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Dehydration and ADH-induced changes in renal stanniocalcin-1 mRNA levels in the rat

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Abstract

STC-1 is expressed in kidney collecting duct cells and targeted to most other nephron segments. However, its function and regulation are both unclear. The present study explored the regulation of *Stc-1* mRNA levels *in vivo* in rat models of dehydration and overhydration. Dehydration caused an upregulation in *Stc-1* mRNA levels in kidney cortex and to a lesser extent the inner medulla. Antidiuretic hormone was identified as a potential mediator of gene induction during dehydration acting through the V2 receptor. In contrast overhydration did not produce changes in renal gene activity. Histological studies revealed no difference in STC-1 receptor distribution along the nephron indicative of similar targeting pathways irrespective of hydration states. Together the findings are suggestive of a novel role for renal STC-1 in the regulation of extracellular fluid volume, in particular during water deprivation.

Key words: Antidiuretic hormone, dehydration, extracellular fluid, hyperosmolality, mRNA expression, rat, real-time polymerase chain reaction, stanniocalcin-1

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.OH	hydroxyl radical
~	approximately
ADH	antidiuretic hormone
ADP	adenosine di-phosphate
ANF	atrial natriuretic factor
ANGII	angiotensin II
ANP	atrial natriuretic peptide
AQP	aquaporin
ATP	adenosine tri-phosphate
Ca^{2+}	Calcium ion
cAMP	cyclic-adenosine monophosphate
CCAC	Canadian Council on Animal Care
CD	collecting ducts
cDNA	complementary deoxyribonucleic acid
Cl^-	chloride
CS	corpuscles of Stannius
D1- receptor	dopamine-1 receptor
DCT	distal convoluted tubule
DDAVP	desmopressin
DEPC	diethyl pyrocarbonate
EDTA	ethylenediaminetetraacetic acid
DEPC	diethyl pyrocarbonate
ENaC	epithelial sodium channels
eNOS	endothelial nitric oxide synthase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFR	glomerular filtration rate
H^+	hydrogen ion
H_2O_2	hydrogen peroxide
hCG	human chorionic gonadotropin
HCl	hydrogen chloride
HCO_3^-	bicarbonate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPO_4^{2-}	hydrophosphate
i.p.	intraperitoneal
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
IP3	inositol 1,4,5-trisphosphate
K^+	potassium ion
MDCK-II	Madin-Darby canine kidney cells
Mg^{2+}	Magnesium ion
MgCl	magnesium chloride
mRNA	messenger ribonucleic acid
Na^+	Sodium ion
NaCl	sodium chloride

NaOH	sodium hydroxide
NH_4^+.	ammonium ion
NHE	Na^+/H^+ exchanger
NKCC2	Na^+-K^+-2Cl cotransporter
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
$O_2^{\bullet}$	superoxide
ONOO	peroxynitrite
PCT	proximal convoluted tubule
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PT	proximal tubule
RAAS	Renin-Angiotensin-Aldosterone System
ROS	reactive oxygen species
SMIT	sodium/myo-inositol cotransporter
SNGFR	single nephron glomerular filtration rate
STC	Stanniocalcin
STC-AP	fusion protein of STC-1 and human placental alkaline phosphatase
TAL	thick ascending limb
TGF	tubularglomerular feedback
TonEBP	tonicity enhanced binding protein
UCP-2	uncoupling protein-2
UTR	untranslated region
V1a-receptor	vasopressin-1a receptor
V2-receptor	vasopressin-2 receptor

1 Introduction

1.1 Stanniocalcin overview

1.1.1 Stanniocalcin in fish

Stanniocalcin (STC) is a polypeptide hormone that was first discovered in bony fish. In fish, it is produced by specific glands called the Corpuscles of Stannius (CS) and is secreted in classical endocrine fashion. The main function of STC in fish is the regulation of calcium homeostasis. The gills, kidney and intestine are the target organs of blood-borne STC. The main effect of STC signalling on the gills is a reduction in calcium influx (Fenwick. 1974). In the kidney, STC increases phosphate reabsorption whereas in the intestine it decreases calcium uptake (Sundell et al. 1992; Lu et al. 1994).

1.1.2 Discovery of mammalian STC and its genetic structure

More recently, STC was discovered in mammals. The first report of mammalian STC showed that protein extracts from human kidney tissue, when injected into fish, had effects on gill calcium transport as fish STC. Furthermore, human kidney extracts were immunoreactive to antisera raised against salmon STC and Western blot analysis revealed a protein of similar size to fish STC (Wagner et al. 1995). These findings stimulated further research on mammalian STC in order to further characterize the protein and its functions. Two types of STC genes were subsequently found in mammals and are now referred to as STC-1 and STC-2. Mammalian STCs have 58% identity in amino acid sequence to one another (DiMattia et al. 1998)). However, they are located on different chromosomes and their tissue distribution and presumptive functions are different (Chang

and Reddel. 1998; Ishibashi et al. 1998; Moore et al. 1999). The present project focuses on STC-1.

The genomic structure of mammalian STC-1 was first described in humans (Olsen et al. 1996). The human *STC-1* gene is located on chromosome 8p11.2-p21 and consists of 4 exons. Exons 1-3 consist of 402, 143 and 212 base pairs respectively and exon 4 contains at least 3125 base pairs. There are two untranslated regions (UTR) with the 5' untranslated region located on exon 1 and the 3' untranslated region representing most of exon 4. Three CAG repeats are found in the 5' UTR and 1 additional set of six CAG repeats are within the 3' UTR. Interestingly, CAG repeats have been associated with genetic diseases such as Huntington's disease (Chang et al. 1998). However, the significance of the CAG repeats in *STC-1* is unknown. *STC-1* has also been studied in the other mammals and high similarities in gene sequences have been observed. For example, it has been shown that the mouse and human *STC-1* transcripts have 85% nucleotide sequence identity in the protein coding regions (Varghese et al. 1998).

Human STC-1 encodes a protein of 247 amino acid residues (human stc-structure), shares 73% amino acid identity with salmon STC and has been shown to be biologically active when injected into fish. Similar to fish, human STC-1 is a 50 kDa glycosylated protein. Its eleven cysteine residues allow for the formation of 10 disulfide linkages, with the 11^{th} unpaired cysteine enabling STC-1 to form a disulfide-linked homodimer. It has been suggested that the biological activity of STC-1 is associated with the N-terminus of the hormone since this region shows the highest sequence similarity among species (Olsen et al. 1996).

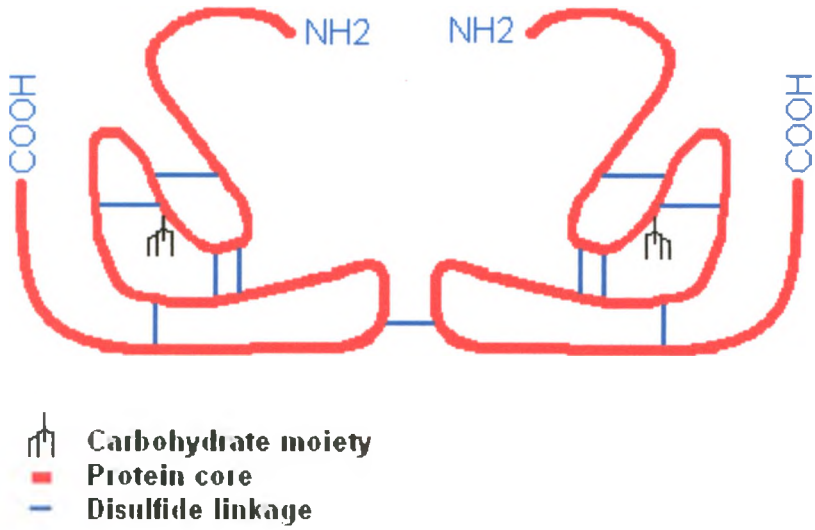

Figure 1. A schematic diagram of STC-1. STC-1 is a 50 kDa homodimer of two identical 25kDa monomers of 247 amino acids.

1.1.3 *Stc-1* gene expression in mammals

Unlike fish, where there is a specific endocrine gland contributing to STC-1 secretion, mammals lack such a gland and STC-1 expression is widely distributed among different organs. *Stc-1* mRNA expression levels are found to be the highest in the thyroid gland, prostate and most notably the ovary. Also, *Stc-1* mRNA is present in kidney, heart, lungs, placenta, certain brain areas and in bone cells (Varghese et al. 1998; Yoshiko et al. 1999; Worthington et al. 1999; Zhang et al. 1998). Interestingly, there are some tissues where the *Stc-1* gene is not expressed, and yet, STC-1 protein is present in abundance. For example, in the ovary, *in situ* hybridization shows that *Stc-1* mRNA is found only in the theca cells. On the other hand, immunocytochemistry reveals STC-1 protein in oocytes and granulosa cells, which do not express the gene in addition to theca cells (Paciga et al. 2003). This phenomenon occurs in a number of organs where *Stc-1* gene expression is localized to one cell type while the protein is also present in nearby cells which do not express the gene. These findings have led to the hypothesis that STC-1 is actually sequestered by its target cells. This sequestration, together with the wide distribution of *Stc-1* gene expression among different organs, suggests that STC-1 signals locally in a paracrine fashion.

1.1.4 Role of STC-1 in the kidney

1.1.4.1 Stc-1 gene expression, protein and receptor distribution in the kidney

In situ hybridization was used to localize *Stc-1* mRNA in the rat and mouse kidney. Staining for *Stc-1* mRNA appeared to be confined to the cortical and medullary

collecting duct cells and was not found in any other tubules (Haddad et al. 1996a; Wong et al. 1998). In contrast, in situ ligand binding studies which were used to localize STC-1 receptors reveal specific ligand binding sites in the cells of thick ascending limb (TAL), collecting ducts (CD) and the distal convoluted tubules (DCT). Some binding was also present on the proximal convoluted tubules (PCT) (McCudden et al. 2002). Immunocytochemical localization of the ligand revealed the presence of STC-1 protein in cortical TAL, DCT and cortical and medullary CD cells. However, unlike the TAL and DCT where immunoreactivity was noted in all cells, the ligand in the CD was localized to principal cells and alpha-type cells only. Beta-type CD cells were negative for STC-1 immunoreactivity. Additionally, STC-1 immunoreactivity has been identified in the proximal straight tubule (Haddad et al. 1996a).

The above studies revealed that STC-1 receptors and protein were present in different cells than *Stc-1* mRNA and identified yet another organ where STC-1 was undergoing sequestration within target cells. On this basis, it has been suggested that STC-1 is produced in CD cells for targeting of and sequestering by cells in other tubule types such as TAL and DCT. Moreover, the CDs which produce STC-1 are also targeted by CD cell-derived STC-1 as they too contain STC-1 receptors.

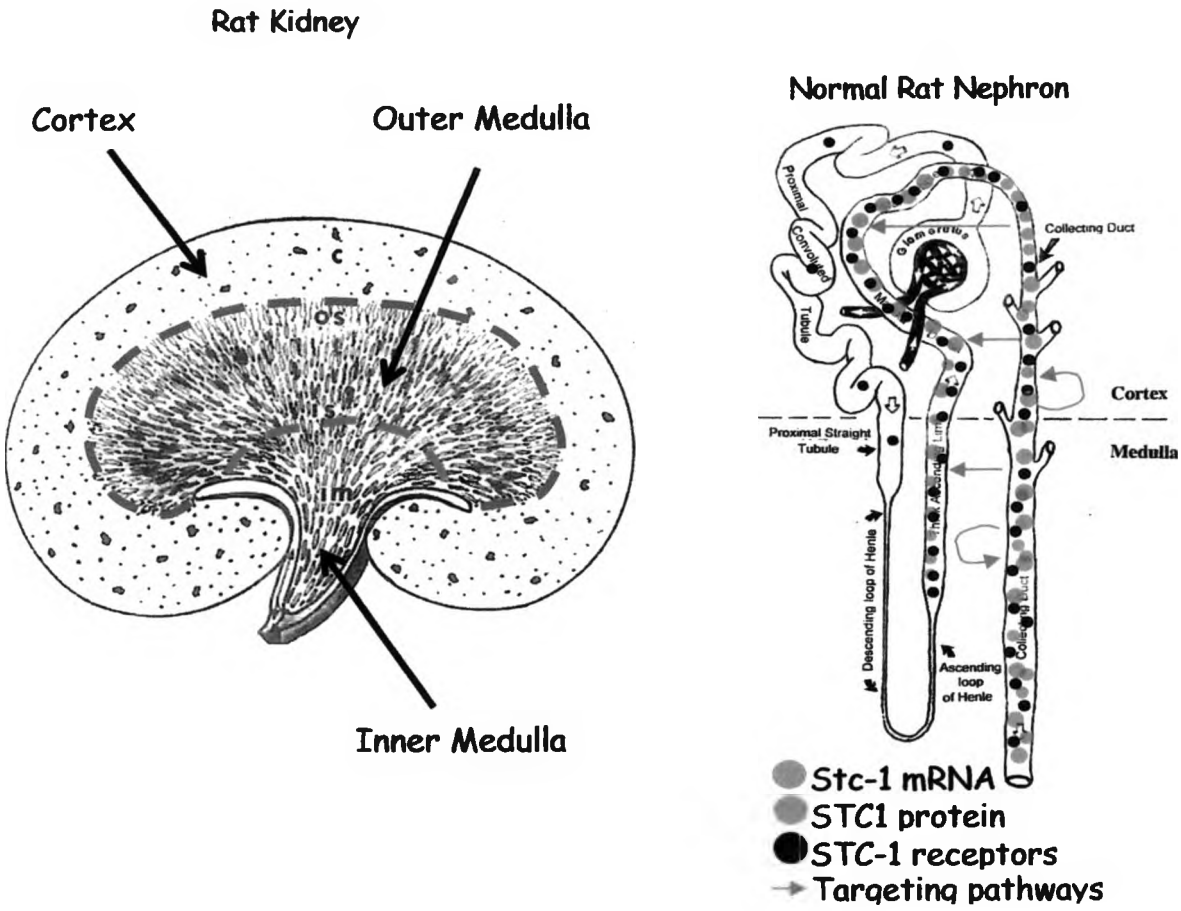

Figure 2. Representative diagram of rat kidney (left) and normal rat nephron (right). In the normal rat nephron, STC-1 receptors (●) and protein (◎) are localized throughout the TAL, DCT and CD. In contrast, *Stc-1* mRNA (◎) is confined to the cortical and medullary CD. Two alleged CD derived STC-1 targeting pathways are indicated by arrows. CD derived STC-1 is thought to target both the TAL and back upon the CD cells (TAL, thick ascending limb; DCT, distal convoluted tubule; CD, collecting duct).

1.1.4.2 Intracellular localization of STC-1 protein and receptor in kidney

Subsequent to the histological identification of STC-1 receptor binding sites on nephron cells, the pattern of receptor distribution at the subcellular level was also assessed. As stated above, STC-1 receptor binding activity was detected throughout the CD system. However, the pattern of binding when viewed histologically, was different in cortex and medulla. Cortical CD cells had more intense binding, which was punctate in appearance (McCudden et al. 2002). This punctate distribution of receptors suggested that they might be confined to specific subcellular structures. Receptor binding on kidney subcellular fractions showed that binding activity was concentrated on both the plasma and mitochondrial membranes (McCudden et al. 2002). Further analysis using electromicroscopy and immunoassays found that the ligand was concentrated within the mitochondrial matrix (McCudden et al. 2002). Furthermore, the majority of ligand and receptor binding sites were located on or within the mitoplast and its associated membrane (McCudden et al. 2002). This indicated that STC-1 targeting to nephron cells was destined for the inner mitochondrial matrix.

1.1.4.3 STC-1 function in kidney

Despite the kidney being the site of STC-1 discovery in mammals, very little is known about STC-1 function in this organ. Therefore, a primary focus of STC research remains the kidney and the available data is constantly changing. Of the few *in vivo* experiments performed on mammalian kidney, it has been shown that STC-1 causes a reduction in renal phosphate excretion due to an increase in sodium-phosphate (Na^+/Pi) cotransporter activity which is located within the proximal tubule brush-border membrane. However, the decrease in phosphate excretion did not change plasma levels of

phosphate or calcium (Wagner et al. 1997). Therefore, the reduction of phosphate excretion is thought to have a purely local significance at the level of nephron cell.

As mentioned earlier, both STC-1 and its receptor are located on and within the mitochondrial matrix. To address the physiologic importance of mitochondrial STC-1, functional studies were performed on isolated mitochondria and cells. The mouse IMCD-3 cell line (inner medullary collecting duct) was chosen for whole cell experiments because it has STC-1 receptors but does not produce large amounts of STC-1 and is therefore, not targeted by significant amounts of endogenous hormone.

The assessment of mitochondrial function was done using a Clark-type electrode for measuring oxygen consumption rates in isolated mitochondria and cells. In both instances, STC-1 had stimulatory effects on oxygen consumption in a time- and dose-dependent manner (Ellard et al. 2007). The effect on oxygen consumption by cells increased with the length of treatment time, to a maximum of 52% after 24 hours and was concentration-dependent between 5-500 nM of the hormone. Interestingly, the increases in oxygen consumption did not result in corresponding increases in ATP production (Ellard et al. 2007). This indicated that while STC-1 increased respiration rate, at the same time it uncoupled oxidative phosphorylation.

The exact mechanism of respiratory uncoupling is not well understood. One report has shown that STC-1 upregulates uncoupling protein-2 (UCP-2) levels in cultured macrophages (Wang et al. 2009a). Whether or not this effect is specific to macrophages or applicable to kidney cells as well remains to be established. It is possible that renal STC-1 also acts through UCP-2, which is highly expressed within the kidney (Fleury and

Sanchis. 1999). If this is true, STC-1 may upregulate UCP-2 in the kidney to increase the permeability of the inner mitochondrial membrane, thereby allowing reentry of protons to the inner matrix and in this way, reduce the electrochemical proton gradient necessary for ADP phosphorylation.

1.1.4.4 Regulation of Stc-1 gene expression and secretion by the kidney

Some studies on renal STC-1 gene regulation have been conducted and can possibly serve as a link towards an understanding of STC-1 function within the kidney.

The first of these studies characterized the regulation of STC-1 gene expression in the rat kidney by dietary calcium and phosphate. It showed that STC-1 mRNA was downregulated in adult rats maintained on a high calcium diet, an effect that was unique to the medulla (Deol et al. 2001). A low phosphate diet decreased mRNA expression whereas a high phosphate diet increased STC-1 mRNA levels. However, these changes took place in the cortex and the outer medulla while transcript levels in the inner medulla were unaffected. Since a low phosphate diet leads to transient hypercalcemia, and *vice versa,* the reduction of STC-1 gene expression by high calcium diet and high phosphate diet in the opposite directions was expected. Why the STC-1 gene is differentially regulated throughout the kidney is not clear. However, findings are indicative of there being different regulatory mechanisms in cortex and medulla (Deol et al. 2001).

