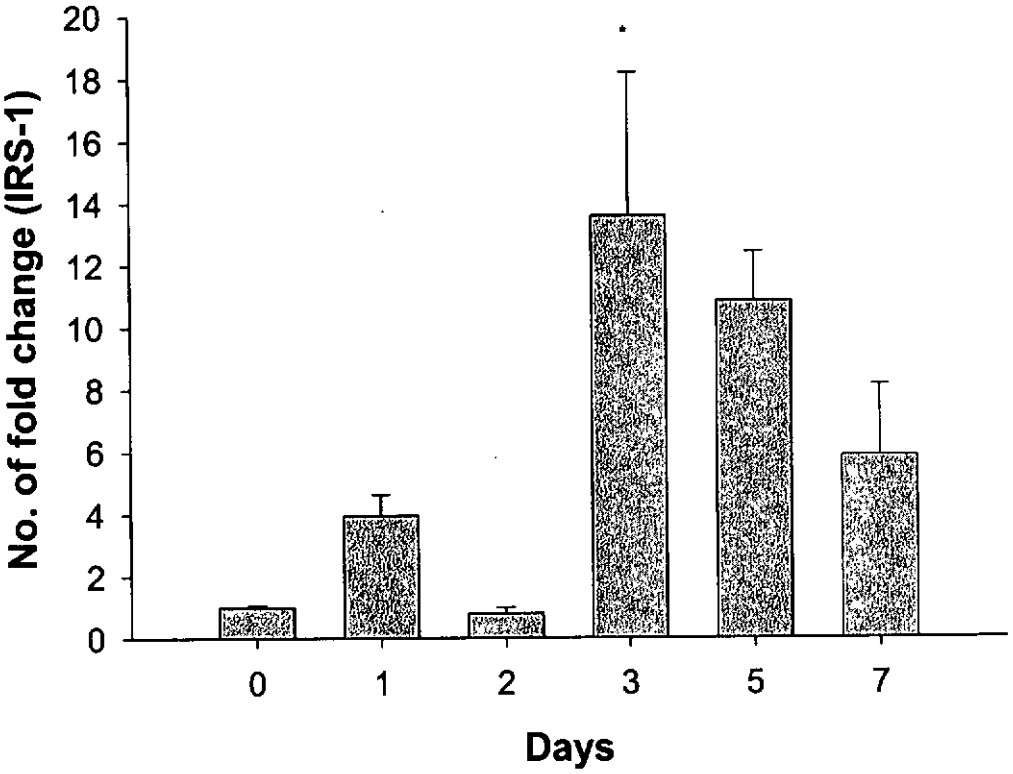

Figure 27: IRS-1 protein expression in adult rats in response to dietary P restriction. Fifty micrograms of renal proximal tubules cell lysates were resolved in 10 % SDS-PAGE analysis and electro-transferred to PVDF membrane for western blotting. The IRS-1 protein expression showed no change in the first two days and reached a maximum on Day 3 (13.59 ± 4.61 folds). The expression showed a gradual decrease from Day 3 to Day 7 (5.87 ± 2.28 folds) Data were analyzed by one-way ANOVA and the posttest, by Tukey's method, for n = 3. A p value of < 0.05 was considered statistically significant. $^{*}p < 0.05$ vs Day 0.

4.3.3 *In vivo* stability of proteins in IGF-I axis in young and adult rats fed normal or low P diet

In the last Chapter, the stability of renal 1-OHase protein was demonstrated to be P- and age-dependent. In this part of study, *in vivo* stability analysis was only focus on the two receptors IGFIR and IR, as undetectable expression level was measured in both downstream signaling proteins, i.e. Shc and IRS-I in young rats. Hence, effect of cycloheximide (CHX) on the expression of IGFIR and IR proteins in renal proximal tubules isolated from young and adult rats were investigated.

The expression of IGFIR protein was decreased in both young groups (NPD fed group, Figure 28A, and LPD fed group, Figure 28B) in response to CHX (Figure 29). The CHX effect was prolonged after 6 hours of CHX treatment in normal P group, while the effect was diminished and the protein expression was restored to the original level after 6 hours in low P condition (Figure 29). The IGFIR protein in LP condition was found to degrade more rapidly and more extensively (after 1 hour, with 77 % total protein being degraded, $p < 0.001$), but showed a higher turnover rate (as indicated by its faster recovery of the expression level at 6 hours) than in the NP condition (after 2 hours, with 73 % total protein being degraded, $p < 0.01$) (Figure 29). Unlike the 1-OHase protein, LPD did not stabilize the IGFIR protein, but seemed to increase the protein turnover rate in young rats. Therefore, enhancement of protein stability might not be the cause for increasing protein expression level upon LPD treatment.

In adult rats, the protein expression of IGFIR in response to CHX treatment was shown in Figure 30 and 31. The effect of CHX was totally different in both adult groups (Figure 31). The protein expression was decreased significantly during the first four hours, and restored to its original level after 6 hours treatment (Figure 31) in LPD fed rats. However, no significant change in protein expression of IGFIR was observed in NPD group in response to CHX treatment. The IGFIR protein was found to be less susceptible in adult rats (with a maximum of about 40 % IGFIR protein degraded, Figure 31) than in young rats (with a maximum of about 80 % IGFIR protein degraded, Figure 29) in the low P condition. Unlike the 1-OHase protein in young rats, IGFIR protein in adult rats was not stabilized by the LPD. These demonstrated that the increase in IGFIR protein expression level by LPD might not be due to the change in protein stability.

