CHAPTER V
DISCUSSION

5.1 Analysis of proteome profile in renal cortex and medulla related with K status in subjects living in northeastern Thailand

The proteomic analysis revealed a number of proteins were differentially expressed in renal cortex and medulla tissues between of the NKD and KD of cadaveric donors. The results indicated that the depletion of K had some effects on the function and pathophysiology of renal tissues. The identified proteins of renal cortex and medulla in this study were classified into many groups. In renal cortex, the identified proteins were classified into six functional groups based on their major roles in biological processes, including metabolic enzymes, signaling proteins, RNA metabolism, cell division, DNA repair and immune responses. Whereas in renal medulla the altered proteins were classified into eight functional groups, including metabolic enzymes, cytoskeletal proteins, transporter proteins, small GTP-binding protein, rho protein signal transduction, transcription regulation, the immune responses, and novel protein. Functions and the properties of the altered proteins had been searched for theirs information by using the well-established protein data base including NCBI (http://www.ncbi.nlm.nih.gov/), HumanCyc (http://humancyc.org/), and Swiss-Prot/TrEMBL (http://expasy.org/) to obtain additional details for their functions. The synonyms and details of theirs metabolic functions, as well as the identified properties involved in renal cortex and medulla, were summarized in Table 12 and 13, respectively.

Table 12 Brief descriptions of some proteins which were significantly altered in KD renal cortex

Spot	**Protein**/Synonyms	**Function**/Catalytic activity
7857	*Signaling protein* **SPARC-like 1**[1,3] Sc1 Ecm2 Hevin Mast9 Sparcl1	Regulate cell growth through interactions with the extracellular matrix and cytokines. Binds calcium and copper, several types of collagen, albumin, thrombospondin, PDGF and cell membranes. There are two calcium binding sites; an acidic domain that binds 5 to 8 Ca^{2+} with a low affinity and an EF-hand loop that binds a Ca^{2+} ion with a high affinity.
8899	*Immune response* **Immunoglobulin heavy chain variable region**[1]	Immune response, antigen binding.
8988	*Immune response* **Immunoglobulin heavy chain variable region**[1]	Immune response, antigen binding.
11929	*Immune response* **T cell receptor variable beta chain**[1]	Immune response, receptor activity.
27745	*Signaling protein* **Protein tyrosine kinase 2-beta isoform 3** [2,3] FAK2, PYK2, RAFTK, CADTK, CAK beta, Calcium-dependent tyrosine kinase, Cell adhesion kinase beta, FADK 2, Focal adhesion kinase 2, Proline-rich tyrosine kinase 2, Related adhesion focal tyrosine kinase	Member of the focal adhesion kinase family; tyrosine kinase that may activate ion channels and MAP_kinase pathway.

Table 12 Brief descriptions of some proteins which were significantly altered in KD renal cortex (Cont.)

Spot	**Protein**/Synonyms	**Function**/Catalytic activity
	RNA metabolism	
27746	**ATP-dependent RNA helicase DDX60** [1] EC 3.6.1 Probable ATP-dependent RNA helicase DDX60 DEAD box protein 60	Its function has not been identified.
	Cell division	
27758	**Spindle and kinetochore-associated protein 1** [1,3] C18orf24	Component of the SKA1 complex, a microtubule-binding subcomplex of the outer kinetochore that is essential for proper chromosome segregation. Required for timely anaphase onset during mitosis, when chromosomes undergo bipolar attachment on spindle microtubules leading to silencing of the spindle checkpoint.
	DNA repair	
27786	**General transcription factor IIH subunit 1** [1,3] Gtf2h1 protein General transcription factor IIH polypeptide 1 TFIIH basal transcription factor complex p62 subunit Basic transcription factor 2 62 kDa subunit	Its involved in nucleotide excision repair (NER) of DNA and, when complexed to CAK, in RNA transcription by RNA polymerase II.
	Metabolic enzyme	
27851	**Glutathione S-transferase P** [1] EC 2.5.1.18 GST class-pi GSTP1-1 FAEES3 GST3	Glutathione S-transferase P is enzymes that play an important role in detoxification by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione. RX + glutathione = HX + R-S-glutathione
	Metabolic enzyme	
27954	**Acyl-CoA thioesterase 9**[1] EC 3.1.2 ACOT9, MT-ACT48, ACATE2, CGI-16	Acyl-CoA thioesterases are a group of enzymes that catalyze the hydrolysis of acyl-CoAs to the free fatty acid and coenzyme A (CoASH), providing the potential to regulate intracellular levels of acyl-CoAs, free fatty acids and CoASH. Active on long chain acyl-CoAs.