STC-1 gene expression is also upregulated by calcitriol, the active form of vitamin D3 which increases calcium reabsorption in the kidney (Honda et al. 1999). Together, the two studies thus sited link renal STC-1 to calcium regulation. However, as STC-1 does

not affect plasma electrolyte levels of calcium or phosphate it is likely involved in the regulation of intracellular levels of calcium and phosphate as opposed to systemic levels.

In addition, it has been noted that renal *Stc-1* mRNA levels in the mouse change during postnatal development. *Stc-1* mRNA levels were found to be the highest on the first day of life and then gradually decreased until the 28th day. Interestingly, aquaporin-2 (a water channel on the apical membrane of CD principal cells) increased over this period of time, indicating a possible correlation between STC-1 and aquaporin-2 expression levels (Deol et al. 2001). It is well established that neonates are incapable of efficient renal water regulation due to the lack of aquaporin-2. Therefore, it is possible that STC-1 plays a role in renal water conservation. This hypothesis was explored further on the kidney cell lines as well as in dehydrated rats, a model in which water conservation is maximized.

1.1.4.5 Regulation of Stc-1 gene expression and secretion in the kidney cells

Several *in vitro* studies have shed light on our present knowledge of *Stc-1* gene regulation. In experiments using MDCK-II cells (Madin-Darby canine kidney cells- a kidney collecting duct cell line capable of STC-1 production), it was shown that after incubation in high osmolality media (produced by added NaCl), *Stc-1* gene expression and protein secretion were both upregulated in a time- and tonicity-dependent manner (Sazonova et al. 2008b; Sheikh-Hamad et al. 2000). Interestingly, the presence of physiological levels of extracellular calcium was necessary for the upregulation of *Stc-1* gene expression (Sheikh-Hamad et al. 2000). Furthermore, the addition of organic osmolytes (betaine and inositol) to the media attenuated the *Stc-1* gene upregulation

occurring in the presence of high osmolality (Sheikh-Hamad et al. 2000; Sheikh-Hamad,D. et al. 1994; Sheikh-Hamad,D. et al. 1996; Sheikh-Hamad et al. 1997).

Besides high osmolality, high calcium concentrations have also been shown to affect *Stc-1* mRNA levels in MDCK-II cells. Increasing concentrations of calcium chloride in the media (2-10μM) downregulated *Stc-1* gene expression (Sazonova et al. 2008a). Also, the effects of several hormones such as angiotensin-2 (ANG2), atrial natriuretic factor (ANF) and antidiuretic hormone (ADH) on STC-1 gene expression were tested in this cell line although none of the hormones significantly affected STC-1 secretion (Terada et al. 1994).

Few details are available regarding the cellular mechanisms by which hyperosmolality upregulates the *Stc-1* gene. One report has shown that hyperosmolality activates protein kinase C (PKC), phospholipase C (PLC) and inositol 1,4,5-trisphosphate (IP3) in the MDCK cell line (Terada et al. 1994) yet it remains to be determined whether STC-1 is affected by this pathway.

In light of the finding that increases in extracellular calcium concentration decreased STC-1 secretion, STC-1 receptor regulation was also tested in the MDCK-II cell line. The results revealed a progressive increase in STC-1 receptor levels in response to high calcium levels. On the contrary, there were no changes in receptor levels in response to the stimulation of STC-1 secreted by NaCl (or by hypertonicity) in the same cell line (Sazonova et al. 2008a). Therefore, the receptor appears to be upregulated in response to low ligand levels but unaffected by ligand excess.

1.1.5 Role of STC-1 in the muscle-skeletal system

Studies on cultured fetal rat calvaria cells have shown that human recombinant STC-1 treatment increases osteogenic development, while STC-1 antisense oligonucleotides have the opposite effect (Yoshiko et al. 2003). STC-1's involvement in osteogenesis was also indicated by studies conducted in mice overexpressing STC-1. Two transgenic mouse models have been developed. In the first model transgene expression was under control of the metallothionein promoter (MT-hSTC1) so gene expression was nearly ubiquitous. In the second model, the transgene was under the control of the myosin light chain promoter (MLC-hSTC1) such that the gene expression was confined to skeletal myocytes (Varghese et al. 2002; Filvaroff et al. 2002).

Both overexpressing lines exhibited growth retardation. In MLC-hSTC1 mice, skeletal changes were described which included shortening of bone length and narrowing of the growth plate, which is an important indicator of bone age. High levels of STC-1 were present in the chondrocytes moving from the proliferative to the hypertrophic zone, suggesting its involvement in chondrocytes maturation and growth plate closure. Additionally, decreases in trabecular bone thickness and density were observed with no change in trabecular number or mineralization. Also, morphological changes in skull thickness were present and revealed decreased osteoclast activity but no changes in osteoblast marker expression (Filvaroff et al. 2002). Skeletal muscles in MLC-hSTC1 mice weighed less than those of age matched controls and had enlarged mitochondria. Functionally, the muscle twitch response was significantly weaker in MLC-hSTC1 mice than in weight matched, younger controls (Filvaroff et al. 2002).

MT-hSTC1 mice had increased serum phosphate levels which was not the case for the MLC-hSTC1 mice in which transgene expression was confined to myocytes. This finding was interesting because earlier studies in wild type rats had shown that acute injections of STC-1 decreased renal phosphate excretion, however, without changing plasma levels of phosphate (Varghese et al. 2002). The difference may be indicative of there being differential effects of low versus high serum levels of the hormone.

It is important to note that while studies on transgenic mice have contributed a great deal to the understanding of STC-1 function, there were some limitations in these models. First, STC-1 was present in high concentrations in the serum of transgenic mice (Filvaroff et al. 2002) when normally, it is undetectable in the serum of wild type animals (aside from during pregnancy and lactation)(Paciga et al. 2002). Secondly, there was a lack of data showing that *Stc-1* gene expression among organs had a similar pattern in transgenic and wild type mice. Therefore, it is possible that tissues most heavily targeted by STC-1 in transgenic mice were unaffected in the normal phenotype and *vice versa*.

1.1.6 Role of STC-1 in neurons and heart

Stc-1 mRNA and protein are present in fully differentiated neurons in both human and mice (Zhang et al. 1998). *Stc-1* is also expressed in pyramidal cells of the cortex and hippocampus as well as in Purkinje cells of the cerebellum, all of which are highly sensitive to ischemia (Chang et al. 2003).

At present, it is thought that STC-1 has cytoprotective properties in all neuron cells. For example, Paju cells (cell line derived from human neural crest) transfected with *Stc-1* cDNA display higher survival rates when exposed to hypoxic stress and lower intracellular calcium levels than non-transfected control. Additionally, treatment of Paju

cells with STC-1 increased the uptake of inorganic phosphate, another finding resembling its actions in the kidney. Consequently, it can be concluded that STC-1 may induce its cytoprotective effects through the lowering of intracellular calcium concentration indirectly via an increase in phosphate uptake (Zhang et al. 2000).

In vivo experiments also explored STC-1's cytoprotective properties. STC-1 expression levels increased after exposure of brain tissue to hypoxic preconditioning (induced chronic hypoxia which increases resistance of involved tissues to acute ischemic damage). This upregulation was found to be mediated by interleukin-6 (IL-6) which is a prosurvival protein involved in cell proliferation, differentiation and apoptosis. Experiments on both cell lines and IL-6 knockout mice confirmed that the STC-1 upregulation was mediated by IL-6. Therefore, it is likely that STC-1 was induced in ischemic brain tissue for cytoprotection (Westberg et al. 2007b). Similar to brain tissue, STC-1 was shown to be upregulated in cardiomyocytes after hypoxic preconditioning. This effect was also found to be mediated through IL-6 protein (Westberg et al. 2007a).

Interestingly, STC-1 is present in fully differentiated neurons but not in immature ones (Zhang et al. 1998). Moreover, other reports have revealed that STC-1 is involved in the neuron cell differentiation (Wong et al. 2002). It has also been shown that less differentiated Paju cells are more susceptible to toxins (Teplova et al. 2004). Therefore, it is possible that the neuroprotective properties of STC-1 are somehow associated with cell differentiation.

1.1.7 Role of STC-1 in ovaries and mammary gland

Mammalian *Stc-1* gene expression is highest in the ovaries likely reflecting a unique action in these organs. Most *Stc-1* mRNA is confined to the thecal-interstitial cells, whereas STC-1 protein is more widely distributed, being found in oocytes and corpus luteal cells in addition to theca cells (Varghese et al. 1998). Such distributions of *Stc-1* mRNA and protein demonstrates once more ligand sequestration and paracrine action of the hormone, which as previously mentioned, has also been described in the other organs such as kidney and liver (Haddad et al. 1996a; Wong et al. 1998; McCudden et al. 2002).

Interestingly, during pregnancy and lactation STC-1 becomes detectable in the serum, indicating that in addition to paracrine signalling, STC-1 has a classical endocrine function during these physiological states. It is likely that serum born STC-1 is produced by the ovaries for targeting of other organs, since STC-1 concentrations in the serum are highly correlated with *Stc-1* mRNA levels in the ovaries (Deol et al. 2000).

Stc-1 mRNA levels were found to be increased in the ovaries during pregnancy and lactation (Deol et al. 2000). In the mouse, ovarian STC-1 secretion was shown to be upregulated by human chorionic gonadotropin (hCG). Human chorionic gonadotropin is capable of binding luteinizing hormone receptors normally targeted by luteinizing hormone during pregnancy. The PKA pathway is also involved in hCG-mediated STC-1 signalling. (Paciga et al. 2002) *Stc-1* gene upregulation is also observed in the uterus and embryo during implantation and the early stages of pregnancy, indicating a possible role in gestation (Stasko et al. 2001). Functional studies have also shown that STC-1 can downregulate the secretion of progesterone by luteal cells in culture (Paciga et al. 2003).

The mechanism entails STC-1 targeting to the cholesterol lipid droplets which possess STC-1 receptors. It has been suggested that in doing so, STC-1 reduces the availability of cholesterol which is essential for steroidogenesis. Therefore, in the intact ovary, theca cell-derived STC-1 may function to regulate progesterone production by nearby luteal cells.

The mammary gland is another organ that has been shown to be affected by STC-1 during pregnancy and lactation. STC-1 receptor distribution is of great interest in the mammary gland because of the unique regulatory changes that occur. In the non-pregnant state, STC-1 receptors are primarily localized to the mitochondria, like in other organs (Haddad et al. 1996b). However, during pregnancy and lactation, these mitochondrial receptors are downregulated and supplanted by marked receptor upregulation in the nuclei of milk-producing alveolar cells. Such a shift in receptor localization has only been demonstrated exclusively in the mammary gland (Hasilo et al. 2005). The role of mammary gland-targeted STC-1 in lactation has also been demonstrated *in vivo* in lactating mice. Animals injected with STC-1 antiserum had no changes in milk output, but the milk contained 40% less triglyceride content. As a consequence, the pups from mice with suppressed STC-1 targeting experienced growth retardation due to the significant reduction in milk caloric value (Zaidi et al. 2006).

1.1.8 STC-1 and cancer

Numerous studies have implicated STC-1 in carcinogenesis in the sense that high levels of *Stc-1* gene expression have been observed in malignant tumours. Examples of cancers where change in *Stc-1* expression have been observed as compared to the normal surrounding tissue include breast adenocarcinoma, ovary, colon, hepatocellular

carcinomas, and MEN2B medullary thyroid carcinomas (Kahn et al. 2000; Welcsh et al. 2002; Gerritsen et al. 2002; Okabe et al. 2001; Fujiwara et al. 2000; Watanabe et al. 2002; Ismail et al. 2000; McCudden et al. 2004). Based on the above, and also the finding that *Stc-1* mRNA has been detected in the blood of cancer patients (Fujiwara et al. 2000), it has been proposed that STC-1 could have value as a molecular marker of carcinogenesis. (Fujiwara et al. 2000).

Interestingly, while STC-1 was upregulated in most types of cancer, one study has found it to be decreased in hormone responsive breast and ovarian cancer cell lines (Welcsh et al. 2002). Moreover, expression of STC-1 is strongly correlated with BRCA1 expression (Welcsh et al. 2002) which is a protein known to repress estrogen induced genes associated with the tumourgenesis (Miki et al. 1994; Thompson et al. 1995). Therefore, it is possible that STC-1 plays some protective role in the estrogen-dependent carcinogenesis (Welcsh et al. 2002).

1.2 Kidney function during dehydration

Alterations in water or electrolyte balance are major sources of stress to an organism. One of the physiologic situations leading to water and electrolyte imbalance is dehydration, a condition that results from greater water loss than water intake.

The major challenges the body faces during dehydration include extracellular fluid volume contraction and an increase in its osmolality (Brazzuna et al. 1975). Since the kidney is the primary organ responsible for water and electrolyte regulation most adaptive

mechanisms directed to normalize the volume and content of the extracellular fluid compartment involve the kidney.

In a dehydrated state, changes in kidney function result in anti-diuresis, natriuresis and a resulting increase in urine osmolality. These changes in water reabsorption and sodium excretion are brought about by complex signaling pathways which include paracrine, endocrine and neuronal input.

The above perturbations pose a stress to the kidneys themselves, in particular the medullary zone, which is required to function under hyperosmotic conditions in order to maximally increase water reabsorption. Therefore, additional local mechanisms are required to adapt medullary nephron segments to this unfavorable environment. The present section summarizes the current knowledge of kidney function and the important changes that occur during dehydration.

1.2.1 Plasma biochemistry during dehydration

Studies have shown that a prolonged period of dehydration leads to significant increases in plasma osmolality and sodium (Na^+) concentration. However, plasma potassium (K^+) levels decrease, and other solute concentrations as well as plasma pH do not change (Brazzuna et al. 1975; Brazzuna et al. 1975). Total body Na^+ and K^+ decreases during dehydration due to the resulting increases in natriuresis and kaliuresis (Bennett and Gardiner. 1987a; Zucker et al. 1982; Merrill et al. 1986).

Antidiuretic hormone (ADH), is increased dramatically during dehydration as it constitutes the major hormone responsible for water conservation in the kidney (Blair-West et al. 1979). It has been shown that dehydration also results in the stimulation of the renin-angiotensin-aldosterone system, resulting in increased levels of plasma renin and

angiotensin II (ANGII) (Blair-West et al. 1979; Bennett and Gardiner. 1987a). In some species such as the dog there is a dissociation occurring between ANGII and aldosterone, such that plasma aldosterone remains unchanged during dehydration despite the high serum levels of renin and ANGII. In the rat, however, serum aldosterone levels are elevated during dehydration (Zucker et al. 1982; Metzler et al. 1986; Ramsay et al. 1988).

1.2.2 Glomerular apparatus during dehydration

The first kidney structure involved in the regulation of water conservation is the glomerulus. The glomerulus is located in the cortical zone of the kidney where the capillary walls come into contact with Bowman's capsule membranes to form a barrier that is permeable to electrolytes and water, but not proteins.

Filtration through the glomerular capillaries is driven by two major forces: hydrostatic pressure and oncotic pressure. Therefore, filtration rate depends on the difference in hydrostatic and oncotic pressures between the glomerular capillaries and Bowman's capsule, as well as degree of membrane permeability which determines the filtration coefficient. In general, substances affecting the glomerular filtration rate (GFR) are numerous and mostly comprise vasoconstrictors and vasodilators which affect arteriole resistance and/or the membrane permeability. Examples include ANGII, ADH, dopamine and prostaglandins (Seldin and Giebisch. 1992) Numerous studies have shown that GFR is decreased during dehydration (Prasad et al. 1988; Hope and Tyssebotn. 1983; Selen and Persson. 1983a) likely due to a decrease in glomerular plasma flow rate and in the glomerular capillary ultrafiltration coefficient, both of which are hormonally regulated (Yared et al. 1985).

The renin-angiotensin-aldosterone system is the most powerful mechanism controlling GFR. Renin release is triggered by dehydration and leads to the formation of ANGII. ANGII affects both afferent and efferent arteriole resistance (Dworkin et al. 1983). During dehydration when kidney perfusion is reduced, ANGII decreases GFR by reducing capillary hydrostatic pressure. This was demonstrated in water deprived rats which were administrated saralasin (a competitive ANGII inhibitor) (Yared et al. 1985). Prostaglandins I2 and E2 are also secreted during dehydration and both have been shown to decrease GFR (Schor et al. 1981).

ADH, acting through the V1a receptor subtype, has been shown to decrease the filtration coefficient, and GFR in the hydrated state (Dworkin et al. 1983). However, during dehydration ADH regulates GFR in the opposite direction. One report indicated that, in water deprived rats, administration of the ADH blocker d(CH2)ATyr(Me)AVP resulted in a decrease in GFR. Possibly, the effect was due to stronger vasoconstrictive action of ADH on the extrarenal vasculature in comparison to the intrarenal vessels with the resulting increase in the intrarenal vascular flow rate (Yared et al. 1985). It has also been shown that dopamine increases GFR during dehydration (Baylis and Brenner. 1978; Goldberg. 1972). Such modulation in GFR by the above hormones is likely directed to maintain a certain level of filtration necessary for effective clearance of metabolic waste products.

Tubuloglomerular (TGF) feedback is yet another important factor regulating GFR (Seldin and Giebisch. 1992). In general, TGF is thought to stabilize GFR and be responsible for the adjustment of single nephron glomerular filtration rate (SNGFR) in accordance to the flow rate in the macula densa that makes up the early distal tubule. It has been shown that SNGFR has an oscillatory behavior and that TGF is the factor

responsible for these oscillations. The macula densa is the sensory part of the feedback system, where luminal sodium chloride (NaCl) concentration is monitored. Studies have revealed that TGF is initiated by changes in NaCl transport through the furosemide-sensitive Na^+-K^+-$2Cl^-$ cotransporter but not by changes in osmolality created by non-ionic substances. Changes in NaCl transport trigger signaling changes in macula densa. Although how exactly signals are passed from the macula densa to the glomerulus is not well understood, it has been suggested that adenosine, prostaglandins, calcium ions, cAMP and local renin-angiotensin system serve as TGF signals. The glomerulus is the effector in the TGF pathway. Signals from the macula densa cause changes in afferent and efferent arteriole pressure as well as filtration coefficient, all of which affect SNGFR. In general increases in tubular Na^+ cause a decrease in GFR, whereas decreases in tubular Na^+ content increase GFR.

During dehydration, the TGF is activated by the rise in tubular Na^+ levels and attempts to reduce GFR. Increased TGF sensitivity was found during dehydration along with a higher magnitude of the TGF response. Possible mechanisms influencing the increase in TGF sensitivity during dehydration include a reduction in interstitial pressure in macula densa and rise in plasma ANGII (Selen et al. 1983).