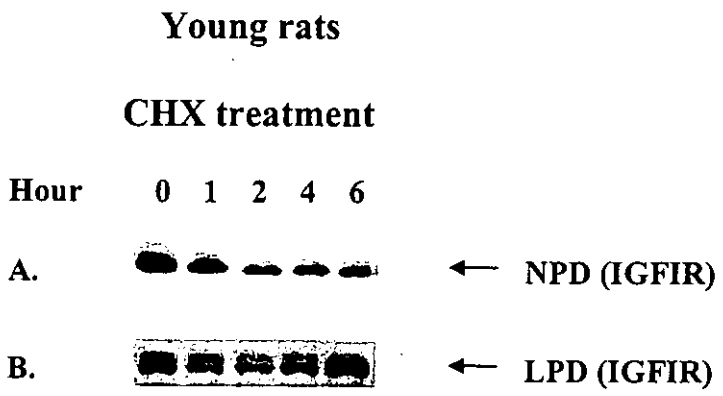

Figure 28: Western blotting analysis showing the effect of cycloheximide on IGFIR protein in young rats fed with either NPD or LPD. The dose of 0.3 mg / 100 g of body weight was injected intravenously through the rat tail. The CHX effect was assessed at different time points, 0 hour, 1 hour, 2 hours, 4 hours and 6 hours. Fifty micrograms of renal proximal tubules cell lysates were resolved in 10 % SDS-PAGE analysis and electro-transferred to PVDF membrane for Western blotting

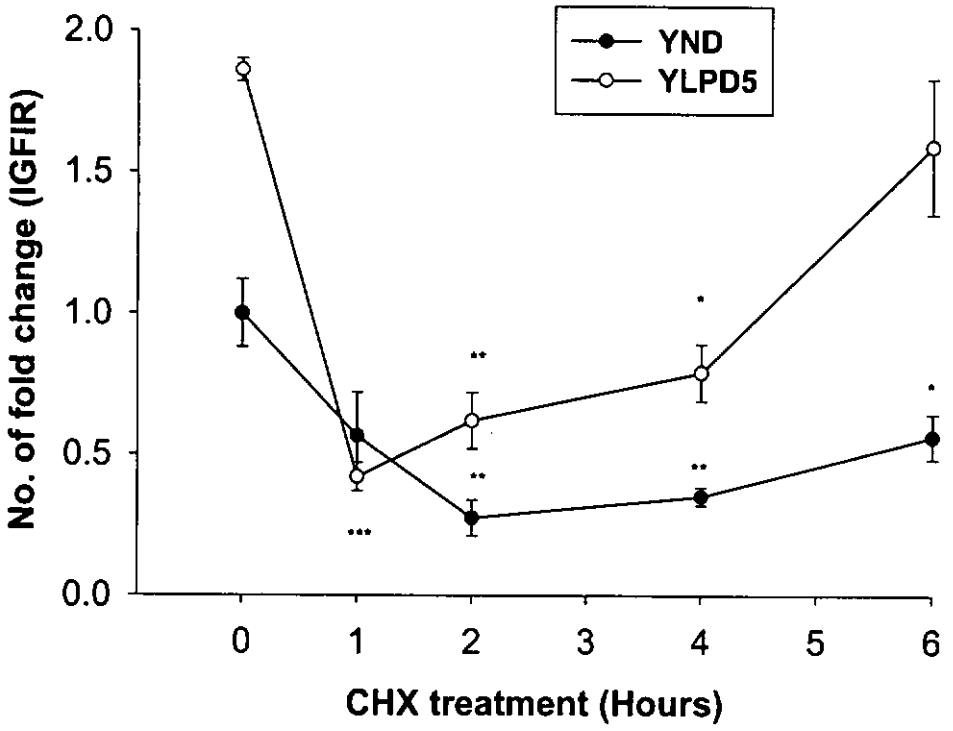

Figure 29: Effect of cycloheximide on IGFIR expression in young rats fed either NPD or LPD. The change in IGFIR protein expression level in response to CHX was shown. The effect of CHX of both NPD and LPD fed rats were similar. The IGFIR protein expression showed a first drop in both diets fed rats. The minimum IGFIR expressions in NPD and LPD fed rats occurred at 2-hour and 1-hour of CHX treatment, with 73 % ($^{**}p < 0.01$) and 77 % ($^{***}p < 0.001$) protein being degraded, respectively. Data were analyzed by one-way ANOVA and the posttest, by Tukey's method, for n = 4. A p value of < 0.05 was considered statistically significant. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs 0 hour in corresponding rat group.
YND represented young rats fed with NPD,
YLPD5 represented young rats fed with 5 days of LPD.

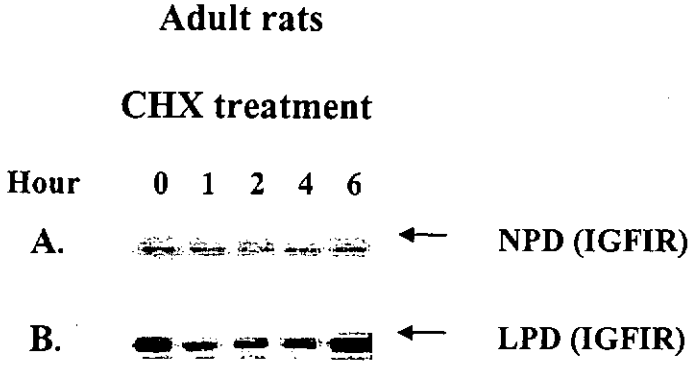

Figure 30: Western blotting analysis showing the effect of cycloheximide on IGFIR protein in adult rats fed with either NPD or LPD. The dose of 0.3 mg / 100 g of body weight was injected intravenously through the rat tail. The CHX effect was assessed at different time points, 0 hour, 1 hour, 2 hours, 4 hours and 6 hours. Fifty micrograms of renal proximal tubules cell lysates were resolved in 10 % SDS-PAGE analysis and electro-transferred to PVDF membrane for Western blotting.