Table 13 Brief descriptions of some proteins which were significantly altered in KD renal medulla

Spot	**Protein**/Synonyms	**Function**/Catalytic activity
	Metabolic enzyme	
1147	**Catalase** [2,3] EC 1.11.1.6 CAT	Catalase converts the reactive oxygen species hydrogen peroxide to water and oxygen and thereby mitigates the toxic effects of hydrogen peroxide.
	Cytosketal protein	
11234	**Actin, gamma 1** [1,3] ACT; ACTG; DFNA20; DFNA26; ACTG1	Actins are highly conserved proteins that are involved in various types of cell motility, and maintenance of the cytoskeleton.
	Transporter protein	
1262	**ADP. ATP translocase** [1,3] Adenine nucleotide translocator ADP,ATP carrier protein, mitochondrial	Catalyzes the exchange of ADP and ATP across the mitochondrial inner membrane.
	Immune response	
1422	**T-cell receptor beta chain** [1,3]	T cell receptors recognize foreign antigens which have been processed as small peptides and bound to major histocompatibility complex (MHC) molecules at the surface of antigen presenting cells (APC).
	Novel protein	
1476	**Riken cDNA 1700001E04**[3]	Its function has not been identified.
	Rho protein signal transduction	
1489	**Guanine nucleotide exchanger factor GEFT isoforms 3** [1,3] RhoA/Rac/Cdc42 guanine nucleotide exchange factor GEFT Rac/Cdc42/Rho exchange factor GEFT p63RhoGEF	The Rho family of small GTPases act as molecular switches to control a wide range of cellular processes.

Table 13 Brief descriptions of some proteins which were significantly altered in KD renal medulla (Cont.)

Spot	**Protein**/Synonyms	**Function**/Catalytic activity
	Transcription regulation	
1558	**Enhancer of polycomb homolog 1 isoforms 2**[1] EPC1	Component of the NuA4 histone acetyltransferase (HAT) complex which is involved in transcriptional activation of select genes principally by acetylation of nucleosomal histones H4 and H2A.
	Metabolic enzyme	
1562	**Cabonic anhydrase 1**[1,3] EC 4.2.1.1 Car1 Carbonic anhydrase B	Reversible hydration of carbon dioxide. Can hydrates cyanamide to urea. $H_2CO_3 = CO_2 + H_2O$
	Metabolic enzyme	
1661	**Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase isoform 1**[1,3] LBP; ECHD; LBFP; PBFE; L-PBE; MGC120586; EHHADH	The protein encoded by this gene is a bifunctional enzyme and is one of the four enzymes of the peroxisomal beta-oxidation pathway.
	Small GTP-binding protein	
1666	**Rab11 family-interacting protein 2**[1,3] NRip11 Rab11fip2	A Rab11 effector protein acting in the regulation of the transport of vesicles from the endosomal recycling compartment (ERC) to the plasma membrane.
	Immune response	
2408	**Immunoglobulin heavy chain complementarity-determining region 3** [1]	Its function has not been identified.

[1]= Swiss –Prot database,[2]= HumanCyc database,[3]= NCBI database

5.2 Technical concerns in proteomic technology and the limitation of proteomic

Some technical issue in this study need to be discussed, we performed proteomic analysis of renal cortex and medulla tissues from 12 different subjects and one of the most concerned issue in gel-based, differential proteomics in the reproducibility /com patibility of 2-D spot pattern across different gels, in which variations may occur and make the analysis more difficult. In previous gel-based, proteome studies analyzing human tissues, cell lines, and body fluids, up to 25% of CV was observed and acceptable for the reproducibility of 2 DE (Hunt et al., 2005; Molloy et al., 2003; Terry and Desiderio, 2003). In our study, we also observed some degree of variations in the kidney tissue obtained from 12 subjects. However, the %CV obtained from this study was within acceptable ranges. Therefore, quantitative intensity analysis in our present study was justified for the compatibility of intensity volume of each spot across different gels.