1.2.3 Proximal tubules during dehydration

The proximal convoluted tubules are the most important sites for the regulation of water, electrolytes and acid balance. They participate in the reabsoption of Na^+, K^+, Ca^{2+}, HCO_3^-, Cl^-, HPO_4^{2-}, glucose, amino acids, citrate and many other solutes, as well as the secretion of H^+, K^+ and NH_4^+.

As much as 70% of the filtered Na^+ and water are reabsorbed by the proximal tubule (PT) cells. Therefore, minimal disturbances in the reabsorptive process can result in major changes in water and electrolyte homeostasis. Sodium reabsorption is regulated by many diverse mechanisms. On the other hand, water reabsorption is passive in this nephron segment and depends almost entirely on the sodium gradient. Water transport in the proximal convoluted tubules takes place through aquaporin 1 channels located on the apical and basal membranes and via paracellular transport.

Sodium transport through the PT is complex because of the number of transporters involved. However, Na^+/H^+ exchangers (NHEs) and the Na^+/K^+-ATPase, both of which are tightly regulated, are the largest contributors to Na^+ reabsorption. For instance, NHE3 is responsible for the reabsorption of two thirds of Na^+ and water while Na^+/K^+-ATPase is the only transporter located on the basolateral cell membrane capable of maintaining the transcellular Na^+ gradient. Therefore, further discussion is warranted on these two transporters (Seldin and Giebisch. 1992).

1.2.3.1 NHE regulation in the proximal tubules during dehydration

There are several known NHE isoforms (NHE1-NHE9). However, only NHE3 and NHE8 are known to be expressed in the luminal side of the PT epithelium and to be involved in Na^+ reabsorption (Biemesderfer et al. 1993; Amemiya et al. 1995). NHE3 is highly expressed in adulthood while NHE8 is the prevalent isoform in neonates (Goyal et al. 2003; Goyal et al. 2005; Becker et al. 2007). NHE1 may also be of some importance during dehydration as it is responsible for nephron cell adjustments to the hyperosmolar environment typical in the dehydrated state. NHE1 is expressed on the basolateral side of

all nephron cells with the exception of macula densa and intercalated cells of the cortical collecting ducts which lack NHE1 completely (Bobulescu and Moe. 2009).

NHE3 function depends on the inward Na^+ gradient across the luminal face of PT cells created the high levels of filtrate sodium (140mM) and low levels of intracellular Na^+ (5 mM) maintained by the basolateral Na^+/K^+-ATPase. Although ATP is not hydrolyzed by NHE3 to power transport, it binds NHE3 and is required for optimal transporter activity (Cabado et al. 1996; Cassel et al. 1986). Because NHE3 is regulated by multiple factors and mechanisms, the net effect of each can be difficult to estimate. However, they include urine acidity, hyperosmolality, ANGII and ANP, all of which are highly relevant to dehydration (Donowitz and Li. 2007).

Low pH has been shown to increase NHE3 activity and during dehydration urine pH rises faster along the nephron due to a decrease in filtrate flow rate combined with spared ammonium secretion. Therefore, higher urine acidity during dehydration is likely to increase NHE3 activity (Yang et al. 2000).

ANGII has a dose dependent action on NHE3. Physiological doses (0.01-0.1 nM range) stimulate NHE3 while higher pharmacological doses (100nM) inhibit it (Harris and Young. 1977). The mechanism of ANG II action on NHE3 is not well understood. However, recent studies have showed that it activates cellular pathways through the AT1 G- protein coupled receptor. Subsequent effects include inhibition of adenylyl cyclase, activation of phospholipases A2, C or D and Ca^{2+} release in response to inositol-1,4,5,-triphosphate or calcium channel opening induced by the arachidonic acid metabolite 5,6,-epoxy-eicosatrienoic acid (Harris et al. 1996). The final steps of the pathway by which physiological doses of ANGII act are attributed to an increase in cytosolic Ca^{2+} and

consequent enhancement of Na^+/H^+ exchange activity (Dominguez et al. 1987; Welsh et al. 1988). Increases in intracellular Ca^{2+} concentration are due to Ca^{2+} entry, and not its release from the intracellular stores (Welsh et al. 1988).

Unlike ANG II, dopamine has inhibitory effects on NHE3. Dopamine is upregulated by the high serum Na^+ levels of dehydration and promotes natriureseis to compensate for the decrease in extracellular volume. Dopamine is produced within the PT cells and signals in autocrine and paracrine fashion. There are two known types of dopamine receptors: D1-like receptors (D1 and D5) and D2-like receptors (D2, D3 and D4). Proximal tubules have been shown to express both D1- and D2-like receptors. Dopamine induces its natriuretic effects mostly through D1-like receptors which are G-protein coupled. Several pathways can be activated by D1-type receptors which may contribute to the decrease in NHE3 abundance. It is necessary to stress the importance of dopamine as a natriuretic hormone during dehydration because it regulates not only NHE3 activity but also Na^+/K^+-ATPase activity, which is essential for maintaining the Na+ gradient that facilitates Na^+ reabsorption (Aperia. 2000).

The next important factor regulating NHE3 activity is hyperosmolality itself. Serum and urine hyperosmolality, high hematocrit and high Na^+ concentrations are among the most important consequences of dehydration. Hyperosmolality has a dual effect on NHE3 activity depending on the time-course. Acute hyperosmolality, which occurs within a few hours of water withdrawal, has inhibitory effect on NHE3 (Nath et al. 1996). In contrast, chronic hyperosmolality extending beyond 48 hours stimulates NHE3 (Ambuhl et al. 1998). The acute inhibitory effects of hyperosmolality can be also secondary to dopamine. Dopamine is upregulated in the kidney by high ECF Na^+

concentrations (Aperia. 2000; Alper and Moore. 1982) Since high Na^+ is the primary contributor to the hyperosmolar state during dehydration, it is likely that dopamine is a part of the natriuresis mechanism induced by hyperosmolality. Finally, it is important to mention ANP, an important regulator of NHE3 in PT cells. ANP indirectly affects Na^+ reabsorption via its ability to stimulate dopamine release for the inhibition of NHE3 (Aperia. 2000). Generally, however, ANP is suppressed during dehydration, and therefore, its natriuretic effects are diminished during water withdrawal (Makino et al. 1996).

As mentioned above acute and chronic forms of NHE3 regulation have been identified. Acute regulation occurs within 24 hours, while chronic regulation takes more than 24 hours. The mechanisms governing acute and chronic regulation are different. The best known acute mechanisms entail NHE3 phosphorylation and trafficking leading to its intracellular redistribution and downregulation on the luminal membrane. Control at the level of transcription and synthesis/degradation of pre-existing protein are responsible for the chronic regulation of NHE3 activity as its half-life is relatively long (20 hours) (Donowitz and Li. 2007).

1.2.3.2 Na^+/K^+-ATPase regulation in the proximal tubules during dehydration

Na^+/K^+-ATPase plays a crucial role in water and electrolyte balance during dehydration. It is the major Na^+ transporter on the basolateral membrane and is present along the entire nephron. Functionally, it creates a transcellular Na^+ concentration gradient between the lumen and the nephron cell cytoplasm and in this way facilitates Na^+ reabsorption by NHE3 across the apical membrane. ATP hydrolysis is required for the function of the transporter, and therefore energy metabolism can influence its activity

(Feraille and Doucet. 2001). Na^+/K^+-ATPase is highly regulated and this regulation varies according to nephron segment. In PT cells, the pattern of regulation resembles that of the NHE3, as both transporters are functionally connected. The major regulators of Na/K-ATPase during dehydration are ANGII and dopamine. Low physiological concentrations of ANGII stimulate the Na^+/K^+-ATPase and high concentrations inhibit it (Bharatula et al. 1998) though the pathway under which the latter occurs is unclear. In contrast, dopamine always acts as an inhibitor of the Na^+/K^+-ATPase and is upregulated to promote natriuresis during dehydration (Aperia. 2000).

1.2.4 Thin segment of Henle's loop during dehydration

The thin segment of Henle's loop is divided into descending and ascending limbs based on epithelium morphology and function and the direction of filtrate flow. The thin segment of Henle's loop always begins at the junction of the outer and inner stripes of the outer medulla but has a variable length depending on nephron subtype. It may end in the inner stripe in the superficial nephrons or extend to the papilla in the case of juxtamedullary nephrons. Water and Na^+ transport both take place in Henle's loop. The descending limb is responsible for both water and Na^+ recycling while the ascending limb is only involved in Na^+ recycling.

The descending limb is highly permeable to water. This nephron segment is not regulated by hormones and water reabsorption fully depends on the osmotic gradient in the medullary interstitium, which varies substantially depending on the location of the loop. The lower it resides in the medulla, the higher the interstitial osmolality and resulting osmotic gradient becomes (Seldin and Giebisch. 1992). The osmotic pressure of the medullary interstitium increases markedly during dehydration and is followed by a

corresponding increase in water reabsorption by this segment (Selen and Persson. 1983b). Na^+ recycling in the descending limb varies depending on epithelial cell type. For example, long loops have higher sodium permeability than short loops, likely reflecting their role in creating medullary osmolar gradient. Transport is passive everywhere, however, with sodium moving out of the luminal filtrate according to its concentration gradient. The sodium recycling rate must be higher during dehydration due to higher sodium concentration in the interstitium and consequently, concentration gradient (Seldin and Giebisch. 1992).

In contrast to the descending limb, the ascending thin limb has a very low permeability to water and high permeability to sodium. Sodium reabsorption is driven by an electrochemical gradient created in part by Cl^- reabsorption. Sodium transport is passive in this portion of the tubule and is thought not to be hormonally regulated. On the other hand, the latest studies have shown the presence of ADH-sensitive adenylate cyclase. Recall, that ADH is highly upregulated during dehydration. However, the action it produces in the thin ascending limb of Henle's loop is unknown. The role of the thin ascending limb is related to the dissociation of Na^+ and water reabsorption. This dissociation leads to creation of the osmotic gradient for passive water reabsorption by CD cells and the descending thin limb, a mechanism referred to as countercurrent system. The thin ascending limb comprises the initial part of the countercurrent system (Seldin and Giebisch. 1992).

1.2.5 Thick ascending limb of Henle's loop during dehydration

The thick ascending limb (TAL) is the final portion of Henle's loop and ends in the macula densa. Therefore, ion transport in the TAL regulates the osmolality of macula

densa filtrate and therefore plays a role in tubular glomerular feedback. Consequently, TAL has also an influence on glomerular filtration rate.

The transport properties of TAL cells differ from those of the thin descending limb in that they are impermeable to water and yet permeable to a number of solutes including Na^+, K^+, HCO_3^-, Cl^-, Ca^{2+} and Mg^{2+} to name only some. About 15% of the total filtered sodium is reabsorbed by TAL cells. Since the TAL is permeable to Na^+ but impermeable to water, it is commonly referred to as the diluting segment and helps drive the countercurrent system (Seldin and Giebisch. 1992).

TAL contains Na^+/K^+-ATPase on the basolateral membrane, which is regulated by numerous hormones. During dehydration, ADH, dopamine and ANGII are the most influential hormones affecting sodium transport in the TAL. ADH acts primarily through V2 receptors which are strongly expressed in medullary TAL and more weakly in the cortical portion, to regulate Na^+ reabsorption. The mechanism of action includes an increase in Vmax of the Na^+/K^+-ATPase and increase in transporter abundance on the basolateral membrane, resulting in an upregulation of sodium transport across the basolateral membrane (Ecelbarger et al. 2001). In contrast, V1a receptors are absent from TAL cells (Tashima et al. 2001). ADH binding to V2 receptors leads to activation of adenylate cyclase, an increase in cAMP production and the activation of protein kinase A (PKA) (Mutig et al. 2007).

Besides ADH, hyperosmolality further upregulates Na^+/K^+-ATPase gene expression and protein production (Sakuma et al. 2005). Dopamine, ANGII and prostaglandins are all inhibitors of TAL Na/K-ATPase activity during dehydration. Also, Na^+/K^+-ATPase activity is obviously inhibited in response to ATP depletion (Chabardes

et al. 1999; de Jesus Ferreira et al. 1998; Hussain and Lokhandwala. 1997; Yu et al. 1996).

Once Na^+/K^+-ATPase activity is maximally upregulated in the TAL, the activity of apical Na^+ transporters can be a crucial limiting factor for Na^+ entry into the cells. Consequently, the net increase in Na^+ reabsorption that occurs in TAL during dehydration is likely due to upregulation of transporters on the apical side of the membrane. For example, the bumetanide-sensitive Na^+-K^+-2Cl cotransporter (NKCC2) and the epithelial Na^+ channel (ENaC) are directly increased by ADH (Ecelbarger et al. 2001). Upregulation of the NKCC2 transporter is essential for Na^+ reabsorption in the TAL and is mediated by the PKA pathway (Kim et al. 1999). The regulation of K^+ recycling and basolateral Cl- removal are also necessary for the maintenance of NKCC2 activity and they too involve PKA signaling (Hebert and Andreoli. 1984; Schlatter and Greger. 1985). It is important to mention, however, that the PKA pathway is only efficient in the presence of a sufficient ATP supply. Thus, in a situation where ATP becomes limiting, PKA is overridden by the phospholipase A2 (PLA2) pathway, resulting in inhibition of both NKCC2 and the Na^+/K^+-ATPase (Escalante et al. 1991).

Dopamine stimulates NKCC2, the main apical sodium transporter in the TAL (Aoki et al. 1996). However, net Na^+ reabsorption is decreased by dopamine in the TAL due to its inhibition of Na^+/K^+-ATPase (Grider et al. 1998). Therefore, the inhibition of Na^+/K^+-ATPase by dopamine outweighs its stimulatory effect on NKCC2.

1.2.6 Distal convoluted tubule during dehydration

The distal convoluted tubule (DCT) extends from the TAL and then gradually transforms into the connecting tubule and finally into the collecting duct (CD).

The cells making up the initial portion of the DCT resemble those of the TAL, in terms of transport properties. The DCT is highly permeable to Na^+ and low to water and is hormonally regulated in a fashion similar to that of the TAL. In contrast to the initial portion of DCT, the function of its distal portion is similar to the CD. It is highly permeable to water and water reabsorption is regulated by ADH (Seldin and Giebisch. 1992). Because the DCT shares structural similarities with TAL and CD it likely exhibits similar function during dehydration.

1.2.7 Collecting duct during dehydration

The collecting duct (CD) is the last segment of the nephron. Between 5-10 nephrons converge on and drain into each CD segment. The CDs commence in the upper cortex and extend down into the inner medulla. Therefore, the osmolar environment surrounding each CD changes considerably along its length. There is also substantial axial heterogenisity among the cells making up CD system.

Depending on location, the CD is divided into cortical, outer medullary and inner medullary segments. Each segment can be characterized by its specific transport properties and function. One of the primary functions is regulated water reabsorption, which explains the importance of this nephron segment during dehydration. Moreover, it is the only site where water reabsorption is not directly linked to Na^+ reabsorption (Seldin and Giebisch. 1992).

1.2.7.1 Regulation of water reabsorption in the collecting duct

Water reabsorption is a passive process in the collecting duct, driven by the osmotic gradient created by countercurrent system described above. Additionally, Na^+ reabsorption in the collecting duct itself contributes to the osmotic gradient.

As in other nephron segments, water reabsorption in the collecting duct is exclusively transcellular. This transepithelial water transport is dependent on specific water channels referred to as aquaporins (AQPs). Four different AQPs (AQP1-AQP4) are responsible for water transport through nephron cells. In the kidney, AQP2, AQP3 and AQP4 are expressed in the collecting duct while AQP1 is present in the proximal tubules and thin descending limbs (Nielsen et al. 1993b). AQP2 is located on the apical side of CD principal cells while AQP3 and AQP4 are found on the basolateral membrane (Nielsen et al. 1993a; Ecelbarger et al. 1995). Basolateral AQP3 and AQP4 are much more abundant then AQP2 on the apical membrane. Therefore, AQP2 is responsible for the changes in water permeability of the collecting duct and consequently, regulated water reabsorption.

ADH is the major hormone regulating water reabsorption in the collecting duct. The expression of AQP2 on the apical membrane is tightly regulated by ADH and both short term and long term regulation has been described. Short term regulation involves regulated trafficking of stored AQP2 from the intracellular compartment to the apical membrane whereas long term regulation occurs through the induction of AQP2 gene expression. ADH signaling in the collecting duct takes place through G-protein coupled V2 receptors located on the basolateral surface of all principal cells. Stimulation of V2 receptors leads to the activation of adenylate cyclase, increased cAMP levels and activation of PKA. Both translocation of AQP2 vesicles and upregulation of AQP2

transcription rely on this pathway (Cheng et al. 2009).Numerous studies have reported that ADH is not the only factor that regulates AQP2 through V2 receptors during dehydration (Cheng et al. 2009). Moreover, the regulation of AQP2 can also occur by other mechanisms (Marples et al. 1998). For example, prostaglandins secretion is induced by ADH through V1a receptor activation, a receptor subtype associated with the vasoconstrictive properties of ADH and expressed in the kidney vasculature and cortical collecting duct (Tashima et al. 2001). Prostaglandins oppose the action of ADH on AQP2 during dehydration. Prostaglandins decrease cAMP levels and PKA activity and are considered to be a part of the ADH negative feedback pathway (Seldin and Giebisch. 1992). ADH can also upregulate nitric oxide synthase (NOS) thereby, increasing nitric oxide (NO) levels (Martin et al. 2002) which stimulates water reabsorption by the CD (Murase et al. 2003). Its importance can be demonstrated by the fact that knockout mice lacking NOS develop nephrogenic diabetes insipidus (Morishita et al. 2005).

Secretin and oxytocin have also both been shown to regulate AQP2 and water reabsorption. The peptide secretin has antidiuretic properties similar to ADH. Studies suggest that it acts through its own receptor to regulate AQP2 (Chu et al. 2007). Oxytocin is the other hormone capable of inducing antidiuresis. Oxytocin can bind the V2 receptor, although with a lower affinity than ADH and its antidiuretic effects is known to be mediated primarily through V2 receptors (Terashima et al. 1999; Chou et al. 1995b; Chou et al. 1995a).

1.2.7.2 Regulation of sodium reabsorption in the collecting duct

Changes in cell water permeability are the most important factor in the regulation of water reabsorption at the site of the CD. However, it is important to highlight that

changes in Na^+ reabsorption in the CD takes place during dehydration as well. Changes in Na^+ reabsorption contribute to the overall osmotic gradient created by the countercurrent system which assists in the passive reabsorption of water.

Na^+ reabsorption by the CD occurs in principal cells and mediated by amiloride-sensitive epithelial sodium channels (ENaC) on the apical membrane, as well as Na^+/K^+-ATPase on the basolateral membrane (Seldin and Giebisch. 1992). ADH binding to the V2 receptor stimulates Na^+/K^+-ATPase and ENaC by the induction of PKA (Feraille et al. 2003; Feraille and Doucet. 2001) resulting in an increase in Na^+ reabsorption. Prostaglandins, dopamine and nitric oxide (NO) modulate ADH action by decreasing CD cell Na^+ reabsorption. They are all induced by ADH and constitute negative feedback pathways (Seldin and Giebisch. 1992; Ortiz and Garvin. 2002; Martin et al. 2002).