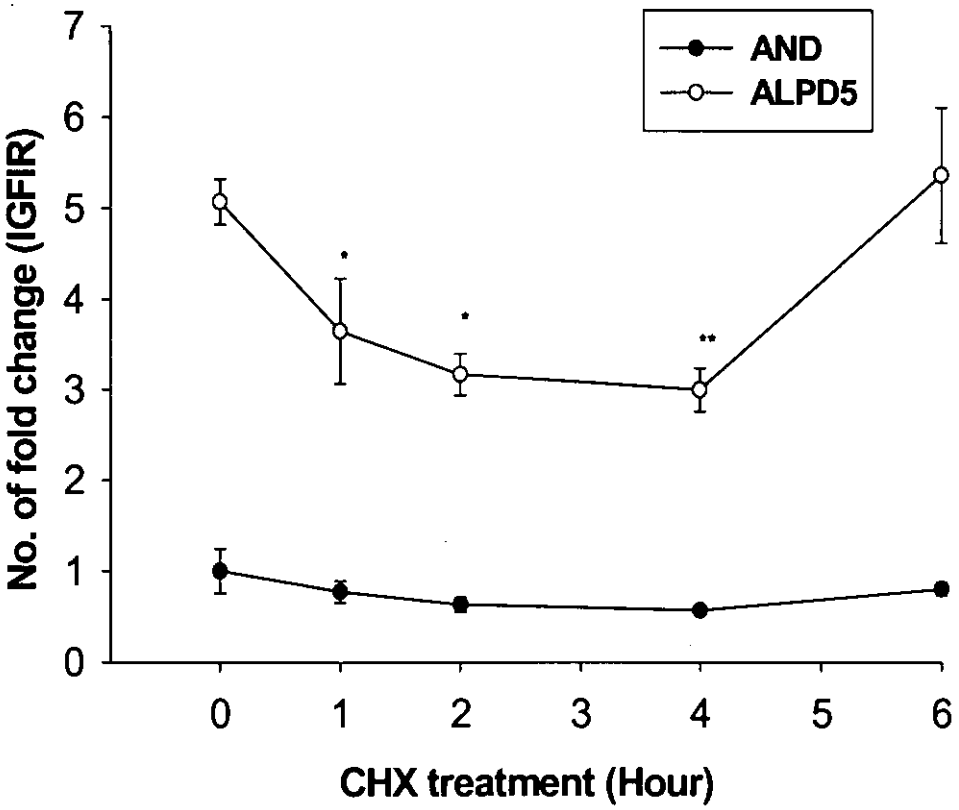

Figure 31: Effect of cycloheximide on IGFIR expression in adult rats fed either NPD or LPD. The change in IGFIR protein expression level in response to CHX was shown. The expression level changed differently in both adult groups in response to CHX treatment. The expression of IGFIR protein was significantly reduced during the first 4 hours and restored to its original level by 6 hours in LPD rats. However, the change in protein expression level was not significant in the NPD groups in response to the CHX treatment. Data were analyzed by one-way ANOVA and the posttest, by Tukey's method, for n = 4. A p value of < 0.05 was considered statistically significant. $^{*}p < 0.01$ and $^{**}p < 0.005$ vs 0 hour in corresponding rat group.
AND represented adult rats fed with NPD,
ALPD5 represented adult rats fed with 5 days of LPD.

In young rats, the response for IR protein expression was totally different in both groups (NPD fed group, Figure 32A, and LPD fed group, Figure 32B). IR protein expression was not affected by the CHX treatment in the NPD group (Figure 32A and Figure 33). On the contrary, over 90 % of IR protein was degraded during the first hour, but the expression level restored to the original level after 6 hours in LPD condition (Figure 32B and Figure 33). This showed that IR protein was rather stable in NPD condition and its stability was lowered by LPD. Unlike the 1-OHase protein in young rats, LPD did not enhance the IR protein stability, but made it becoming more susceptible during the CHX treatment. This indicated that the increase in protein stability might not be the reason for the increase in protein expression level by LPD.

The response to CHX of IR protein in both adult groups (NPD fed group, Figure 34A, and LPD fed group, Figure 34B) was extremely different from that of the young groups. The IR protein was rather stable that was not degraded during the course of CHX treatment (Figure 35). The IR protein expression was steady and was not changed, suggesting that the increase in protein expression level by LPD might not be due to the enhancement of protein stability.

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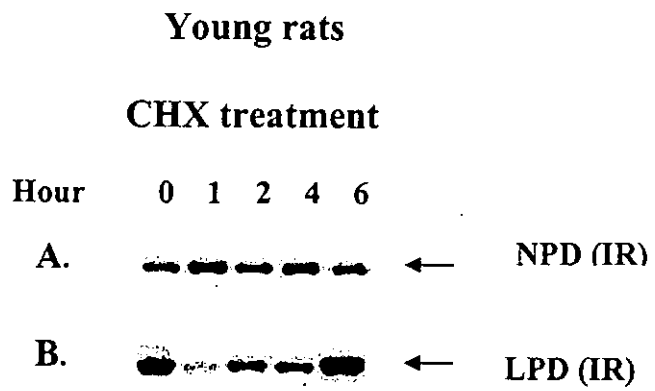

Figure 32: Western blotting analysis showing the effect of cycloheximide on IR protein in young rats fed with either NPD or LPD. The dose of 0.3 mg / 100 g of body weight was injected intravenously through the rat tail. The CHX effect was assessed at different time points, 0 hour, 1 hour, 2 hours, 4 hours and 6 hours. Fifty micrograms of renal proximal tubules cell lysates were resolved in 10 % SDS-PAGE analysis and electro-transferred to PVDF membrane for Western blotting.