The total number of protein spots visualized in the present study was relatively small for the entire kidney proteome. This limitation might be because (i) we used the small format 2-D gel (using 7-cm-long IPG strip) in the present study that has some limitation in protein separation; much more spots should have been resolved using a large format (using 18-to 24-cm-long IPG strip); (ii) Coomassie Blue stain was employed instead of silver or fluorescence stains that are more sensitive in protein detection; and (iii) we used the highly stringent criteria or parameters for detection of "true protein spots" in the present study; much more spots are expected to be detected when the lower stringent criteria or parameters are used.

5.3 Schematic propose for the mechanism involving in HN

The mechanism of KD involving in HN are proposed and shown in Figure 13. Chronic K depletion may results from low K intake and high sweat loss. The causes of K depletion among the Northeastern Thais (NE) have proposed to be related with two main factors, including low K intake and non-renal K loss due to excessive sweating (Sriboonlue et al., 1998). Sriboonlue et al (1998) demonstrated that people in this rural area normally consume traditional food that are of low K content (Sriboonlue et al., 1998). The effects of chronic K depletion and concomitant with the increase accumulation of intracellular Na^+could lead to the production of both a chemical and electrical gradient across the cell membrane of the renal tissues (Lingrel et al., 2003). The ion gradients formed by the Na^+-K^+ATPase are necessary for Na^+ coupled transport of nutrients and amino acids into the cells, osmotic balance, cell volume regulation and for maintenance and restoration of the resting membrane potential in excitable cells (Geering, 1997; Pavlov and Sokolov, 2000). As long as, there are no external of K supplies, the concentration gradient for Na across the plasma membrane will remain decreased. This effects may also have implication of Mg co-depletion. Mg is a cofactor of Na^+-K^+ ATPase, its depletion therefore could bring about the reduction in activity of Na^+-K^+ ATPase (Bovornpadungkitti et al., 2000; Dorup, 1994; Dorup and Clausen, 1993; Dorup et al., 1988; Prasongwatana et al., 2001). This will interfere with the transport processes dependent on this gradient and its potential energy. Thus, the extrusion of H ions will be impaired via the Na/H exchange mechanism, leading to intracellular acidosis and extracellular alkalosis (Clausen, 1992). Similarly, both reduced K and raised intracellular Na contents may favour the cellular accumulation of free calcium ($[Ca^{2+}]i$) due to activation of voltage-gated Ca^{2+}channels and a reverse operation of the Na^+-Ca^{2+} exchanger (Archibald and White, 1974; DiPolo and Beauge, 1991; Xiao et al., 2002) and can lead to the mitochondria swelling and cellular damage (Gissel, 2005; Knochel, 1982; Knochel and Schlein, 1972).

Two possible mechanisms of these altered proteins may linked to the pathophysiology of HN. Firstly, increased intracellular Ca could leads to increased production of ROS, causing peroxidation of membrane lipids, and possibly decrease in ATP synthesis. Secondly, Ang II increases several inflammatory proteins including

MCP-1 and TGF-β involved in cell growth and matrix regulation and could activate renal RAS and therefore participated in renal injury (Figure 11).