Aldosterone is another major hormone regulating Na^+ reabsorption in the CD. It is targeted primarily to the cortical CD to increase Na^+ reabsorption (Seldin and Giebisch. 1992). However, the role it plays during dehydration is not clear. Some studies conducted on dogs have shown that its concentration is not increased in plasma during dehydration (Zucker et al. 1982; Metzler et al. 1986; Ramsay et al. 1988). On the other hand, studies performed on water-deprived rats do show that serum aldosterone levels are upregulated as compared to hydrated controls (Bennett and Gardiner. 1987b; Ezzarani et al. 1985).

1.2.7.3 Inner medullary cells adaptive mechanisms to hyperosmolar environment

The osmolality in kidney tubule cells and the surrounding interstitium can vary substantially, especially in the inner medulla. To adapt to conditions of high osmolality such as those occurring during dehydration, CD cells employ a unique strategy. Osmolytes capable of increasing intracellular osmolality accumulate within the cell which

prevents cell shrinkage. These osmolytes include inositol (myo-inositol), betaine, and sorbitol(Sone et al. 1995) and their accumulation is facilitated by the production of specific osmolyte transporters such as the sodium/myo-inositol cotransporter (SMIT), and the sodium/chloride/betaine cotransporter (BGT1) (Garcia-Perez et al. 1989). The transcription of these cotransporter genes is regulated by the transcription factor, tonicity enhanced binding protein (TonEBP) (Miyakawa et al. 1999; Cha et al. 2001). TonEBP is distributed equally in the cytoplasm in isotonic conditions but is redistributed to the nuclei during conditions of hyperosmolality (Dahl et al. 2001) along with an increase in total TonEBP mRNA and protein (Woo et al. 2000).

The response of cells to the hyperosmolar state is not limited to osmolyte accumulation and TonEBP dependent signaling. It has been shown that under conditions of high osmolality, endothelial NOS (eNOS), inducible NOS (iNOS), neuronal NOS (nNOS) mRNA and protein are all increased in the inner and outer medulla (Shin et al. 1999). The function of NOS in a hyperosmolar state appears to be cytoprotective as NO is known to reduce the damaging effects of reactive oxygen species (ROS) which some studies have pointed out are increased by hypertonicity. (Evans and Fitzgerald. 2005a).

It has also been shown that tonicity upregulates heat shock protein-70 (HSP70) mRNA and protein which has an antiapoptotic role in the inner medulla. Reductions in medullary HSP70 expression induce cellular apoptosis in dehydrated rats, indicating that HSP70 has a protective role in cells during conditions of dehydration (Neuhofer et al. 2004).

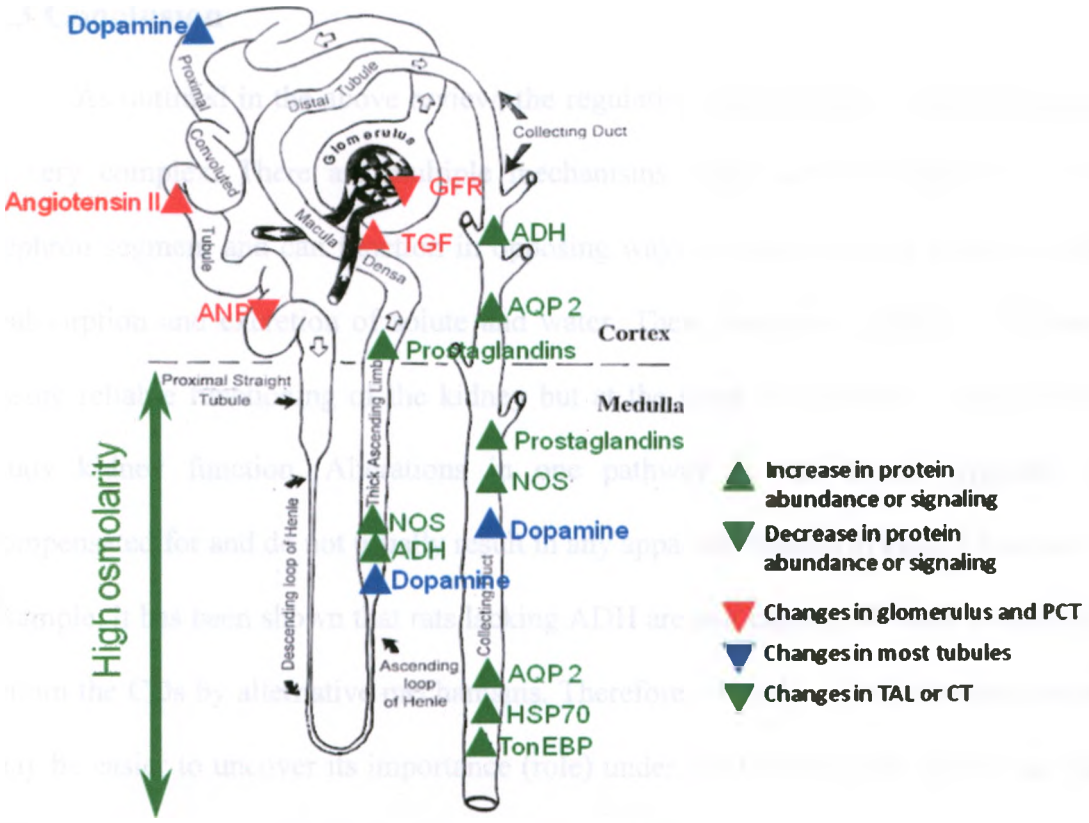

Figure 3. Representative diagram of rat nephron during dehydration. There is a decrease in GFR and increase in TGF; decrease in ANP signaling in the PCT, increase in angiotensin II signaling in PCT; increase in ADH signaling, prostaglandins, NOS in the TAL and CD; increase in AQP 2 along the entire CD; increase in HSP70 and TonEBP in the inner medullary CD. Increase dopamine signaling takes place along PCT, TAL and CD.

1.3 Conclusion

As outlined in the above review, the regulation of water and sodium reabsorption is very complex. There are multiple mechanisms which act simultaneously in each nephron segment and can function in opposing ways to achieve a fine balance between reabsorption and excretion of solute and water. These numerous feedback mechanisms assure reliable functioning of the kidney but at the same time make it very difficult to study kidney function. Alterations in one pathway or another are typically well compensated for and do not usually result in any apparent changes in kidney function. For example, it has been shown that rats lacking ADH are still capable of water concentration within the CDs by alternative mechanisms. Therefore, to study a particular mechanism it may be easier to uncover its importance (role) under conditions which pose a significant stress on both the primary and compensatory mechanisms. Dehydration is an example of the stress conditions because it places demands on all mechanisms responsible for conservation of water and salts.

1.3 Rationale for studies and hypothesis

Rationale

The rationale for this study is based upon a series of observations we and others have made in the MDCK kidney cell line, whereby high osmolality upregulates both *Stc-1* gene expression and secretion (Sazonova et al. 2008b; Sheikh-Hamad et al. 2000). As earlier studies have revealed that STC-1 has cytoprotective properties (Zhang et al. 2000; Westberg et al. 2007a) the increase in STC-1 levels in MDCK cells could be helpful in cell adjustment to hyperosmolar stress. The *Stc-1* gene, and its receptor are also highly expressed in the kidney *in vivo* (Haddad et al. 1996a; McCudden et al. 2002). However, few things are known about the role of STC-1 in this organ. As hyperosmolar stress is commonly experienced by cells within the inner medulla, we wanted to determine if hyperosmolarity also increased *Stc-1* mRNA levels *in vivo*. Over- and under-hydration were chosen for this study because of their marked contrasting effects on interstitial fluid osmolality in the inner medulla.

Hypothesis

Hyperosmolarity induced by dehydration increases *Stc-1* mRNA *in vivo*.

2 Methods

2.1 Experimental animals and reagents

Adult male Wistar rats (250g) were obtained from Charles River Laboratories, Montreal, QC, Canada and were and were allowed a one week acclimatization period before being used in studies. Animals were kept on a 12 hour light/dark cycle and received food and water *at libitum*. During the studies, access to water was modified as outlined in the experimental designs described below. All experimental procedures were carried out in accordance with the Guidelines on the Care and Use of Laboratory Animals as published by the Canadian Council on Animal Care (CCAC) and approved by the Animal Care Committee at The University of Western Ontario. All reagents used in the experiments were purchased from VWR International, Mississauga, Ontario, Canada.

2.2 Experimental design

2.2.1 Dehydration study

The purpose of the study was to evaluate the effect of dehydration on *Stc-1* mRNA, protein and receptor expression in the kidney. Control and experimental rats were placed into separate cages and weighed prior to each study. Experimental rats were deprived of water for 12, 24 or 48 hours. Control rats had access to water at all times. Animals were weighed every 12 hours to monitor the degree of dehydration until sacrifice. A 15% weight loss was accepted as the maximum permitted weight loss due to dehydration in accordance with CCAC guidelines.

2.2.2 Overhydration study

This study was designed to evaluate the effect of overhydration on *Stc-1* mRNA expression in the kidney. Control and experimental rats were placed into separate cages and weighed. Experimental rats were provided with drinking water containing 20% sucrose for 48 hours in order to increase water intake. Control rats received regular drinking water for the same period of time. At the end of a 48 hour experimental period animals were weighed and sacrificed.

2.2.3 Dehydration study with V1a or V2 ADH receptor blockade

This model was developed to study the possible regulation of *Stc-1* by ADH through V1a and V2 receptors. Competitive V1a or V2 receptor antagonists were used. OPC-21268 was used to block V1a receptors. And OPC-31260 was used for V2 receptor blockade. Both antagonists were provided by Otsuka Pharmaceutical Co., Ltd., Kanda-Tsukasamachi, Chiyoda-ku, Tokyo, Japan. Both OPC-21268 and OPC-31260 compounds were resuspended in 5% gum Arabic in water and administered to the rats by oral gavages at a dose of 40 mg/kg. Rats were weighed every 12 hours to monitor the degree of dehydration.

The study was conducted as follows:

Group #1: Hydrated control rats which had access to drinking water at all times.

Group #2: Dehydrated control rats which were deprived of water for 48 hours.

Group #3: Dehydrated rats which were deprived of water for 48 hours and received OPC-21268 compound in the last 12 hours of the water deprivation period.

Group #4: Dehydrated rats which were deprived of water for 48 hours and received OPC-31260 compound in the last 12 hours of the water deprivation period.

2.2.4 DDAVP study

Rats were anesthetized with and Ketamine/Domitor (8 mg per 100 g body weight Ketamine and 0.02 mg per 100 g body weight Domitor, i.p). An incision was made between the shoulder blades and osmotic minipumps (Alzet, Durect Corporation, Cupertino, CA, USA) containing DDAVP in PBS (pH 7.4) PBS alone were placed under the skin of experimental and control rats. Then the skin was sutured. Both solutions were released at the rate of 10 μl/hour which resulted in the administration of DDAVP at the dose of 5 μg/kg/day. The treatment was continued for 48 hours before the animals were sacrificed.

As a closing remark I would like to acknowledge an extensive help of Jeffrey Turner with this experiment.

2.3 Tissue preparation

Control and experimental animals from the above studies were anesthetized with a mixture of Ketamine and Domitor (8 mg per 100 g body weight Ketamine and 0.02 mg per 100 g body weight Domitor, i.p). The depth of narcosis was monitored using corneal and spinal pain reflexes. Blood for serum analysis was obtained by cardiac puncture.

Urine was obtained from all animals by bladder puncture. Kidneys were removed and placed on ice. Part of the inner medulla and cortex were macrodissected for total mRNA isolation. The remaining kidney was used for isolation of subcellular fractions such as nuclei, mitochondria and membranes (procedure described below).

2.3.1 Perfusion and fixation for histological studies

Some experimental and control rats from the studies described above were perfusion fixed and the kidneys were paraffin embedded for histological studies. Perfusion fixation was performed on anesthetized animals by inserting an 18 gauge needle into left ventricle which was then advanced into the aortic arch and secured with forceps. Perfusion with cold PBS (pH 7.4) was then initiated at a flow rate of 40 ml/min using a peristaltic pump. A total of 250 ml PBS was perfused followed by the perfusion of 400 ml of cold 4% paraformaldehyde dissolved in PBS. Perfusion efficacy was assessed by liver blanching, the analysis of muscle twitching and general body stiffness. Fixed kidneys were dissected, paraffin embedded and 5μm sections were cut and mounted on positive charged, Superfrost slides.

2.3.2 Total RNA isolation for real-time RT-PCR

One milliliter of TRIzol (Invitrogen, Carlsbad, CA, USA) per 100 μg of tissue was used for total RNA isolation. TRIzol was added immediately after the tissues were removed and tissues were then homogenized using a motorized pestle homogenizer

(VWR). Total RNA was isolated according to the manufacturer's instructions. Isolated RNA was resuspended in DEPC-treated water (Invitrogen, Carlsbad, CA, USA), aliquoted and stored at -80°C.

RNA was quantified at 260 nm using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA) and 260/280 absorbance ratio was obtained to assess its purity. An absorbance ratio of 1.5-2.0 was considered acceptable for PCR reactions. Integrity of the isolated RNA was assessed on a 1% denaturing agarose gel. An example of RNA quality is demonstrated in Figure 4.

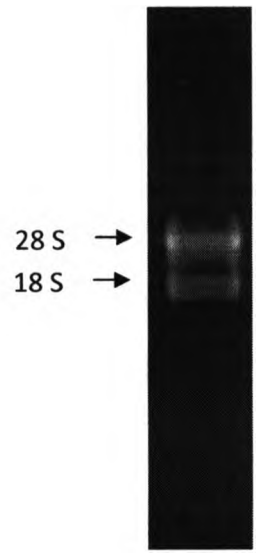

Figure 4. Representative example of extracted total RNA. RNA quality is demonstrated by the integrity of the 18S and 28S ribosomal RNA bands which are visualized in the RNA sample with ethidium bromide.

2.4 Real-time RT-PCR for *Stc-1* gene expression

Stc-1 mRNA levels were assessed by quantitative real-time RT-PCR. Relative quantification by the comparative cycle threshold method (delta-delta-C_T) was performed using GAPDH as a reference gene. Stable GAPDH mRNA levels were established between the control and experimental groups as a necessary pre-condition to its use as a reference gene.

TaqMan *Stc-1* and GAPDH gene expression assays with TaqMan one-step RT-PCR master mix were employed according to the manufacturer's directions (Applied Biosystems, Foster City, CA, USA). Twenty-five ng of total RNA was used for each reaction. Reactions were prepared in quadruplicate on a clear 384 well plate and run on an ABI Prism 7900 HT sequence detector which was set for the following program:

Step#1: 30 minutes at 48°C- reverse transcription

Step#2: 10 minutes at 95°C- activation of AmpliTaq Gold® DNA Polymerase

Step#3: 40 cycles of amplification reaction consisting of 15 seconds at 95°C (Denature) with the subsequent 1 minute at 60°C (Anneal/Extend).

Data analysis was performed using Sequence Detection Software 2.0 (Applied Biosystems, Foster City, CA, USA). Quality of reactions was assessed using standard curve analysis. Efficiencies no less than 90% were deemed acceptable.

2.5 Receptor binding and radioimmunoassays

Receptor binding assays were carried out on each subcellular fraction using a fusion protein of STC-1 and human placental alkaline phosphatase (STC-AP) as previously described. (McCudden et al. 2002), whereas human placental alkaline phosphatase (AP) was used to estimate the nonspecific binding.

Triplicate 100 μg aliquots of total protein from each subcellular fraction were incubated in 1.5ml eppendorf tubes with 500 mU/ml of STC-AP or AP for 1.5 hours at room temperature in Hanks Balanced Salt Solution with bovine serum albumin (HBHA. Unbound STC-AP or AP was removed by 3 washes in HBHA. The HBHA buffer was then replaced with 200μl of lysis buffer (1% Triton X-100 in 10 mM Tris, pH 8.0), pellets were resuspended and samples incubated at 65°C for 45 min to destroy any endogenous alkaline phosphatase activity. Detection was performed by adding p-Nitrophenyl phosphate as a substrate (Sigma-Aldrich Inc., St. Louis, MO, USA) in ethanolamine buffer (6.3 mg/ml p-Nitrophenyl phosphate in 1 M ethanolamine, 0.5 M $MgCl_2$, 0.5 mg/ml bovine serum albumin). The reaction was allowed to proceed for 40 min, after which time the reaction was stopped by the addition of 100 μl of 3M NaOH. The reactions were quantified spectrophotometrically at 405 nm and estimates of specific binding were determined as previously described (McCudden et al. 2002). The nonspecific binding of AP was taken into account and the final results were expressed as amount of specifically bound STC-AP (pmoles) per mg of protein.

Subcellular fractions from control and dehydrated cortical kidney samples (400 μg/each) were assayed in triplicate by radioimmunoassay for STC-1 protein content. The

radioimmunoassay has been characterized for specificity, does not significantly crossreact with other peptide hormones, and has a detection limit of 0.2 ng/ml. (De Niu et al. 1998).

2.6 Histological studies

2.6.1 *In situ* ligand binding studies

The STC-1 fusion protein (STC-AP) was also used for histological localization of STC-1 receptor as previously described. (McCudden et al. 2002) Paraffin embedded kidney sections were deparaffinized in xylene, rehydrated in a series of alcohols and then equilibrated in HBHA buffer. Slides were then incubated with STC-AP or AP alone (for non specific staining) for 1.5 hours in HBHA buffer at room temperature. After incubation, the slides were washed in HBHA containing 0.2% Tween for 5 min and then fixed for 30 sec in 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer containing 60% acetone and 3% formaldehyde. Slides were then rinsed in Higgins detection buffer (0.1M Tris-Cl, 0.1M NaCl, 5mM MgCl, pH 9.0) and incubated at 65°C for 40 min to destroy any endogenous alkaline phosphatase activity. Finally, detection was carried out using 0.34% 4-Nitro blue tetrazolium chloride (NBT) and 0.34% 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) as substrates (Roche Diagnostics GmbH, Indianapolis, IN, USA) added to the detection buffer. The reaction was stopped after 30 minutes.

2.6.2 Immunocytochemistry

Slides containing kidney sections were deparaffinized in xylene and rehydrated in a series of alcohols. Endogenous peroxidase activity was eliminated by incubation in 0.3% H_2O_2 for 30 min. A blocking step was then performed with 3% bovine serum albumin and 3% normal goat serum in PBS (pH 7.4) for 1 hour at room temperature after which the slides were incubated with a polyclonal serum to recombinant human stanniocalcin-1 (dilution 1:2000) at 4°C overnight (De Niu et al. 2000). Visualization of antibody binding was performed with the Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA) according to the manufacture's instructions.