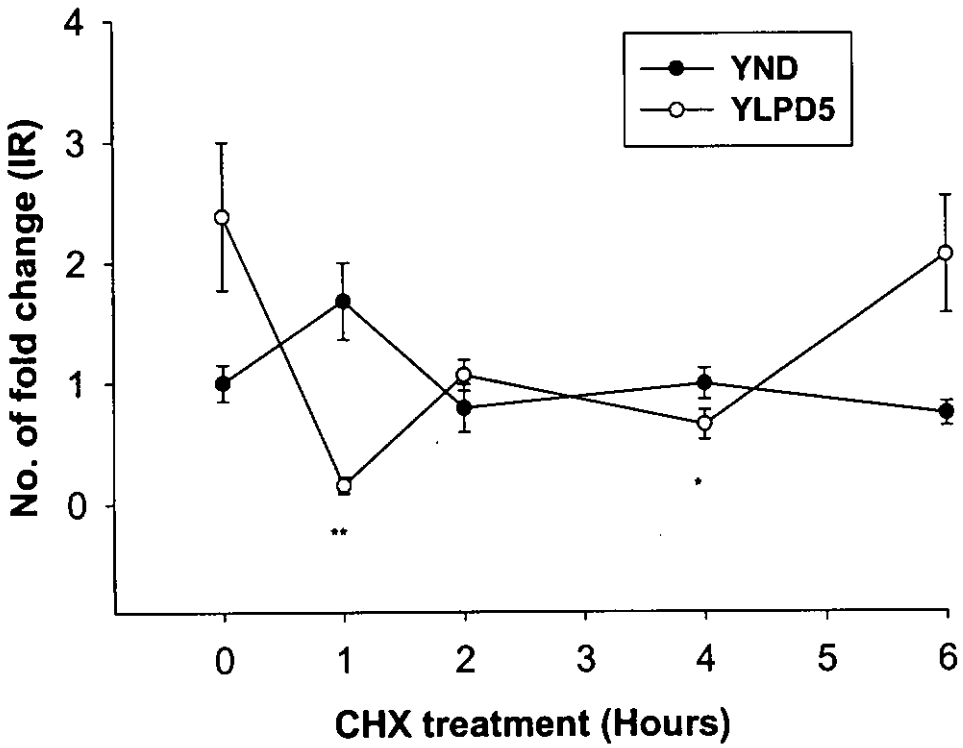

Figure 33: Effect of cycloheximide on IR expression in young rats fed either NPD or LPD. The change in IR protein expression level in response to CHX was shown. No significant change was found in NPD fed rats. However, during the first and fourth hour of CHX injections, up to 94% ($^{**}p < 0.005$) and 73 % ($^{*}p > 0.05$) of IR protein was significantly degraded in the LPD fed rats, respectively. The protein level returned to normal level after 6 hours in these rats. Data were analyzed by one-way ANOVA and the posttest, by Tukey's method, for n=4. A p value of < 0.05 was considered statistically significant. $^{*}p < 0.05$ and $^{**}p < 0.005$ vs 0 hour in corresponding rat group.
YND represented young rats fed with NPD,
YLPD5 represented young rats fed with 5 days of LPD.

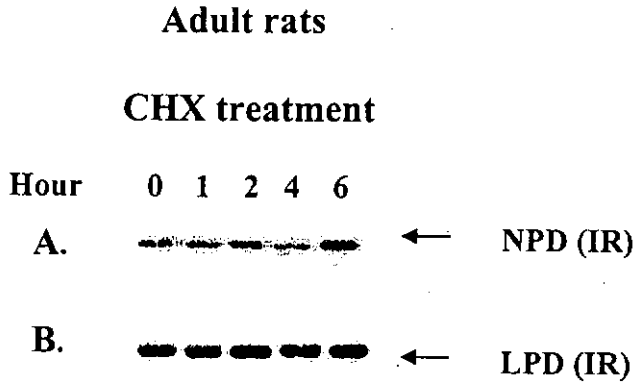

Figure 34: Western blotting analysis showing the effect of cycloheximide on IR protein in adult rats fed with either NPD or LPD. The dose of 0.3 mg / 100 g of body weight was injected intravenously through the rat tail. The CHX effect was assessed at different time points, 0 hour, 1 hour, 2 hours, 4 hours and 6 hours. Fifty micrograms of renal proximal tubules cell lysates were resolved in 10 % SDS-PAGE analysis and electro-transferred to PVDF membrane for Western blotting.