Firstly, Ca^{2+} is one of the major intracellular second messengers. Cytoplasmic Ca^{2+}varies in dynamic manner as a results of Ca^{2+} release from intracellular stores (endoplasmic/sarcoplasmic reticulum/mitocondria). The vicious cycle model (Figure 10) describes how the above-mentioned factors act in concert, creating a self re-inforcing cycle that can lead to necrosis and apoptosis (Gissel, 2005). An increase in Ca influx across the cellular membrane is observed in many situations. Influx of Ca will lead to local increase in [Ca] in the sub-sarcolemmal compartments, which may lead to local activation of calpain. The sarcoplasmic reticulum (SR) is capable of storing quite large quantities of Ca. However, buffer capacity differs between different fiber types. The SR of fast-twitch fibers is capable of increasing their Ca content threefold, whereas the SR of slow-twitch fibers is almost saturated at resting $[Ca]_c$. Thus in the fast-twitch fibers, the SR represents a large buffer capacity, whereas alow-twitch fibers most likely rely on their more abundant mitochondria for Ca buffering. In addition fast fibers from lower vertebrates and small mammals contain high concentrations of parvalbumin which may act as a significant buffer for Ca. The mitochondria will participate actively in the Ca handling, particularly at the time of SR saturation. Mitochondria are capable of storing large amounts of Ca; however, if the storage capacity is exceeded, detrimental consequences may occur. Increased Ca load leads to increased production of ROS, causing peroxidation of membrane lipids, and possibly decrease in ATP synthesis. Mitochondria Ca overload also increases the risk of opening of the permeable transition-pore, which will ultimately lead to apoptosis and necrosis. Local increases in $[Ca]_c$ may lead to PLA_2 activation, resulting in degradation of cellular membranes of organelles. This leads to an increase in production of ROS in the mitocondria (Gissel, 2005).

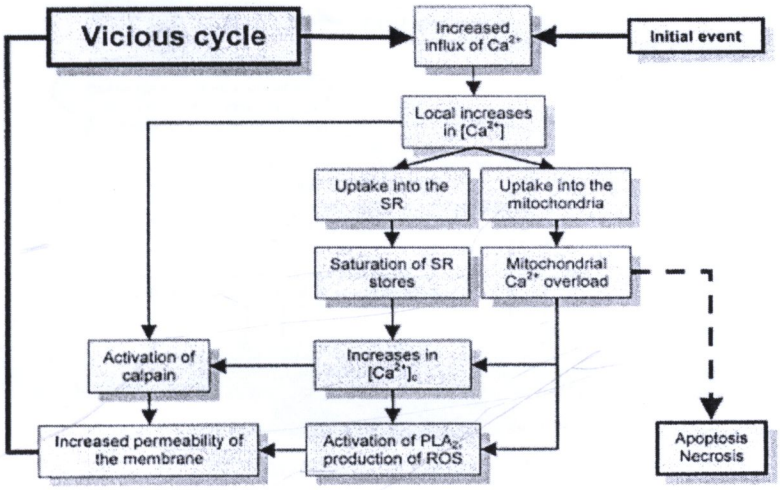

Figure 10 The " viscous cycle" model of how and initiation event or condition leading to increased influx of Ca^{2+}may result in renal cell apoptosis and necrosis (Gissel, 2005)

Secondly, Ang II, the main peptide of the rennin angiotensin system (RAS) is renal growth factor, including hyperplasia/hypertrophy depending on the cell type. This vasoactive peptide activates mesangial and tubular cells and interstitial fibroblasts, increasing the expression and synthesis of extracellular matrix proteins. Some of these effects seem to be mediated by the release of growth factors, such as TGF-β, PDGF. Ang II is chemotactic factor for inflammatory cells and increases chemokines and adhesion molecules in both resident and infiltrating cells. This inflammatory respond could be directed by MCP-1 and TGF-β production, and indirect, by activation of resident cells by macrophages-related factors, and therefore contributes to the progression of fibrosis. In renal cells, Ang II increases several proteins involved in cell growth and matrix regulation. Proteinuria could activate renal RAS and therefore may participate in renal injury (Mezzano et al., 2001).