2.8 Osmolality measurements

Blood collected from rats by cardiac puncture was allowed to clot over night at 4°C. Samples were then centrifuged at 1000 x g and the clear supernatant (serum) was obtained for analysis. Urine was also centrifuged at 1000 x g and the clear supernatant was used for osmolality measurements. Osmolalities were measured on a VAPRO 5520 vapor pressure osmometer (Wescor Inc.) using a sample volume of 10 ul.

2.9 Statistical analysis

All data are expressed as the mean ± SEM. Comparisons of two means were performed with a two-tailed, paired t-test. One-way analysis of variance (ANOVA) was employed for comparison of multiple means. A Bonferroni's post-hoc test was used for

comparison of multiple means when necessary. A p value of < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 4 software (GraphPad software Inc.).

3 Results

3.1 Dehydration study

3.1.1 Body weight, serum and urine parameters

The body weights of dehydrated animals and their hydrated controls were monitored in order to assess the degree of dehydration (Table 1). As expected, body weight was decreased by dehydration 5.3 % after 12 hours, 9.6 % after 24 hours and 11.3% after 48 hours. This progressive decrease in body weight was attributed to a loss of total body water. In contrast, hydrated animals gained 0.7, 3.5 and 2.8% of body weight during 12, 24 and 48 hours of the experimental period, respectively.

End point urine and serum osmolalities were also measured for a more accurate estimation of dehydration severity. The urine osmolality data is presented in Figure 5. Urine osmolality increased 1.6 fold after 12 hours of dehydration, 1.5 fold after 24 hours and 1.8 fold after 48 hours, indicating activation of the urine concentrating mechanisms in order to conserve water.

Table 1: Mean body weight of male Wistar rats before and after the course of dehydration.

Treatment Group	Before (g)	After (g)	Change (%)
12 hr hydrated (N=6)	305.2 ± 6.4	307.3±6.8	0.7 ±0.7
12 hr dehydrated (N=6)	307.8 ± 3.7	291.7±9.2**	-5.3± 0.8
24 hr hydrated (N=6)	246.2±3.8	255.2±4.3***	3.5 ±0.5
24 hr dehydrated (N=6)	246.2±5.6	224.7±5.3**	-9.6±0.6
48 hr hydrated (N=8)	325.0± 22.4	333.4± 20.8**	2.8± 0.8
48 hr dehydrated (N=8)	314.1 ± 20.8	284.5± 22.9***	-11.3± 1.6

Values are shown as means ± SEM. **p<0.01, ***p<0.001, paired t-test vs. starting weight, N-sample size.

% change indicates the difference between initial and final body weights.

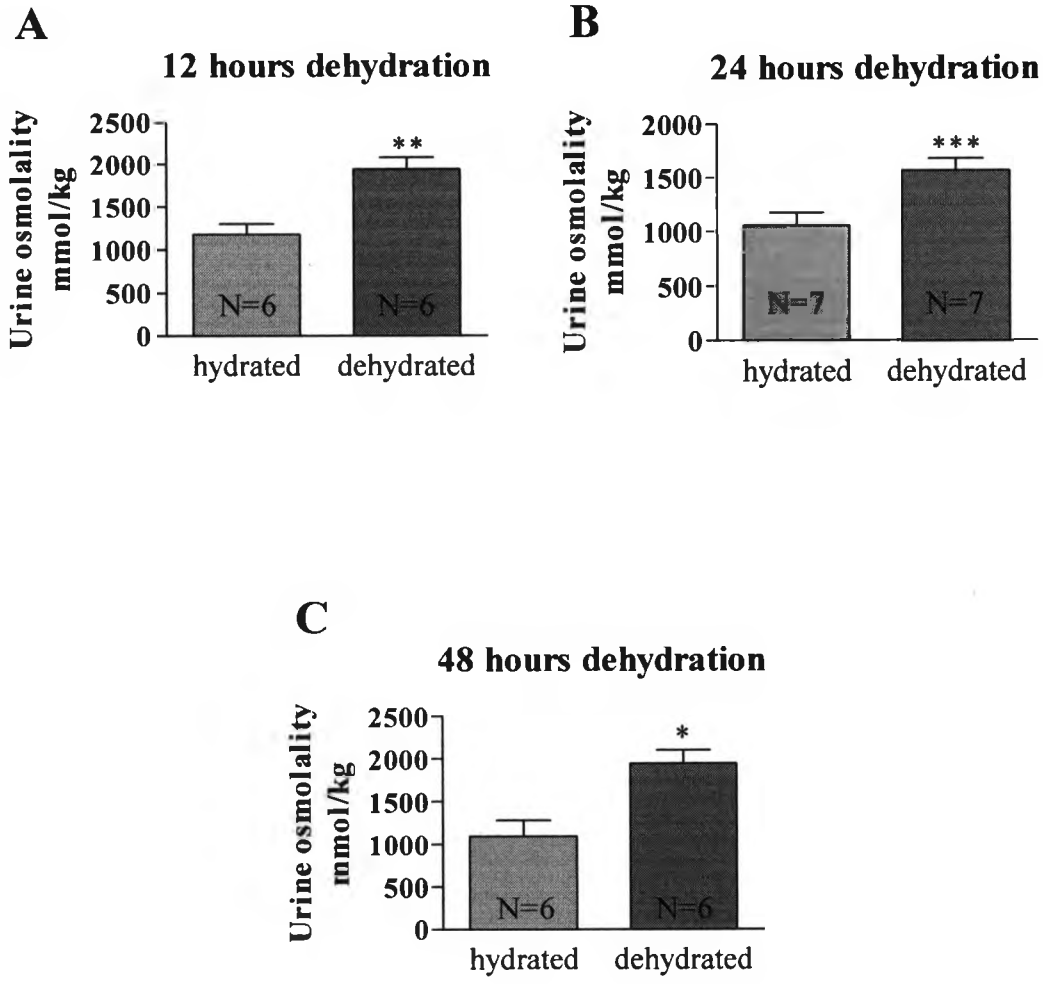

Figure 5. Urine osmolality in rats after dehydration. Urine osmolality was increased following 12 (A), 24 (B) and 48 (C) hours of dehydration as compared to the respective hydrated control animals (* $p<0.05$, **$p<0.01$, *** $p<0.001$, paired t-test; N-sample size).

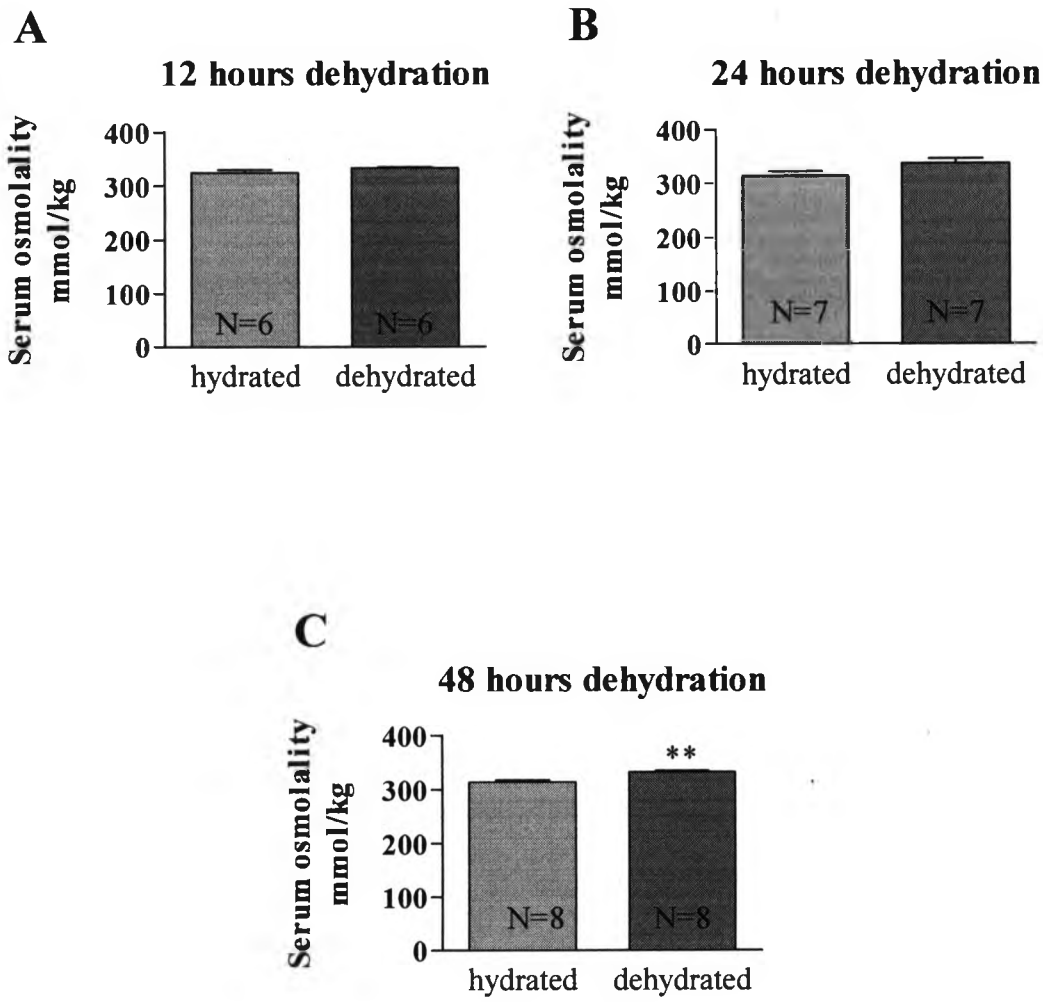

Figure 6. Serum osmolality in rats after dehydration. There was no change in blood plasma osmolality following 12 (A) or 24 (B) hours of dehydration. Increased serum osmolality was demonstrated following 48 (C) hours of dehydration when compared to hydrated control animals (**$p<0.01$, paired t-test; N-sample size).

Serum osmolalities are shown in Figure 6. Serum osmolality was not significantly different from hydrated control animals after 12 or 24 hours of dehydration. However, increase in serum osmolalities were demonstrated after a 48 hour dehydration course.

The similarities between serum osmolalities in 12 and 24 hour rats indicated they had a similar level of dehydration, despite their different lengths of water deprivation. This may be explained by their circadian drinking habits. The entire 12 hour dehydration took place during the night when rats are most actively drinking, whereas the 24 hour dehydration included these same 12 nighttime hours and 12 hours of daytime inactivity, when little drinking takes place (Johnson and Johnson. 1990).

3.1.2 *Stc-1* mRNA levels

Stc-1 mRNA levels were assessed after water deprivation in the kidney cortex and inner medulla by real-time RT PCR. In the cortex, *Stc-1* mRNA levels were ~ 4 fold higher in dehydrated rats as compared to hydrated controls following 12 hours of water deprivation. The same magnitude of *Stc-1* gene upregulation was observed after 24 hours of dehydration (~ 3.7 fold increase). A 48 hour dehydration caused an even greater increase in *Stc-1* mRNA levels that reached a ~ 6.3 fold maximum as compared to hydrated controls. The similar degrees in gene upregulation after 12 hour and 24 hours can be attributed to the circadian effects on dehydration as described above. Recall as well that, the assessments of weight loss and urine and serum osmolalities also showed that these animals were dehydrated to the same degree after 12 and 24 hours (Figure 5 &6, Table 1).

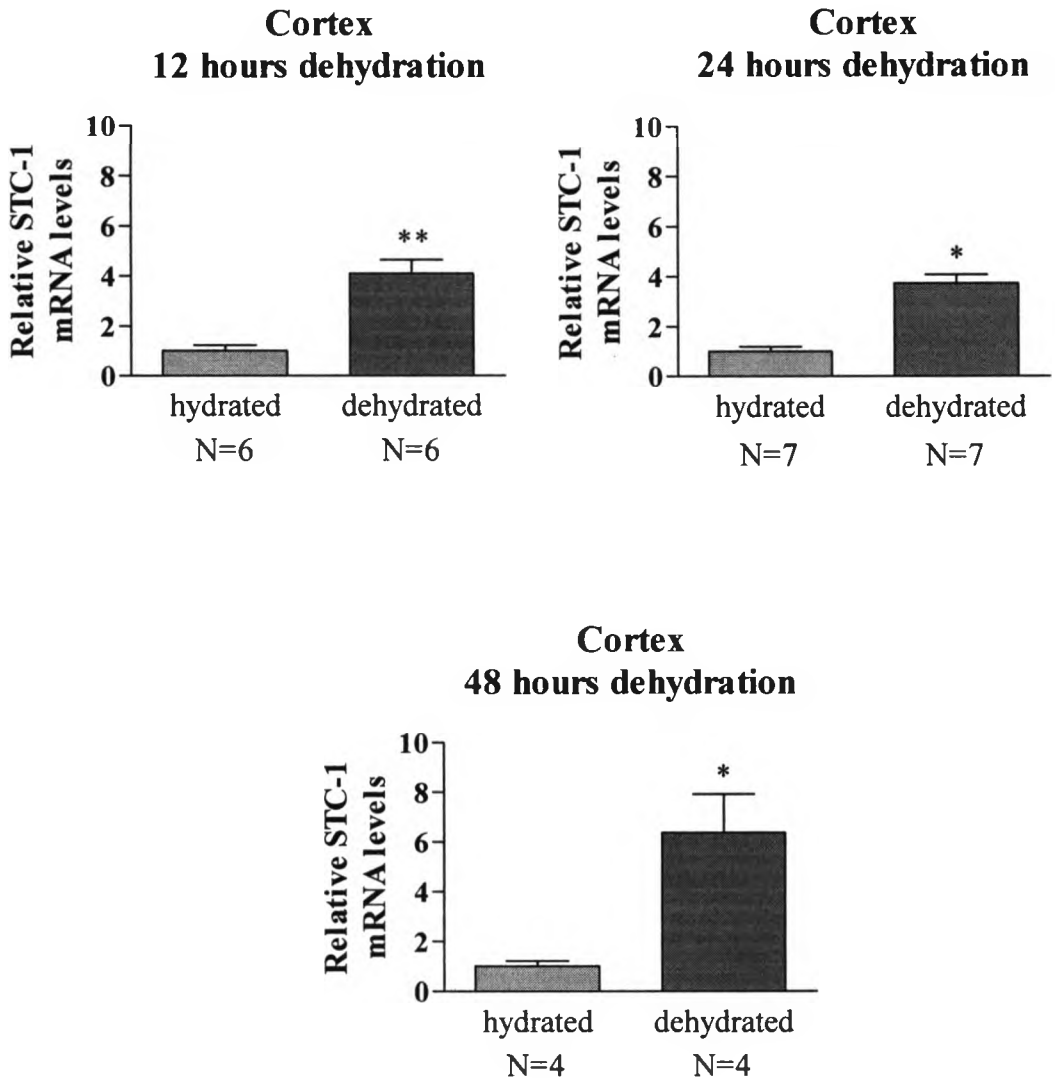

Figure 7. Relative *Stc-1* mRNA levels in rat kidney cortex following 12, 24 and 48 hours of dehydration. Rats were deprived of water for 12, 24 or 48 hours. Relative *Stc-1* mRNA levels were increased in all dehydrated groups as compared to hydrated control animals (* $p<0.05$, **$p<0.01$ *** $p<0.001$, paired t-test; N-sample size).

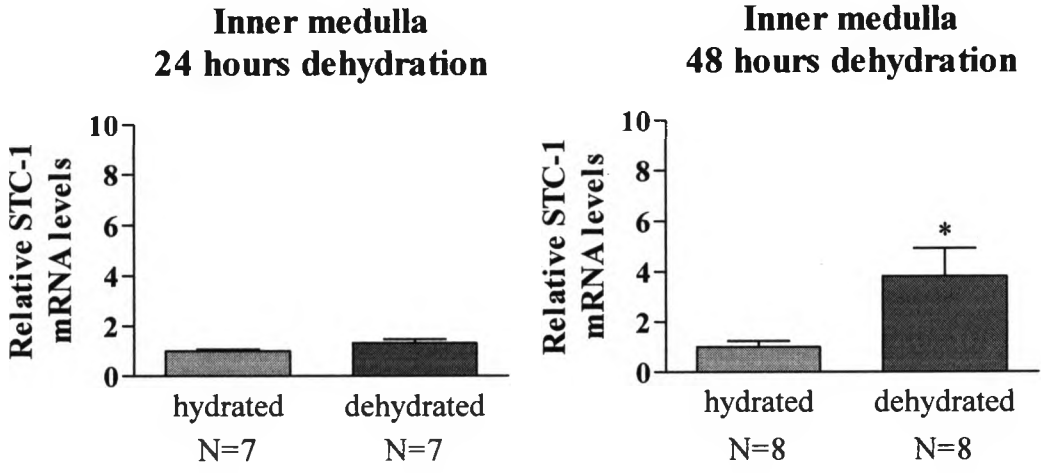

Figure 8. Relative *Stc-1* mRNA levels in rat inner medullary kidney following 24 and 48 hours of dehydration. Rats were deprived of water for 24 or 48 hours. Relative *Stc-1* mRNA levels were significantly increased following a 48 hour dehydration period but not a 24 hr period when compared to hydrated control animals (* $p<0.05$, paired t-test; N-sample size).

Figure 7 depicts the changes in *Stc-1* mRNA levels in the cortex after 12, 24 and 48 hours of dehydration.

As shown in Figure 8, upregulation in *Stc-1* mRNA levels following dehydration was also observed in the inner medulla. However, unlike the response seen in the cortex, the increase in medullary *Stc-1* mRNA levels was much smaller in magnitude and delayed in time. Here, mRNA levels were increased only after a 48 hour dehydration (~ 3.8 fold). The 24 hour dehydration did not produce a significant change in medullary mRNA levels.

Results indicated that STC-1 protein levels were also increased during dehydration (Figure 10). STC-1 protein was measured using STC-1 radioimmunoassay to look at various subcellular fractions including mitochondria, nuclei and plasma membranes following a 24 hour water deprivation. The results showed an increase in STC-1 protein in all subcellular fractions, with the greatest increase in the mitochondrial fraction. This finding allowed us to conclude that the upregulation in *Stc-1* mRNA levels indeed resulted in an increase in STC-1 production.