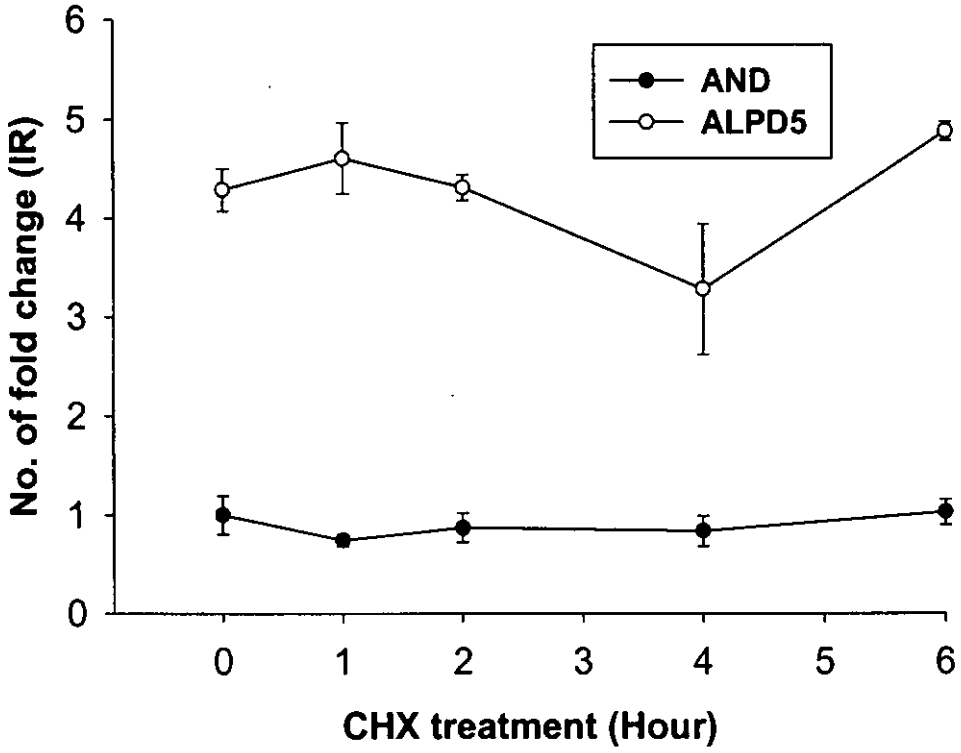

Figure 35: Effect of cycloheximide on IR expression in adult rats fed either NPD or LPD. The change in IR protein expression level in response to CHX was shown. No significant change of IR protein expression was found in both NPD and LPD fed rats. Data were analyzed by one-way ANOVA and the posttest, by Tukey's method, for n = 4.
AND represented adult rats fed with NPD,
ALPD5 represented adult rats fed with 5 days of LPD.

4.3.4 Regression analysis

As demonstrated in Section 3.3.4, renal 1-OHase protein expression was negatively correlated to serum P level in young rats. In this section, expression level of both receptors (IGFIR and IR) in young rats were correlated with serum P level. The data from adult rats was not showed as no correlation was found between 1-OHase protein expression and serum P level in adult rats (in Chapter 3), thus correlation analysis for adult rats was not performed.

Similarly, both IGFIR [IGFIR = (-0.11 ± 0.42) x serum P + (2.51 ± 0.47), $p < 0.05$, $r^2 = 0.21$] and IR [IR = (-0.17 ± 0.047) x serum P + (3.34 ± 0.49), $p < 0.005$, $r^2 = 0.40$] protein expression showed a negative correlation with serum P level in young rats (Figure 36). By comparing the r^2 value, it was noticed that IR showed a stronger relationship with serum P than IGFIR. When comparing the regression lines of the three proteins from young rats (Figure 37), the lines for IR and 1-OHase were nearly superimposed to each other, i.e. they shared similar slope (-0.17 and –0.18, respectively) and similar y-intercept (3.34 and 3.38, respectively). This suggested that their relationship with serum P level was very close to each other. In addition, it was found that the change in protein expression of 1-OHase with IR in young rats [IR = (0.82 ± 0.17) 1-OHase x (0.30 ± 0.27), $p < 0.0005$, $r^2 = 0.54$] showed a stronger positive relationship than that with IGFIR protein [IGFIR = (0.69 ± 0.20) 1-OHase x (0.23 ± 0.32), $p < 0.005$, $r^2 = 0.37$] in response to LPD (Figure 38).