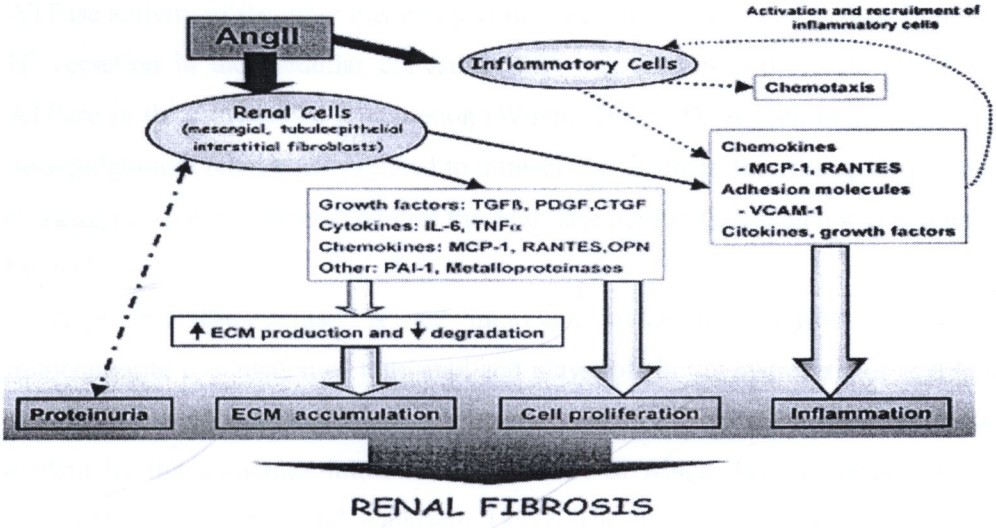

Figure 11 Cells and mediators involved in Ang II-indued renal fibrosis. (Mezzano et al., 2001)

The altered proteins in these K depleted subjects may response via these many systems. In the present study, we have focused our attention on changes in particular metabolic and signaling proteins as these changes may explain pathophysiological processes that occur in HN, especially acid-base balance and tubular injury.

Carbonic anhydrase which reversibly catalyze the hydration of CO_2 to HCO_3^- and H^+, is widely contributed in mammalians tissues and important roles in acid-base regulation, gas transport, and various secretory functions in tissues (Aliakbar and Brown, 1996; Tashian, 1989). Carbonic anhydrase isoform I (CAI, CA-B) is one among 11 isoforms in α-class of CA found in mammals (Tripp et al., 2001). We identified an up-regulation of renal CA I in KD subjects. CA I is responsible for reversible catalyzing reaction of hydration of carbon dioxide ($CO_2+H_2O \leftrightarrows H_2CO_3$). CA I is the major non-hemoglobin in human red cells. It is also found in several other tissues such as colon, but it is not as widely distributed as CAII (Sly and Hu, 1995). In addition, Funakoshi and Deutsch reported that kidney medulla contains both CA-B and CA-C (Funakoshi and Deutsch, 1970). K depletion normally causes decreased intracellular pH attributable to cellular H^+influx and lead to intracellular acidosis (Melnick et al., 1996). Helou et al (1994) have shown that KD rats increased H-K

ATPase activity of the inner medullary collecting duct (Helou et al., 1994). In rabbits, H^+ secretion in the medullar collecting duct was decreased by inhibition of H-K ATPase in the presence of K depletion (Wingo, 1989). These data indicated that the up-regulation of renal CAI is related to intracellular acidosis, thus the up-regulation of this enzyme may response to control the acid-base homeostasis which was maintained by the kidneys.

Acyl-CoA thioesterase is an important metabolic enzyme involved in mitochondrial β oxidation of fatty acid and plays role in the hydrolysis of acyl-CoAs to free fatty acid and CoASH. The central role of free fatty acids and acyl-CoAs is evident by the numerous intracellular processes in which these molecules are key component (Faergeman and Knudsen, 1997). The physiological functions of most acyl-CoA thioesterases are not clearly understood. The ability of thioesterases to regulate acyl-CoA concentration in the cell may provide a mechanism for control of lipid metabolism (Knudsen et al., 1975; Libertini and Smith, 1978). In addition, thioesterases also control the acylation state of some proteins, such as signal transduction proteins, and their intracellular localization (Casey, 1995). Our present study identified the increase levels of Acyl-CoA thioesterase 9 in KD subjects, suggesting their roles in the replenishment of energy source in the low-K condition. However, it role in HN remain poorly understood.