3.1.3 Kidney receptor levels and subcellular distribution during dehydration

Receptor binding assays were performed on various kidney subcellular fractions to assess changes in STC-1 targeting during dehydration. Figure 9 shows the amount of STC-1 binding on plasma membranes, mitochondria and nuclei of hydrated animals (control) and animals dehydrated for 48 hours. In both the dehydrated and hydrated state,

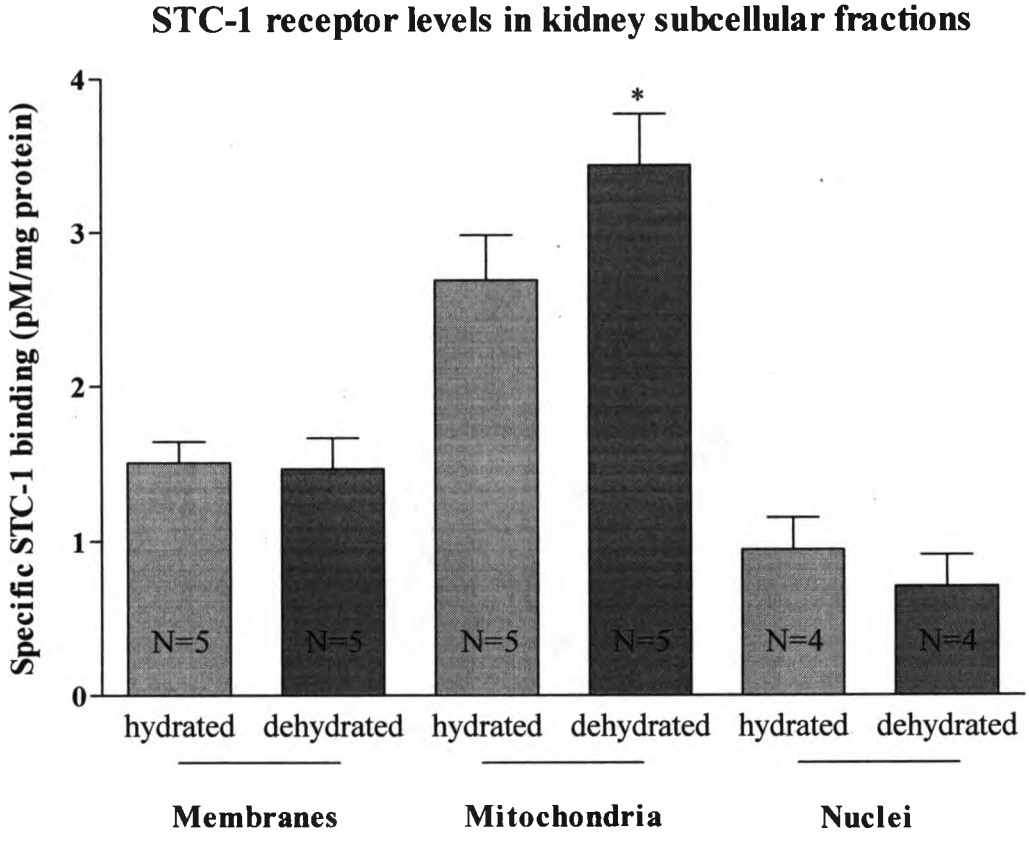

Figure 9. STC-1 receptor levels on kidney plasma membrane, mitochondria and nuclear subcellular fractions following a 48 hour dehydration period. A significant increase in receptor levels was seen only in the mitochondrial fraction of dehydrated animals, whereas those in the other fractions remained unchanged following dehydration (* $p<0.05$, paired t-test; N-sample size).

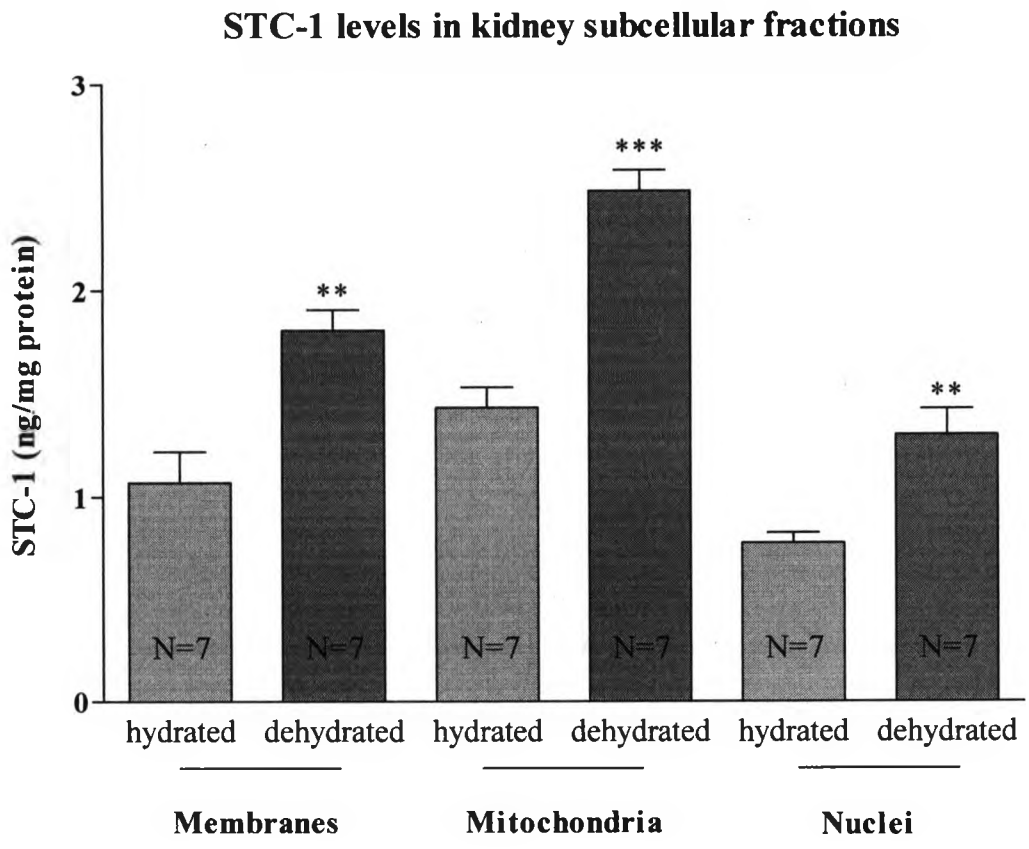

Figure 10. STC-1 levels in kidney plasma membrane, mitochondria and nuclear subcellular fractoins following a 48 hour dehydration period. A significant increase in STC-1 levels was seen in all subcellular fractions of dehydrated animals with the greatest increase in mitochondrial fraction (* $p<0.05$, paired t-test; N-sample size).

the highest degree of binding was observed in the mitochondria followed by the plasma membranes. The lowest binding was present in the nuclear fraction under both conditions.

After 48 hour dehydration the number of STC-1 binding sites in the nuclear and membrane fraction was unchanged. However, a small but statistically significant increase in binding was evident in the mitochondria of dehydrated animals, indicative of the increased targeting to the mitochondria during dehydration. This is of great interest because the mitochondria are the primary focus of STC-1 targeting and function in kidney tissue.

3.1.4 Histological localization of STC-1 receptor and protein during dehydration

Figure 11 shows *in situ* STC-1 receptor binding sites in hydrated animals and in those subject to a 48 hour dehydration. In both hydrated and dehydrated rats, the highest level of STC-1 binding was observed in the collecting duct and the lowest levels were in the proximal convoluted tubules (S1 segment) and glomeruli. Similar binding characteristics were also seen in the kidneys of rats dehydrated for 12 or 24 hours (data not shown). In summary, there were no apparent changes in STC-1 receptor density or distribution along the nephron in response to dehydration, which agrees with the previously described receptor binding data.

The immunostaining of kidney sections from rats dehydrated for 48 hours revealed similar distribution patterns of STC-1 protein as seen in control animals (Figure 12). The highest amount of STC-1 was present in the collecting duct and the lowest levels were once more seen in the proximal convoluted tubules and glomeruli. Similar results

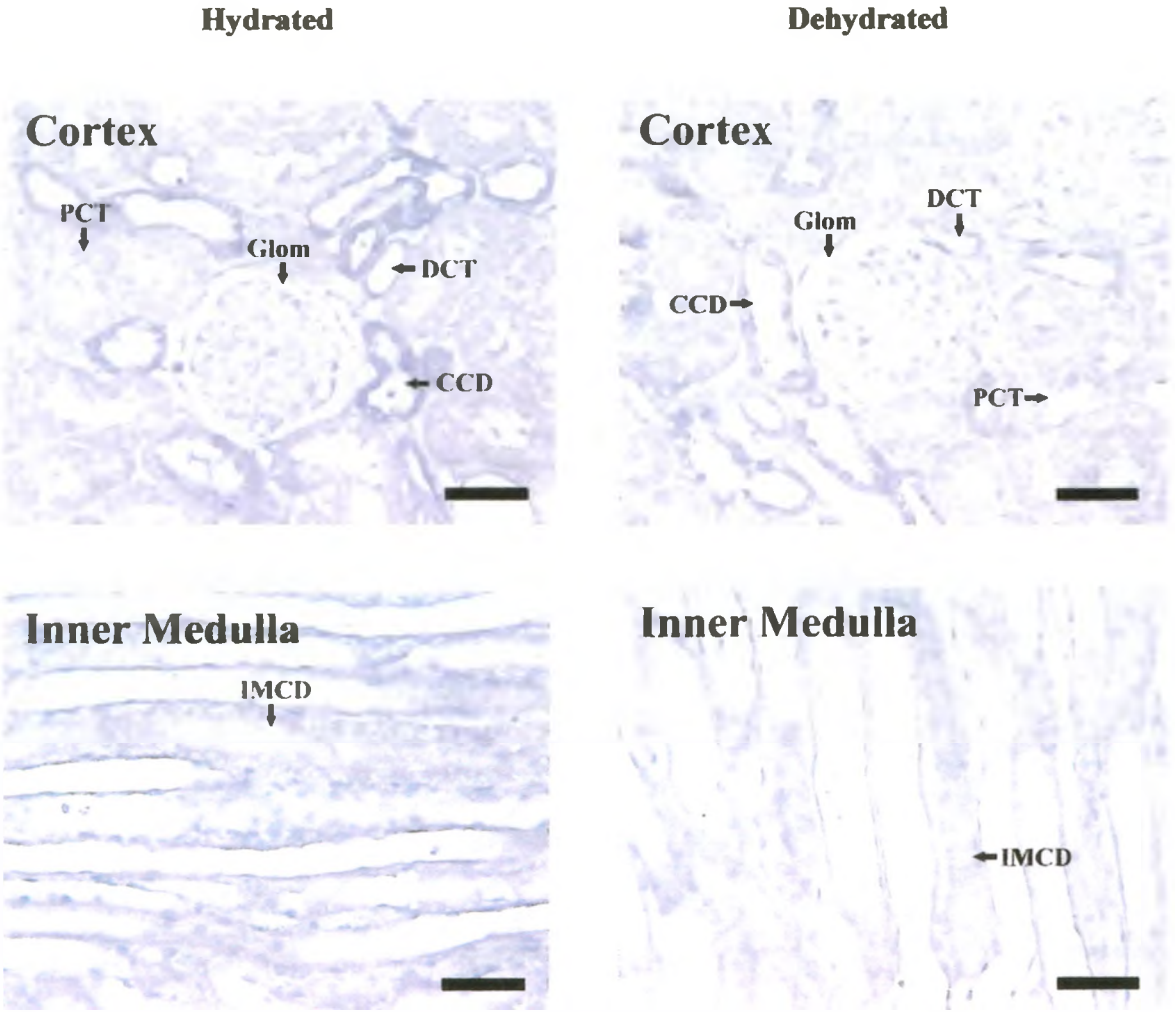

Figure 11. *In situ* ligand binding for STC-1 receptor in the kidney cortex and inner medulla of rats following 48 hours of dehydration. There was no observable difference in STC-1 receptor localization in nephron segments of hydrated and dehydrated rats. Scale bar 50μm.

(Abbreviations: CCD-cortical collecting duct; DCT- distal convoluted tubule; Glom-glomerulus; IMCD- inner medullary collecting duct; PCT-proximal convoluted tubule).

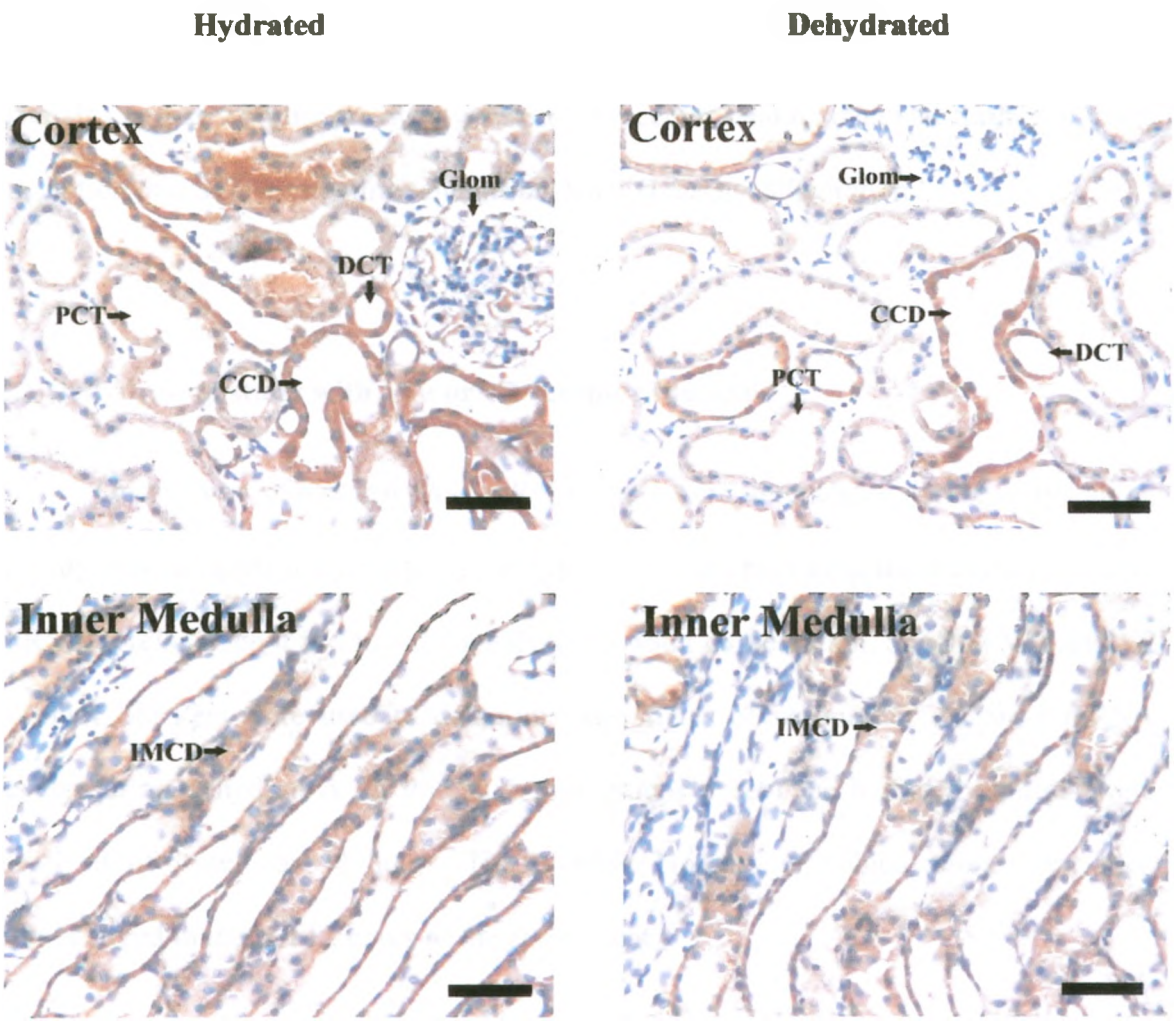

Figure 12. Immunostaining for STC-1 protein in the rat kidney cortex and inner medulla following 48 hours of dehydration. There was no observable difference in STC-1 protein localization in nephron segments of hydrated and dehydrated rats. Scale bar 50μm.

(Abbreviations: CCD-cortical collecting duct; DCT- distal convoluted tubule; Glom-glomerulus; IMCD- inner medullary collecting duct; PCT-proximal convoluted tubule).

were seen in the kidneys of rats dehydrated for 12 or 24 hours (data not shown). This data also agrees with the previously described receptor binding data and further supports the notion that STC-1 targeting remains unchanged during dehydration.

3.2 Dehydration study with V1a or V2 receptor blockade

Once it was shown that *Stc-1* mRNA levels were upregulated during dehydration, a study was designed to evaluate the contribution of vasopressin to the induction of *Stc-1* mRNA levels, given it is a major contributor to water conservation. Specific V1a and V2 receptor blockers were used to determine the effects of vasopressin on *Stc-1* mRNA levels during dehydration. In this study, all groups except the hydrated controls were deprived of water for 48 hours. The receptor blockers or vehicle alone were then administered in the last 12 hours of the experiment.

3.2.1 Body weight, serum and urine osmolalities during dehydration + vasopressin blockade

Rats dehydrated for 48 hours and administered vehicle alone lost 13.6 % of their starting body weight, while hydrated rats gained an average of 6.9%. Similarly, a significant weight loss was also observed in dehydrated rats that received either the V2 or V1a receptor blocker in the last 12 hours (Table 2). There was no significant difference in the degree of weight loss between rats which were treated with V1a or V2 receptor blockers in comparison to the dehydrated rats that received vehicle only. Again this

decrease in the body weight can be attributed to loss of total body water. Therefore, the results indicate that all dehydrated rats, regardless of the receptor blocker administered, had similar degrees of total body water contraction.

The assessment of urine osmolality revealed that 48 hours dehydration resulted in a 2.2 fold increase over controls (Figure 13), which is consistent with previous studies. The administration of V1a or V2 receptor blockers in the last 12 hours of dehydration did not cause a statistically significant drop in urine osmolality as expected, although a trend towards lower urine osmolalities was observed.

Serum osmolalities in all groups of dehydrated rats were higher than in hydrated rats as expected (Figure 14). There was also a trend toward a greater increase in serum osmolality in dehydrated rats that received either the V1a or V2 receptor blocker as compared to dehydrated, untreated animals. However, this trend was not statistically significant (Figure 14)

Table 2: Mean body weight of male Wistar rats before and after 48 hours of dehydration.

Treatment Group	Before (g)	After (g)	Change (%)
Hydrated, Vehicle (N=8)	266.6± 13.0	286.0±12.5**	6.904±0.8
Dehydrated, Vehicle (N=8)	259.3± 15.5	228.1± 13.3***	-13.59±0.5
Dehydrated, V1a (N=8)	264.0 ±12.9	233.3± 11.9***	-13.26± 0.6
Dehydrated, V2 (N=8)	261.6 ±12.4	227.9± 10.8***	-14.83± 0.5

Values are shown as means ± SEM. **p<0.01, ***p<0.001, paired t-test vs. starting weight, N-sample size.

% change indicates the difference between initial and final body weights.