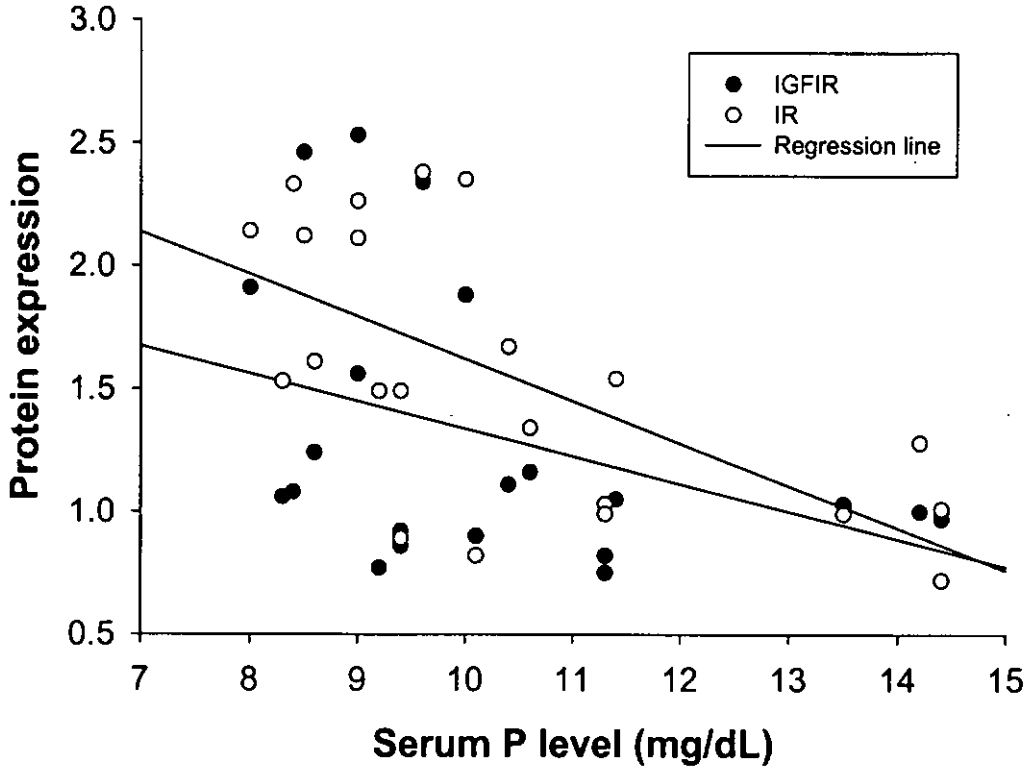

Figure 36: Measurement of IGFIR and IR protein expression of individual rats as a function of serum P level for rats fed LPD for 0-7 days. Negative relationship between the receptor protein expression and serum P level was built in young rats.

Lower regression line: IGFIR [IGFIR = (-0.11 ± 0.42) x serum P + (2.51 ± 0.47), $p < 0.05$, $r^2 = 0.21$];

Upper regression line: IR [IR = (-0.17 ± 0.047) x serum P + (3.34 ± 0.49), $p < 0.005$, $r^2 = 0.40$].

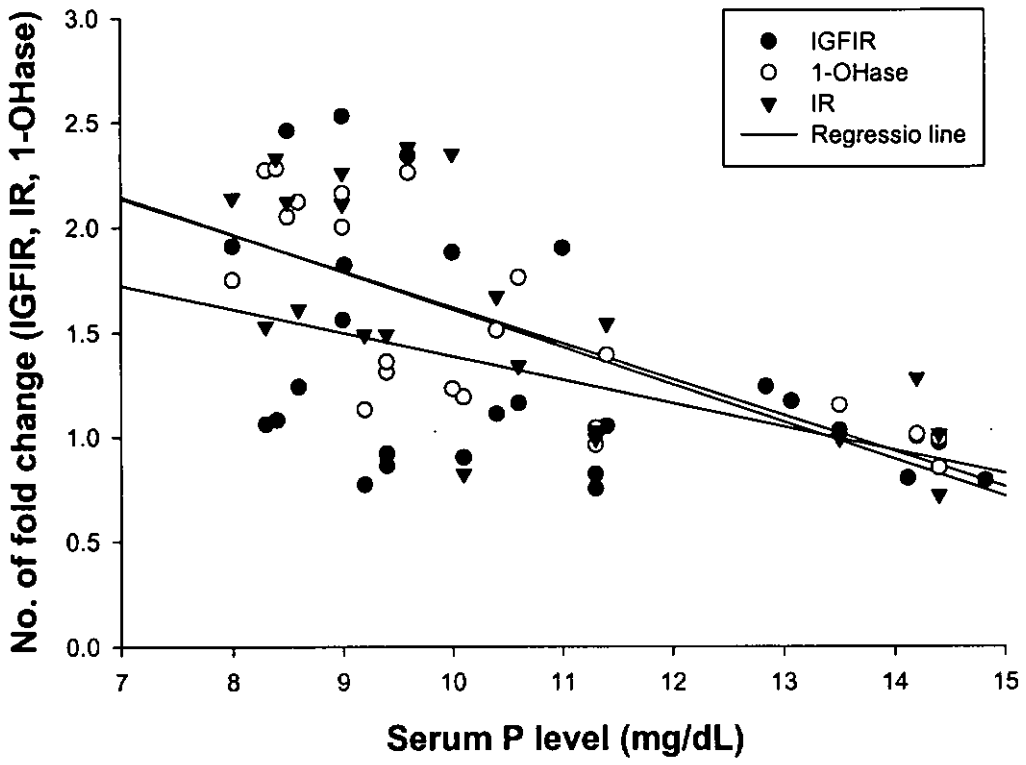

Figure 37: Measurement of IGFIR, IR and 1-OHase protein expression of individual rats as a function of serum P level for rats fed LPD for 0-7 days. Negative relationship was found in these three proteins with serum P level in young rats.

Lower regression line: IGFIR [IGFIR = (-0.11 ± 0.42) x serum P + (2.51 ± 0.47), $p < 0.05$, $r^2 = 0.21$];

Upper regression lines: IR [IR = (-0.17 ± 0.047) x serum P + (3.34 ± 0.49), $p < 0.005$, $r^2 = 0.40$] and 1-OHase [1-OHase = (-0.18 ± 0.037) x serum P level + (3.38 ± 0.39), $p < 0.0001$, $r^2 = 0.54$].