ADP.ATP translocase, a major component of the inner mitocodrial membrane. This is a transport protein of 297 amino acids which takes ATP from the mitochondrial matrix across the inner membrane to the intermembrane space and carriers ADP back (Klingenberg, 1985). In the present study, we identified the up-regulation of ADP.ATP translocase in KD subjects. Its up-regulation have linked to ATP depletion. The primary role of mitochondrial Ca^{2+} in normal physiology is stimulation of ox-phos (oxidative phoshporylation) (Balaban, 2002; Das and Harris, 1990; Hansford and Zorov, 1998; McCormack and Denton, 1993; Mildaziene et al., 1995), this occurs at many enzymes, including allosteric activation of TCA cycle and oxidative phoshporylation, including activation of pyruvate, isocitrate dehydogenase, and α-ketoglutarate dehydogenase (Mildaziene et al., 1995), as well as stimulation of the ATPsynthase, α-glycerophosphate dehydogenase (Wernette et al., 1981), and the adenine nucleotide translocase (ANT) (Mildaziene et al., 1995). Overall effect of

elevated $[Ca^{2+}]_m$ is the coordinated up-regulation of the entire ox-phos matchinery, resulting in higher ATP output can be changed to meet the cellular ATP demand. Conversely, an excessive of intracellular Ca^{2+} $[Ca^{2+}]_i$ effects to abnormalities in energy production and utilization (Dhalla and Temsah, 2001) lead to impaired of ATP production, which related to the oxidative stress and cell damage (Newton et al., 2009; Wang et al., 2003). Therefore, ADP.ATP translocase up-regulation in the KD subjects might reflect replacement of ATP during prolonged K depletion.

Glutathione S-tranferase P (GST class-pi) is one of GSTs, a ubiquitous class of enzymes with potential antioxidant properties. GSTs are best known for the detoxification of xenobiotics such as herbicides (Edwards et al., 2000), but they can also act as antioxidants by tagging oxidative degradation products (especially from fatty acids and nucleic acids) for removal or by acting as a glutathione peroxidase directly scavenge peroxides (Dixon et al., 2002). In addition to reduce lipid peroxides directly, GSTs may act to remove lipid peroxidation. GSTs act by catalyzing the conjugation of reduced glutathione (GSH) with electrophilic, often hydrophobic toxic compounds to form derivatives that can be secreted from the cell, sequestered in the vacuole, or catabolized (Eyer and Schneller, 1983). The up-regulation of renal GTPs has been observed in various models of renal tubular injury (Branten et al., 2000; Cressey et al., 2002; Desmots et al., 2002; Thongboonkerd et al., 2006). Its increased renal expression and urinary excretion may potentially be a marker for the injury at proximal renal tubules (Branten et al., 2000; Cressey et al., 2002; Desmots et al., 2002). We identified the up-regulation of GST class-pi in the renal cortex of KD group. The up-regulation of this enzyme in the renal cortex of cadaveric donors might reflect renal tubular injury.

Catalase is a major enzyme involved in detoxification of H_2O_2 from cells. The relationship between the reduction of renal CAT and oxidative stress were reported in various renal models (Cvetkovic et al., 1998; Kinter et al., 1999; McCabe et al., 1994; Nath et al., 1998; Singh et al., 1993). In this study, we identified the down-regulation of renal CAT in KD subjects. The down-regulation of this enzyme may be related to accelerate H_2O_2 generation by increasing O_2^- into H_2O_2 which causes cellular oxidative damage such as lipid peroxidation and/or cell death (Yabuki et al., 1999).

This data suggest that KD reflect to an impaired antioxidant defense system result in oxidative stress.

The signal protein SPARC-like 1, is a matricellular proteins comprise a group of macromolecules that influence cell-extracellular matrix (ECM) interactions, in some instances through receptor mediated intracellular signaling events (Bornstein, 1995; Sage and Bornstein, 1991). In animal models indicated that the SPARC decreased is associated with renal enlargement but the mechanism is not well understood. However, there are several data suggested that the reduction in SPARC may be associated with the increase activity of PDGF, bFGF, IGF-I and possibly TGF-β thereby exacerbating the effect of the increased of these growth factors and enhancing cell proliferation and ECM deposition in kidney (Raines et al., 1992; Hasselaar and Sage, 1992; Lane and Sage, 1994; Border et al., 1994). Our present study identified decreased levels of SPARC-like 1 in KD subjects, suggesting its roles might reflects kidney growth in the low-K condition.