3.2.2 *Stc-1* mRNA levels

Figure 15 shows *Stc-1* mRNA levels in the kidney cortex of hydrated rats, dehydrated control rats and dehydrated rats that received either the V1a or V2 receptor blocker. In the cortex, *Stc-1* mRNA levels were increased after 48 hour of dehydration. An increase in *Stc-1* mRNA levels was also observed in dehydrated rats that received either receptor blocker. However, *Stc-1* mRNA levels in the group of rats given the V2 receptor blocker were significantly lower than in dehydrated controls. This result suggests that V2 receptor blockade, during the last 12 hours of dehydration, attenuated the upregulation of *Stc-1* mRNA levels following dehydration. More importantly this signifies the involvement of vasopressin as a regulator of *Stc-1* gene expression through the V2 receptor during dehydration. In contrast, *Stc-1* mRNA levels in rats administrated the V1a receptor blocker were not significantly different than the dehydrated controls, thereby ruling out the V1a receptor in cortical gene upregulation.

In the inner medulla (Figure 16), *Stc-1* mRNA levels were upregulated in dehydrated animals that received vehicle alone compared to hydrated controls. In this case, however, the administration of V1a or V2 receptor blockers did not attenuate the rise in *Stc-1* mRNA levels in response to dehydration.

3.3 *Stc-1* mRNA upregulation by DDAVP

To confirm that vasopressin does indeed upregulate renal levels of *Stc-1* mRNA, a study was carried out whereby DDAVP, a potent V2 receptor agonist, was administered

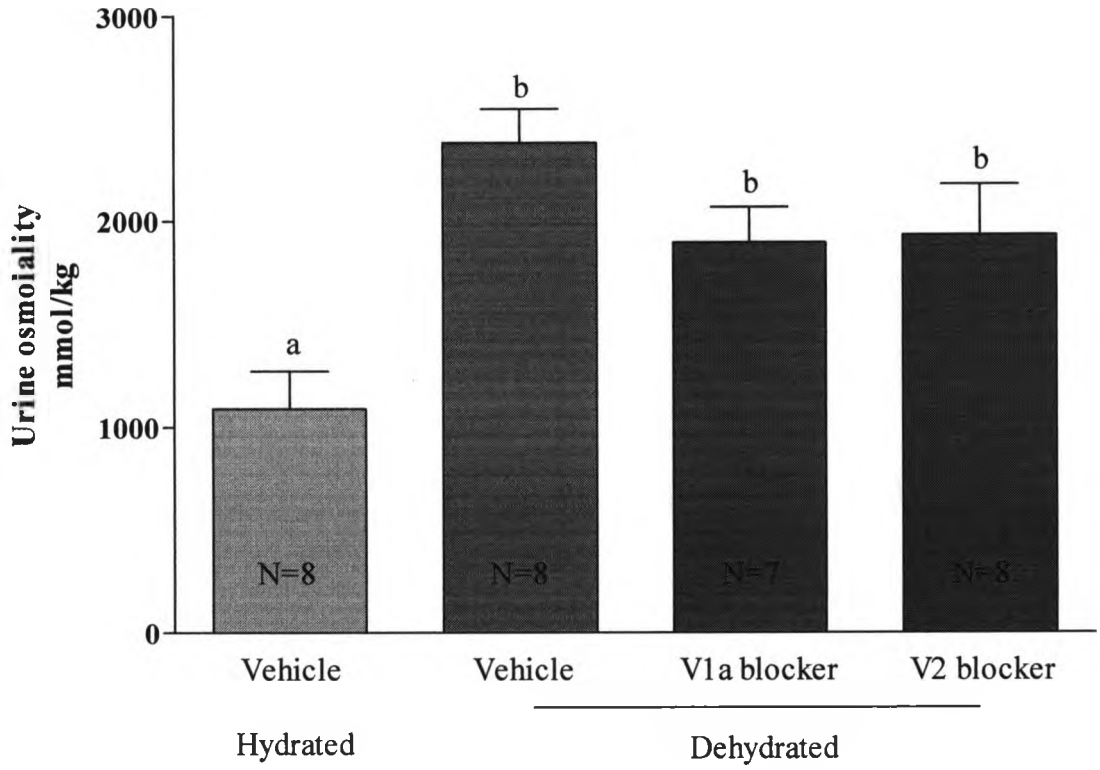

Figure 13. Urine osmolalities in rats following a 48 hour dehydration period with or without vasopressin receptor blockade. Urine osmolality was increased in all dehydrated groups when compared to hydrated controls. The increase in urine osmolality in the groups administered either vasopressin receptor blocker (V1a or V2 blocker) appeared smaller than in the vehicle-treated dehydrated rats, but did not reach statistical significance (groups with different letters are significantly different than one another at the $p<0.05$ level; one-way ANOVA followed by Bonferroni's multiple comparison test; N-sample size).

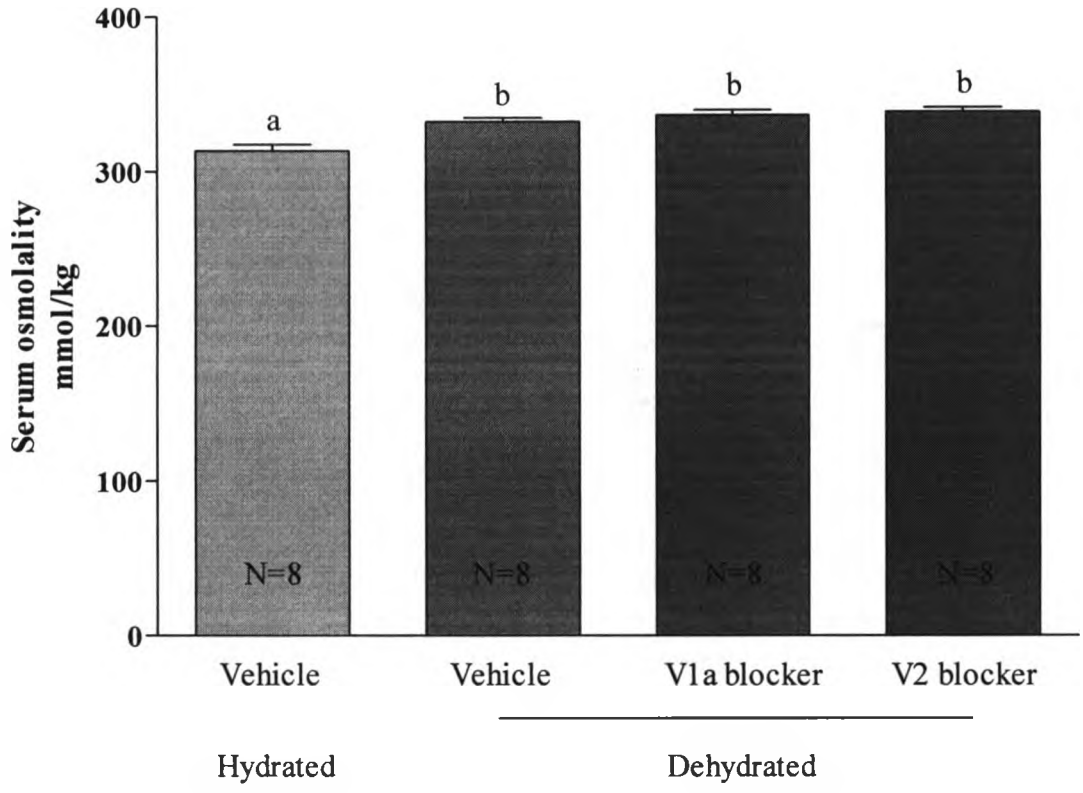

Figure 14. Serum osmolalities in rats following a 48 hour dehydration period with or without vasopressin receptor blockade. Serum osmolality was increased in all dehydrated groups compared to hydrated controls. The increase in serum osmolality in groups administered either vasopressin receptor blocker (V1a or V2 blocker) appeared higher than in vehicle-treated dehydrated rats, but was not statistically significant. (Groups with different letters are significantly different than one another at the $p<0.05$ level; one-way ANOVA followed by Bonferroni's multiple comparison test; N-sample size.)

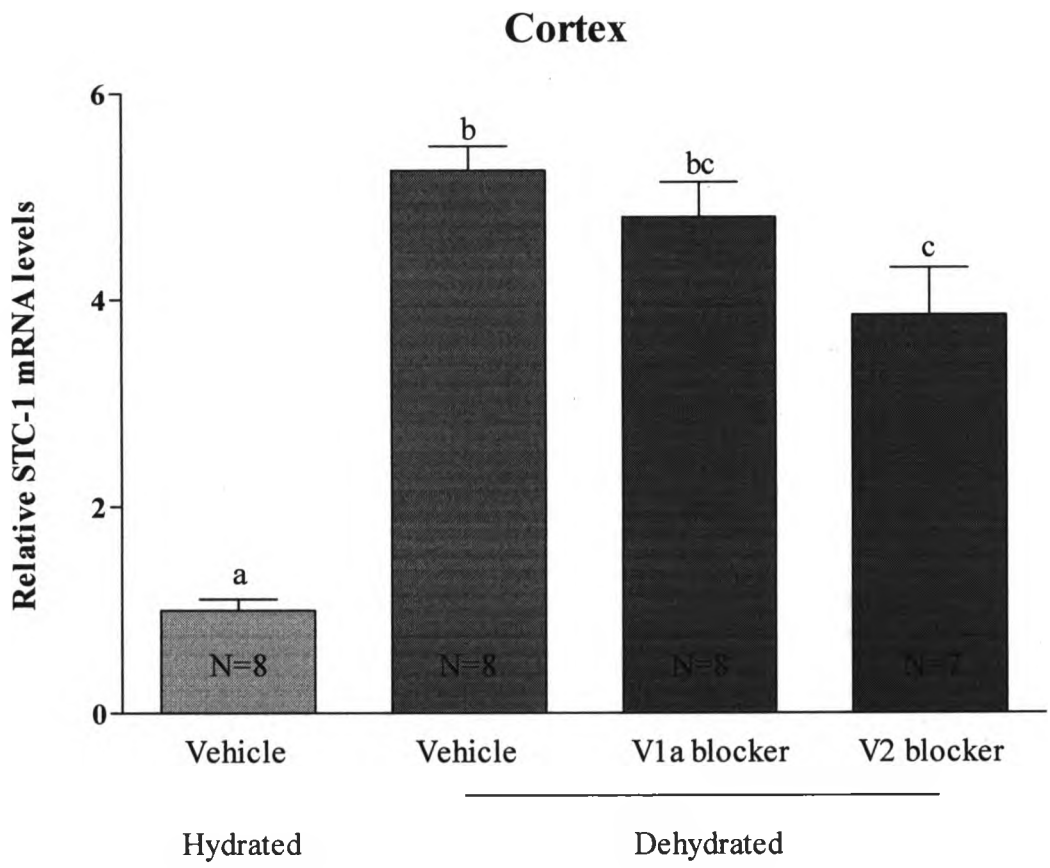

Figure 15. Relative *Stc-1* mRNA levels in rat kidney cortex following a 48 hour dehydration period with and without vasopressin receptor blockade. *Stc-1* mRNA levels were increased in all dehydrated groups. In the group administered the V2 receptor blocker, *Stc-1* mRNA failed to increase to the same extent as in the control dehydrated rats. The V1a receptor blocker had no effect on the magnitude of induction as compared to vehicle-treated control animals (Groups with different letters are significantly different than one another at the $p<0.05$ level; one-way ANOVA followed by Bonferroni's multiple comparison test; N-sample size.)

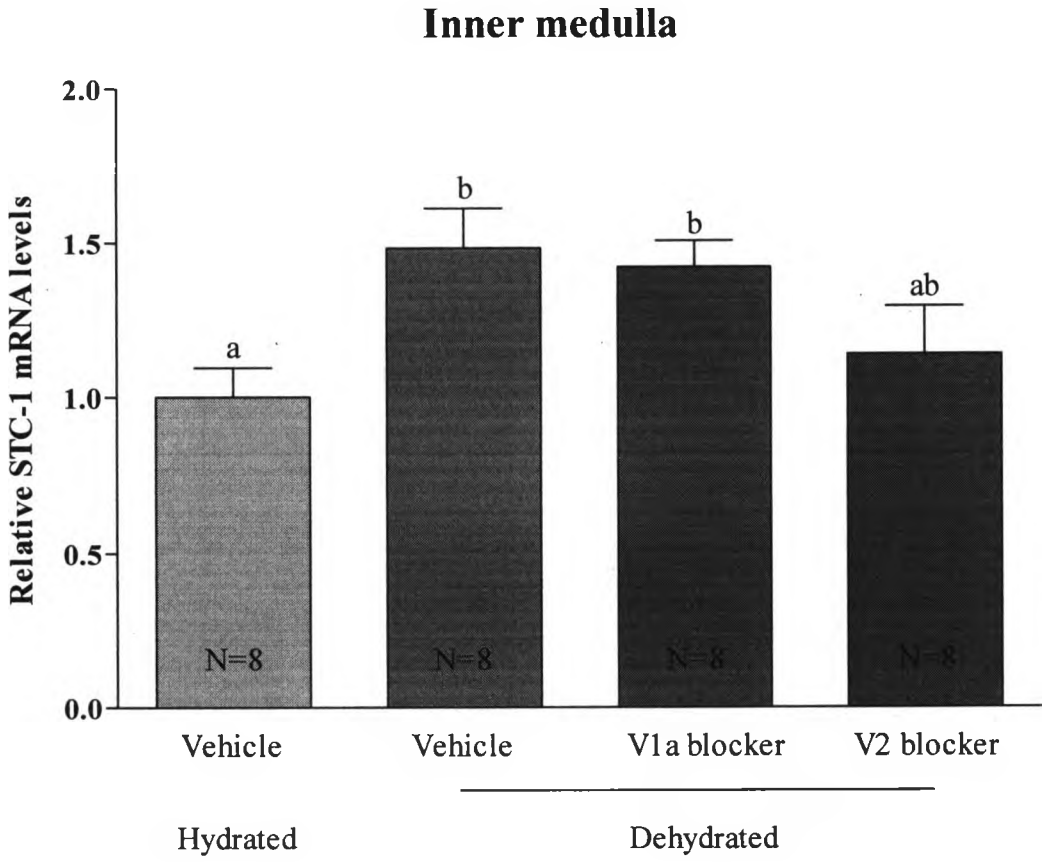

Figure 16. Relative *Stc-1* mRNA levels in rat inner medullary kidney following a 48 hour dehydration period with or without vasopressin receptor blockade. *Stc-1* mRNA levels in rats administrated the V1a receptor blocker were not significantly different than dehydrated controls. *Stc-1* mRNA levels in rats administered the V2 receptor blocker were not significantly different than the hydrated or dehydrated controls. (one-way ANOVA followed by Bonferroni's multiple comparison test; groups with different letters are significantly different than one another at the $p<0.05$ level; N-sample size).

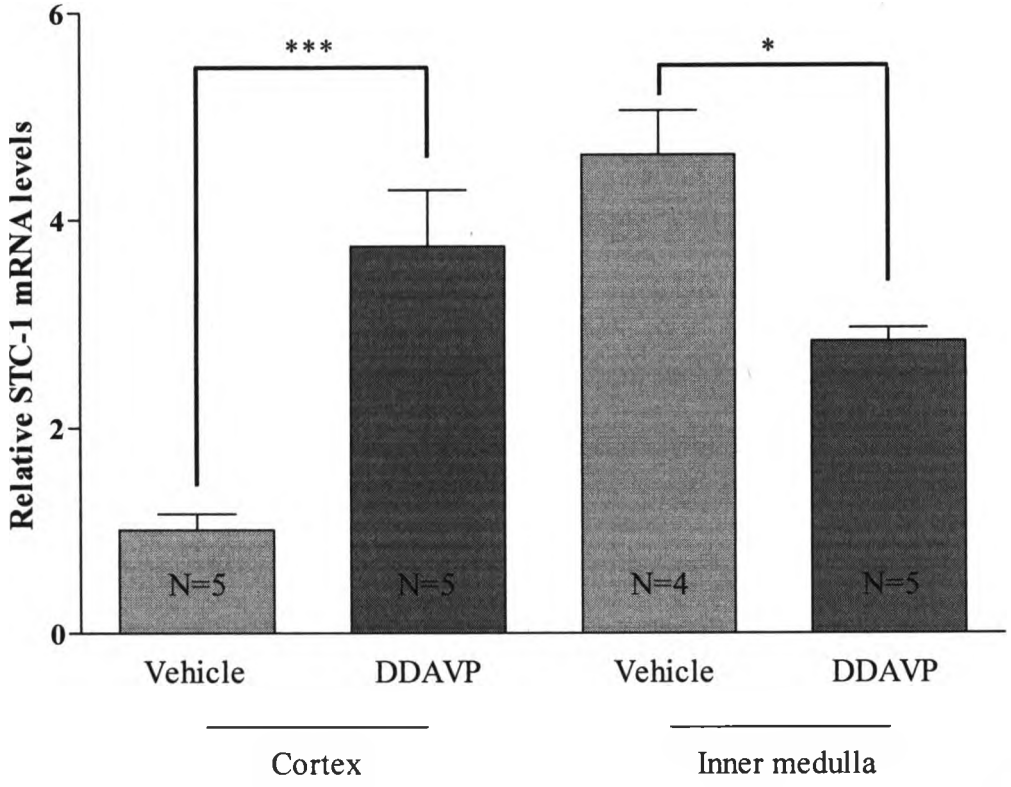

Figure 17. Relative *Stc-1* mRNA levels in rat kidney following a 48 hour DDAVP infusion. Rats were treated with DDAVP (5 ug/kg/day) or vehicle alone for 48 hours. Cortical *Stc-1* mRNA levels were 3.7 fold higher in DDAVP-treated animals compared to vehicle-treated controls. In contrast, medullary mRNA levels were 1.7 fold lower in DDAVP-treated animals (* $p<0.05$, *** $p<0.001$, one-way ANOVA followed by Bonferroni's multiple comparison test; N-sample size).

to hydrated rats for 48 hours. *Stc-1* mRNA levels from the kidney cortex and inner medulla were measured and compared to control animals which received vehicle only. A ~3.7 fold upregulation in *Stc-1* mRNA levels was observed in the kidney cortex after DDAVP treatment (Figure 17). This finding showed that vasopressin, acting through the V2 receptor was capable of upregulating *Stc-1* mRNA levels and likely contributed to gene upregulation during dehydration. Interestingly, a ~1.7 fold decrease in *Stc-1* mRNA levels was observed in the inner medulla of DDAVP treated rats (Figure 17). This suggests that the gene is regulated differently here and implies that some other factor is responsible for the upregulation of the inner medullary gene during dehydration.

3.4 Overhydration study

Both control and overhydrated rats gained weight during the course of the study. However, no difference in weight gain was observed between the two groups indicating that overhydration did not significantly affect extracellular fluid volume (Table 3). Figure 18 shows urine osmolalities of control and overhydrated rats. Urine osmolality declined ~ 2 fold as a consequence of overhydration, indicating that overhydration was effective.