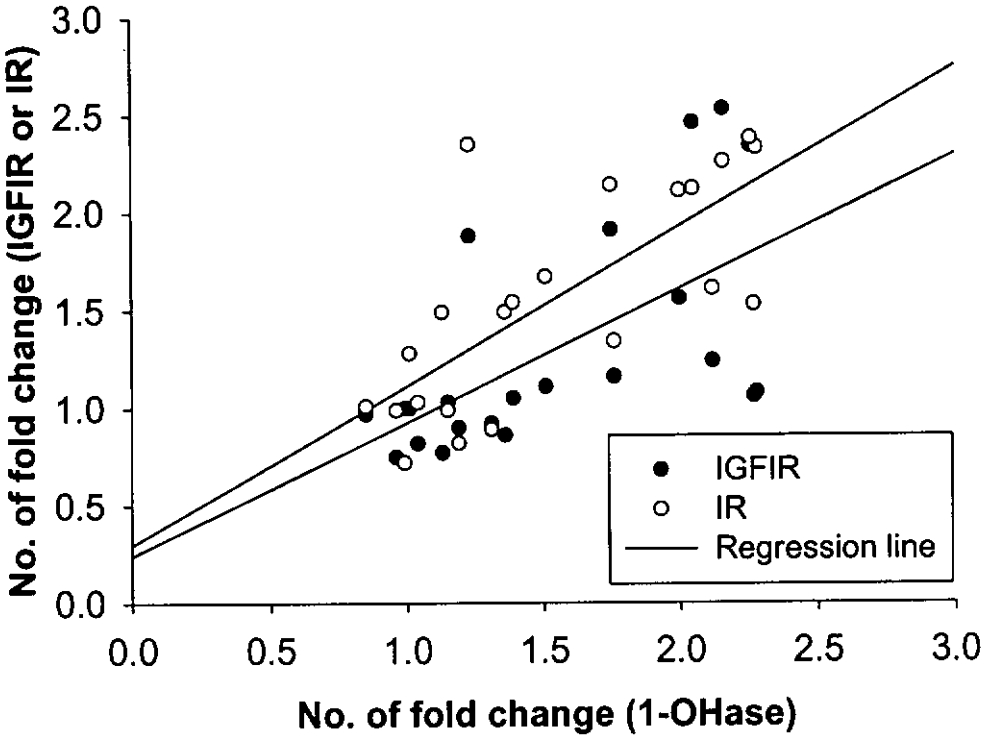

Figure 38: Measurement of IGFIR and IR protein expression of individual rats as a function of 1-OHase protein expression for rats fed LPD for 0-7 days. Positive relationship was found in both receptors with serum P level in young rats.
Lower regression line: IGFIR [IGFIR = (0.69 ± 0.20) 1-OHase x (0.23 ± 0.32), $p < 0.005$, $r^2 = 0.37$]
Upper regression lines: IR [IR = (0.82 ± 0.17) 1-OHase x (0.30 ± 0.27), $p < 0.0005$, $r^2 = 0.54$].

4.4. Discussions

In this chapter, the effect of dietary P restriction on the expression of proteins in IGF-I axis was evaluated. In addition, we have attempted to determine the *in vivo* stability of these proteins in rats fed either NPD or LPD. Results indicated that different mechanisms might be involved in the regulation of these proteins during LPD and that the regulation was age-dependent.

The decrease in serum P level induces a change in the expression of specific proteins in the IGF-I axis of both young and adult rats. LPD regulated IGF-I axis differently in the two age groups. The present study has shown that LPD elicited a more rapid and a more extensive effect on the expression of receptor proteins. In young rats, a significant increase of IGFIR expression was found on Day 5; however, upregulation was observed in adult rats as early as Day 1. In addition, the increase was up to 5 folds in adult rats (vs 1.86 fold in young rats). Similar interpretation also applied to IR protein. This indicated that the regulation of IGF-I axis by LPD was age-dependent. Besides, regulation of the docking proteins, i.e. the Shc and IRS-1, by LPD also showed a dramatic difference in both age groups, suggesting that the age-dependent regulation of IGF-I axis by LPD not only occurs at the receptor level, but also at the level of expression of downstream signaling proteins.

The *in vivo* protein stability experiments was also performed to determine the potential mechanism involved in the up-regulation of proteins in IGF-I axis. In Chapter 3, it was found that the up-regulation of 1-OHase protein was the

result of the enhancement of its *in vivo* stability upon LPD treatment in young rats. As mentioned in the beginning of this chapter, the regulation of 1-OHase was closely related with IGF-I. It was speculated that the increase in the IGFIR and IR protein expression (part of the IGF-I axis) by LPD might undergo the same mechanism as 1-OHase protein in young rats. However, the *in vivo* analysis indicated that the up-regulation of the two receptors was more likely due to other mechanisms, other than the one under tested, i.e. the enhancement of *in vivo* protein stability.

Serum IGF-I level was found to be decreased in response to dietary P restriction in young rats, but increased in adult rats. The LPD induced decrease in the circulating IGF-I could be compensated by increasing the IGFIR protein expression level for maintaining the usual metabolic functions in young rats. However, the serum IGF-I level was found to be increased in adult rats, at the same time, the expression of the proteins in IGF-I axis was also up-regulated dramatically during LP condition in adult rats. It was not known why the enhancement of both circulating IGF-I level and the expressions of receptors and their signaling proteins were needed for adaptive response of adult rats during LPD treatment.

The findings from the present study have showed that LPD up-regulated IGF-I axis in both young and adult rats and this up-regulation was age-dependent. The up-regulated proteins might be involved in the adaptive response of renal proximal tubules to LPD and thus involved in the phosphate homeostasis. In 2002, Cheung *et al.* reported a decrease in the total number of proteins in renal

proximal tubule cells in rats upon LPD treatment. The authors suggested that the change in some protein expression might be specific for adapting the change in serum P. Hence, the up-regulated proteins in the IGF-I axis might play a specific role in the regulatory pathway of phosphate homeostasis.