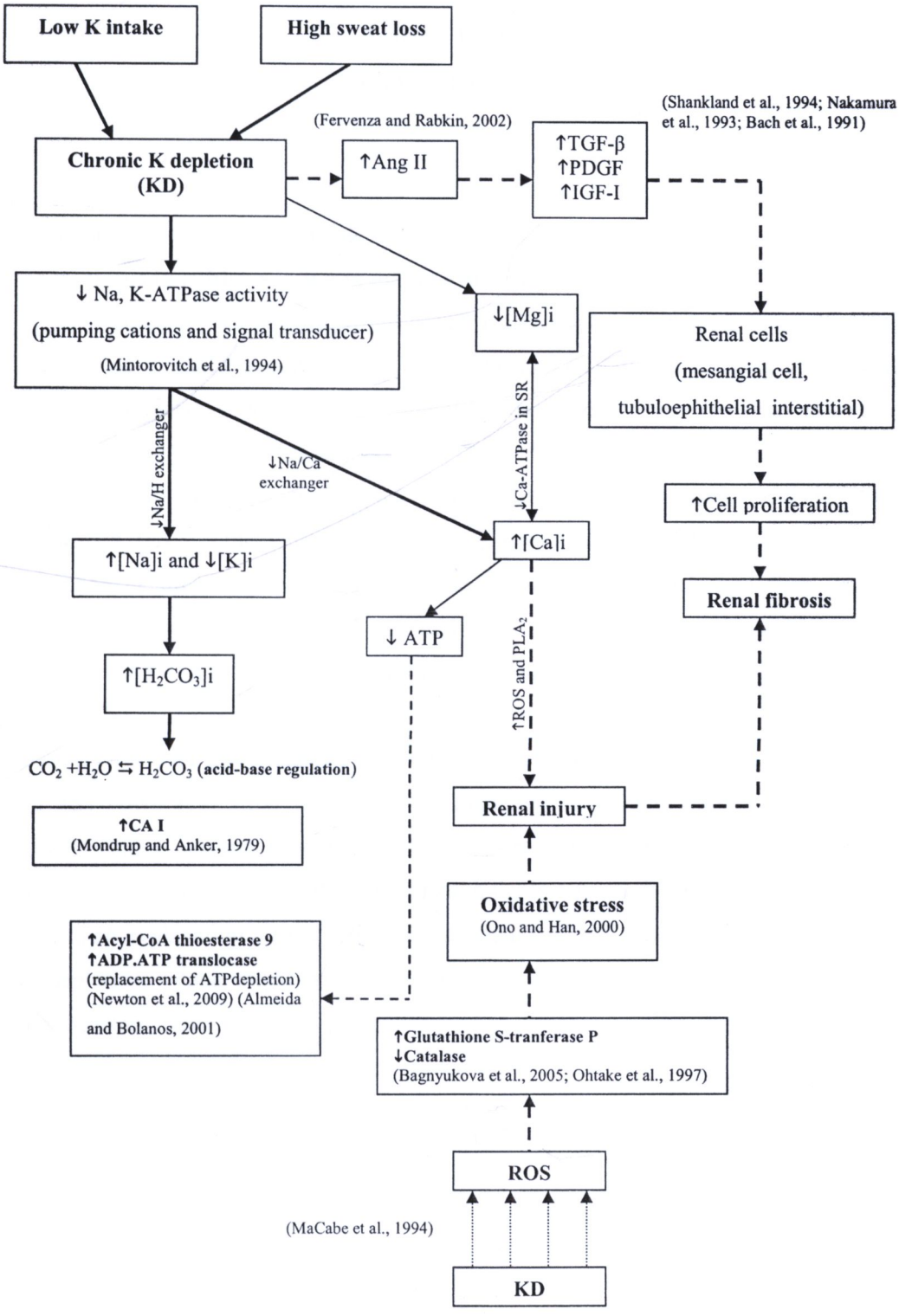

Figure 12 Schetamic propose for the mechanism of K depletion involving in HN (↑= increase and ↓=decrease)