4.5 Conclusions

Dietary P restriction alters the expression of proteins in IGF-I axis in an age-dependent manner. Young rats up-regulated the expression of IGFIR and IR proteins significantly in response to 5 days of LPD. The expression of the downstream signaling proteins, shc and IRS-1, were not detectable and their expression was not up-regulated in young rats. While in adult rats, the response to LPD was more dramatic for both receptors and signaling proteins. They were all up-regulated at a earlier time point and to a greater extent than that in young rats. The *in vivo* stability analysis have shown that the up-regulation of these proteins in both age groups were not caused by an enhancement of protein stability upon LPD, suggesting other mechanisms might be involved.

Discussions and Conclusions

Discussions

It is well known that the active metabolite of vitamin D_3, 1,25-$(OH)_2D_3$, as well as IGF-I are involved in P homeostasis. Moreover, many previous studies (as cited in Section 1.4) have demonstrated the importance of IGF-I on 1-OHase regulation. These studies showed a close relationship between the IGF-I peptide and the regulation of enzyme activities for vitamin D biosynthesis under LPD. The results of the present study further suggest that the regulation of receptors and signaling proteins in IGF-I axis might be involved as well.

The present study has demonstrated LPD regulations of IGFIR, IR, shc and IRS-1 as well as 1-OHase proteins in rat renal proximal tubules and their regulations change with age. The results indicated that the responsiveness of rat renal proximal tubules to IGF-I might change upon LPD treatment. In addition, the present study demonstrated that the regulation of renal 1-OHase protein by LPD in young and adult rats were similar to the regulation of its enzyme activity as reported in the studies by Wong *et al.* (Wong *et al.*, 1997), suggesting that the blunted response of 1-OHase activities to LPD in adult rats was due to inability of LPD to up-regulate 1-OHase protein. However, the increase in the overall pool of circulating 1,25-$(OH)_2D_3$ level in adult rats in response to LPD revealed

that regulating factors, other than the 1-OHase, might be involved in the control of vitamin D metabolism.

The regression analysis in both Chapter 3 and 4 have shown that 1-OHase, IGFIR and IR protein were negatively correlated with the change in serum P level in young rats. IR was preferentially correlated with the change in serum P level and the change in 1-OHase protein expression (as indicated by a higher r^2 value than that of IGFIR, Figure 36-38). The phenomenon was quite surprising as IGF-I was preferentially bound to IGFIR. It was hypothesized that IGF-I involved in 1-OHase regulation through IGFIR. The findings suggested that IR might be preferentially involved in 1-OHase regulation, hence, further investigations are needed to prove this hypothesis. For example, we can use a specific inhibitor, alpha2-HSG, of IR tyrosine kinase (IR-TK) that inhibits the mitogenic pathway without affecting the metabolic arm of insulin signal transduction. It has no effect on EGF- or IGF-I- induced cognate receptor autophosphorylation (Mathews *et al.*, 2000). This can determine whether IR is preferentially involved in renal 1-OHase regulation by blocking its tyrosine kinase activity.

The present study has only showed the effect of dietary P restriction on the regulation of 1-OHase protein and some specific proteins in IGF-I axis and their correlations. Although both systems, i.e. the IGF-I axis and vitamin D, were regulated differently upon the LPD treatment and in different age models, this did not tell about their actual relationship. Therefore, experiments for determining their direct interaction would be required. For example, studies will be designed to determine if the activities of IGFIR (autophosphorylation and tyrosine phosphorylation of downstream signaling proteins) increase with LPD and to determine if blockage of the tyrosine kinase activity by genistein, a tyrosine kinase inhibitor, affects the 1-OHase regulation.

The expression patterns for the four IGF-I axis proteins (IGFIR, IR, Shc and IRS-1) in response to LPD were different in both age groups. The present data showed that these proteins in adult rats were more responsive to the LPD stimulation. However, the response of the proteins in IGF-I axis was parallel to the change in 1-OHase protein in young rats (both showed an up-regulation on Day 5), but not in adult rats, suggesting that regulations of both the IGF-I axis and renal 1-OHase were closely related in young rats. Our studies also indicated that loss of up-regulation of 1-OHase by LPD was not due to a blunted

response of the 1GF-I axis to LPD. It remains to be determined if the increase in response of these proteins in IGF-I axis to LPD might be secondary to the blunted response of target 1-OHase. However, it was only a speculation, further studies will be needed to clarify this. For example, we can use some blockers, e.g. JB-1 to block IGFIR; PD98059 to block MAP kinase function, to see any blockage of 1-OHase synthesis.

Conclusions

Dietary phosphate (P) restriction regulated 1-OHase protein and some specific proteins in IGF-I axis (IGFIR, IR, Shc and IRS-1) in an age-dependent manner. Regulation of 1-OHase showed a parallel alteration with the IGF-1 axis only in young rats, but not in adult rats. The mechanism for up-regulating the renal 1-OHase protein was caused by an enhancement of *in vivo* protein stability, however, that of the proteins in IGF-I axis in young rats was not explained by this, suggesting other regulating processes might be involved. In addition, LPD treatment did not enhance the stability of proteins in IGF-I axis in both age groups. Regression analysis between different measurements were made in young rats as no correlation was found between serum P level and renal 1-OHase protein expression level. The analysis have shown that a negative

relationship was found between the 1-OHase, IGFIR and IR protein expression levels with serum P levels, and a positive relationship was reported between the expression level of 1-OHase with IGFIR and IR protein. Results have shown a closer correlation between 1-OHase and IR, implying that IR was preferentially involved in 1-OHase protein regulation than IGFIR. In depth understanding of the regulation of this vitamin D enzyme, 1-OHase, would enhance our knowledge in the pathogenesis of osteoporosis, and hence, the treatment of this bone disease.

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