Real-time RT PCR showed no difference in cortical or medullary *Stc-1* mRNA levels after 48 hours of overhydration when compared to control animals (Figures 19 & 20). *In situ* ligand binding showed no noticeable changes in the receptor levels or distribution in overhydrated rats in comparison to hydrated controls. In addition, there were no changes in the immunostaining patterns for STC-1 protein in the PT, CD, TAL or DCT of either overhydrated and control animals (results are not shown).

Table 3: Mean body weight of male Wistar rats before and after the course of overhydration.

Treatment Group	Before (g)	After (g)	Change (%)
48 hr hydrated (N=8)	262.3 ±10.1	279.5±9.1***	6.3±0.7
48 hr overhydrated (N=8)	261.6 ±11.7	278.5 ±11.4***	6.1±1.2

Values are shown as means ± SEM. , ***p<0.001, paired t-test vs. starting weight, N-sample size.

% change indicates the difference between initial and final body weights.

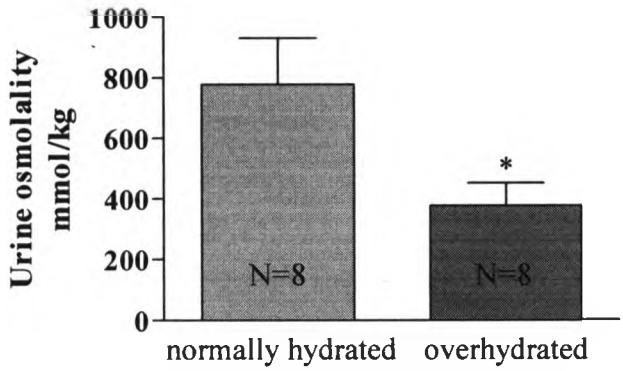

Figure 18. Urine osmolality following a 48 hour overhydration period. Urine osmolality decreased after 48 hours of overhydration when compared to hydrated animals (*p<0.05, paired t-test; N-sample size).

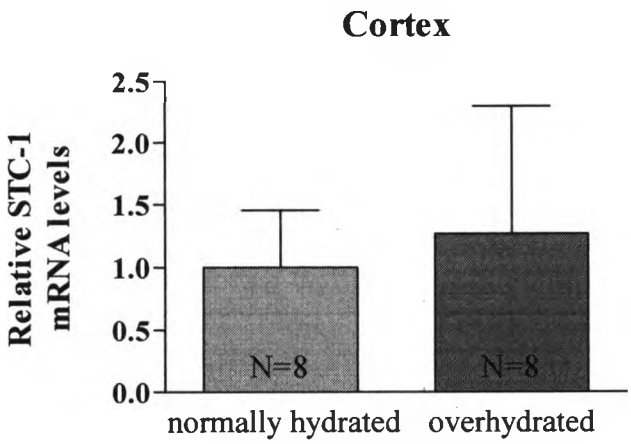

Figure 19. Relative *Stc-1* mRNA levels in rat kidney cortex following 48 hours of overhydration. *Stc-1* mRNA levels were no different in overhydrated and normally hydrated rats (paired t-test; N-sample size).

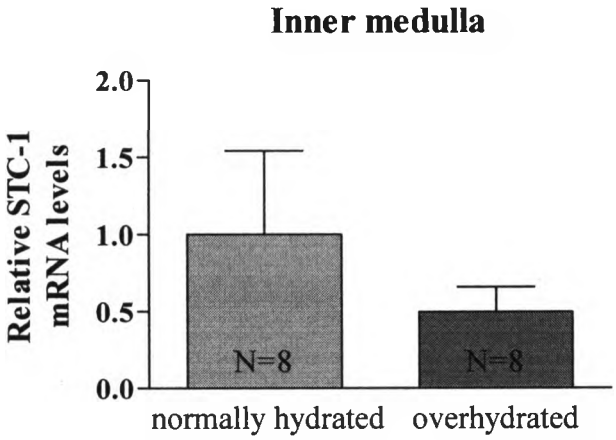

Figure 20. Relative *Stc-1* mRNA levels in rat kidney inner medulla following 48 hours of overhydration. *Stc-1* mRNA levels were no different in overhydrated and normally hydrated rats (paired t-test; N-sample size).

4 Discussion

4.1 Summary of results

The first novel finding of the present study was the significant upregulation in *Stc-1* mRNA levels in kidney cortex and inner medulla in response to dehydration. This suggested that STC-1 function in the kidney might be connected either to water conservation mechanisms or nephron cellular adjustment to hyperosmolar stress. It also opened the door to studies on specific mechanisms that regulated the gene under these conditions, in particular, the possible influences of ADH and hyperosmolality. This was explored in a subsequent project (Turner J.K., et al. 2010). The study also suggested that the *Stc-1* gene was regulated differentially in cortex and inner medulla, a notion which was subsequently confirmed (Turner J.K., et al. 2010). *Stc-1* mRNA levels were also studied during overhydration, where water balance was altered in the opposite direction. However, in this case no changes in *Stc-1* mRNA levels were observed in cortex or inner medulla. This may have been due to the *Stc-1* gene response to osmolar stress being unidirectional. Alternatively, baseline levels of *Stc-1* mRNA may already have been sufficiently low that downward changes could not be reliably measured.

The other focus of the study was STC-1 targeting following alterations in water balance. STC-1 targeting along the nephron segments and to different subcellular organelles were both addressed. The results showed that STC-1 was targeted to the same nephron segments during dehydration, overhydration and in normally hydrated rats. Also, no major shifts in STC-1 receptor levels were seen at the subcellular level, where the ligand is known to preferentially target the mitochondria fraction (McCudden et al. 2002).

However, a small increase in STC-1 targeting to mitochondria was observed during dehydration.

The second important finding described in the thesis is that *Stc-1* mRNA levels in kidney cortex proved to be regulated by ADH acting through the V2 receptor. This was revealed in two different experimental models: receptor blockade and agonist studies.

Finally, the data suggested that the cortical and inner medullary genes were differentially regulated.

4.2 Dehydration as a newly identified inducer of the renal *Stc-1* gene

The present study has shown that dehydration causes an increase in *Stc-1* mRNA levels in both cortex and inner medulla. In the cortex, 12, 24 and 48 hours of dehydration resulted in 4, 3.7 and 6.3 fold increases in *Stc-1* mRNA levels respectively. It was concluded that *Stc-1* mRNA levels increased along with the severity of dehydration. In accordance with this conclusion, a follow-up study has revealed that *Stc-1* mRNA levels are increased even further (8 fold) in response to 72 hours of water deprivation (Turner J.K., et al. 2010).

The 12 and 24 hour water deprivation resulted in similar degrees of gene induction due to there being similar degree dehydration, as confirmed by measurements of serum osmolality. Circadian rhythm is most likely responsible for the similar degrees of dehydration, because the 12 hour dehydration was conducted entirely during the night

when rats drink the most, whereas the 24 hour dehydration encompassed both the night and the daytime when rats drink little (Johnson and Johnson. 1990).

In general, there is limited information available on *Stc-1* gene regulation in the mammalian kidney. It has been shown that a high Ca^{2+} diet downregulates of *Stc-1* mRNA levels (Deol et al. 2001). One other study has shown that calcitriol, the active form of vitamin D3, increases renal *Stc-1* mRNA levels (Honda et al. 1999). Although both of these studies linked STC-1 to its possible involvement in Ca^{2+} homeostasis, functional studies have failed to show any effects of STC-1 injections on serum Ca^{2+} levels (Wagner et al. 1997). Moreover, as Ca^{2+} homeostasis is not altered during dehydration (Bennett and Gardiner. 1987a; Zucker et al. 1982; Merrill et al. 1986), the present findings highlight the notion that renal STC-1 must have functions other than those related to calcium. Since dehydration is a complex condition that triggers multiple adaptive mechanisms, the factors responsible for STC-1 upregulation under these circumstances became the focus of the project.

As mentioned earlier, high osmolality is one component of dehydration thought to be responsible for the induction of *Stc-1* mRNA levels. The evidence in support of high osmolality comes from the studies on MDCK cells, a mammalian kidney cell line. The level of *Stc-1* mRNA and ligand secretion are both upregulated in MDCK cells upon incubation in a media made hypertonic with non-permeant ions such as NaCl and raffinose. As both solutes caused an increase in *Stc-1* mRNA levels and secretion, this indicated that the hyperosmolar effect was not Na^{+} specific (Sazonova et al. 2008b; Sheikh-Hamad et al. 2000).

In vivo progressive dehydration increases interstitial fluid osmolality in the inner medulla. In contrast, osmolalities in the kidney cortex rise in tandem with those of serum. However, because the adaptive mechanisms are directed to limiting the increase in serum osmolality during dehydration rise in cortical interstitial fluid osmolality is modest as compared to inner medulla (Seldin and Giebisch. 1992; Selen and Persson. 1983c).

In marked contrast to the osmolality changes outlined above, *Stc-1* mRNA levels were upregulated to a much greater extent in the cortex than the medulla. Indeed, the inverse would have been anticipated where osmolality is the primary factor affecting gene expression. Furthermore, *Stc-1* mRNA levels were increased by 24 hours of dehydration without significant changes in serum osmolality. Therefore, it must be concluded that factors other than osmolality were driving the increase in cortical gene activity.

Also surprising, was how large the difference was in gene induction between cortex and inner medulla 6.3 vs. 3.8 fold (following 48 hours of dehydration). In part, the difference might be explained by the fact that baseline *Stc-1* mRNA levels are higher in inner medulla to begin with (3 fold higher), such that the requisite rise in *Stc-1* mRNA levels was achieved that much faster. Alternatively, it is possible that *Stc-1* mRNA levels are differentially regulated in these two regions and evidence of the latter emerged in the studies on ADH and specific receptor agonists.

4.3 ADH as a possible regulator of *Stc-1* mRNA levels in the kidney cortex

In the attempt to identify factors responsible for *Stc-1* gene induction during dehydration, ADH was viewed as a possible candidate. ADH plays a major role during

dehydration by regulating most of the mechanisms involved in the processes of water conservation, controlled natriuresis and cellular adjustments to hyperosmotic stress (Seldin and Giebisch. 1992), as described in the Introduction. Therefore, ADH was potentially responsible for upregulating *Stc-1* mRNA levels during dehydration through one or more receptor subtypes.

Specific drugs that competitively block V1a or V2 receptors were first employed to assess the possible involvement of ADH. Rats were dehydrated for 48 hours and V1a or V2 receptors were blocked by the administration of antagonists during the last 12 hours. The results showed that V2 receptor blockade significantly attenuated the upregulation of *Stc-1* mRNA levels in kidney cortex following dehydration, suggesting that the cortical gene was regulated through the V2 receptor. V1a receptor blockade failed to suppress the gene, suggesting that it was not involved.

In the inner medulla, the results were less clear. Here, it was not possible to confirm the involvement of either the V1a or V2 receptor subtype. *Stc-1* mRNA levels were no different in the dehydrated rats administered either the V1a or V2 blockers, although those that received the V2 blocker were no longer significantly different than hydrated controls. Therefore, while the data does not necessarily rule out the role for the V2 receptor in inner medulla, the case was not as strong as in the cortex.

In view of the findings obtained with these antagonists, it was also important to address the issue of the effectiveness in the blockade. The efficiency of receptor blockade was monitored by changes in body weight, as well as urine and serum osmolalities. The question as to whether the receptors were effectively blocked has been raised because

neither antagonist had any significant differences in the body weights, serum or urine osmolalities. It was expected that urine osmolalities would drop and serum osmolalities would rise in response to V2 receptor blockade. Trends were evident in rats treated with OPC 31260. However, statistically significant changes were not achieved. Taking into account the fact that V2 blockade suppressed the increase in cortical *Stc-1* mRNA levels, it must be concluded that V2 receptor blockade was indeed effective. However, the minor changes in urine and serum osmolality in the face of V2 blockade may be explained by the existence of parallel mechanisms that operate independently of the V2 receptor, and continued to promote urine concentration. Others have shown that, dehydration continues to promote translocation of AQP2 to the apical membrane of the CD cells and water reabsorption even in the presence of V2 blockade (Marples et al. 1998). Secretin has been identified as being responsible for the AQP2 translocation, essentially bypassing the V2 receptor (Chu et al. 2007).

It is likely because of these parallel pathways that it was not possible to completely suppress the water conservation mechanism. Also, it should be noted that V2 receptor blockade caused only a small decrease in cortical *Stc-1* mRNA levels in comparison to the rise brought about by dehydration. Because of these limitations further studies were carried out using a specific V2 receptor agonist.

The specific V2 receptor agonist, DDAVP, was employed to further evaluate the involvement of V2 receptors. The results showed that following DDAVP infusion, *Stc-1* mRNA levels were increased 3.7 fold in cortex, but decreased 1.7 fold in inner medulla. This confirmed that the cortical gene was indeed being regulated through the V2 receptor. The results also highlighted the fact that the gene was differentially regulated in the

cortex and medulla. Importantly, the fact that inner medullary gene activity was increased during dehydration but was reduced by V2 activation implies that other factors must overcome the V2 -dependent downregulation during water deprivation. These factors have yet to be identified.

Both the V1a and V2 receptors, through which ADH acts, are expressed in CD cells where the *Stc-1* gene is also expressed, and normally these receptors are more concentrated in the inner medulla. However, the literature shows that the number of functional ADH receptors is increased considerably in cortical CD cells during dehydration, in comparison to the inner medulla where they remain unchanged. Such a shift in receptor expression levels implies an increase in ADH targeting to the cortical segment during dehydration (Fadool and Aggarwal. 1990). Therefore *Stc-1* mRNA upregulation during dehydration could follow the shifting pattern in ADH targeting.

The question also remains as to whether the V2 receptor dependent mechanism is the only one that upregulates *Stc-1* mRNA levels in the cortex? In answer to this, subsequent studies have been able to show that high osmolality produced by either mannitol or sodium chloride also upregulated the cortical gene. However, the fold changes brought in by osmolality alone were not so high as those due to dehydration (Turner J.K., et al. 2010). Therefore, osmolality may be an important factor in regulating the gene in addition to ADH. Also, further studies with specific V1a receptor agonist, should be considered to clarify a role for this receptor subtype in *Stc-1* gene regulation.

4.4 STC-1 targeting during dehydration

In situ ligand binding and immunocytochemistry were performed on kidney sections of control and dehydrated animals to assess STC-1 receptor and protein distribution patterns along the nephron. The results showed that there were no changes in either receptor or protein localization patterns, indicative that STC-1 was targeted to the same nephron segments during dehydration as in the normal, hydrated state (namely PCT, cortical and medullary TAL, cortical and medullary CD).

Previous studies have shown that in the normally hydrated state, STC-1 production is confined to the CD system, as STC-1 mRNA is present exclusively in this segment. From CD cells, STC-1 is targeted to upstream nephron segments (PT and TAL) and sequestered there within the cells (Haddad et al. 1996a; Wong et al. 1998; McCudden et al. 2002). Whether these same paracrine targeting pathways are preserved in their entirety during dehydration remains to be established. The receptor and protein localization patterns would seem to suggest that this is the case. However, in situ hybridization is also needed to address this question more fully. If STC-1 gene activity also remains confined the CD system, this would suggest that the same paracrine targeting pathways are operative along the nephron irrespective of hydration status, but that their amplitude is increased during dehydration.

STC-1 receptor localization was also studied at the subcellular level to assess ligand targeting to different organelles. In both hydrated and dehydrated rats the receptors appeared to be concentrated on the same organelles. The highest number of binding sites was observed on the mitochondria, followed by the membrane fraction and the cell

nuclei. A significant increase in mitochondrial binding was seen in dehydrated rats, however, suggestive of an increase in mitochondrial targeting. This notion was supported by there being a significant increase in STC-1 protein levels within the mitochondrial fraction.

4.5 Proposed functional effects of STC-1 during dehydration

STC-1 targeting to the mitochondria results in respiratory uncoupling (Ellard et al. 2007) which has recently been shown to decrease reactive oxygen species (ROS) in macrophages (Wang et al. 2009b). As such, ROS reduction could be one role of renal STC-1 during dehydration. Collectively, ROS comprises superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (.OH) (Garvin and Ortiz. 2003). As ROS plays multiple roles in the kidney, any changes in its levels by STC-1 could have numerous consequences. One of them would be a reduction in ROS-induced apoptosis (Circu and Aw. 2010). It is known that ROS production is increased during oxidative stress (Circu and Aw. 2010). The high extracellular fluid osmolalities present during dehydration have been shown to induce oxidative stress and increase ROS production (Zhou et al. 2005; Zhou et al. 2006; Hizoh and Haller. 2002). Subsequently, ROS damages the cells and initiates apoptotic pathways (Circu and Aw. 2010). Renal STC-1 upregulation might sufficiently attenuate the increase in ROS and thus act as a prosurvival factor. Notably, the cytoprotective actions of STC-1 have also been demonstrated in other tissues such as cardiomyocytes and neurons (Zhang et al. 2000; Westberg et al. 2007b; Westberg et al. 2007a).

In kidney, however, the effects of ROS are even broader. ROS are now being commonly viewed as signaling molecules, and have been shown to increase Na^+ reabsorption in the TAL (Garvin and Ortiz. 2003). As such, by reducing ROS, STC-1 could conceivably decrease Na^+ reabsorption during dehydration. Natriuresis is one of many compensatory mechanisms that are operating during dehydration to accommodate the fall in extracellular fluid volume (Merrill et al. 1986). Therefore, it is possible that STC-1 upregulation is the component of this adaptive mechanism that prevents sodium overloading during negative water balance.

In addition, ROS, more specifically $O_2^{\bullet -}$, directly influences NO levels. Whereas NO production is directly dependent on NOS activity, $O_2^{\bullet -}$ regulates its metabolism. $O_2^{\bullet -}$ reacts with NO at a very high rate to produce peroxynitrite ($ONOO^-$) which lacks the physiological effects of NO. Hence, the balance of NO levels depends on both $O_2^{\bullet -}$ and NOS (Evans and Fitzgerald. 2005b). Because STC-1 is capable of regulating ROS levels it can likely also modulate NO levels through $O_2^{\bullet -}$.

During dehydration, NO is known to have an important role in promoting both natriuresis and water reabsorption (Ortiz and Garvin. 2002; Murase et al. 2003; Morishita et al. 2005). At present, it is known that NOS is upregulated during dehydration which results in more NO production (Shin et al. 1999). The upregulation of STC-1 during dehydration could possibly reduce $O_2^{\bullet -}$, slow NO catabolism and in this way contribute to higher steady-state levels of NO. Interestingly, ADH has been shown to induce NOS during dehydration (Martin et al. 2002) and the present project has identified ADH as a newly defined regulator of STC-1. Therefore, it can be hypothesized that in addition to its

regulatory effects on NOS, ADH may upregulate STC-1 as an additional means of NO control.

The other consequence of respiratory uncoupling involves stimulation of Ca^{2+} uniport activity and mitochondrial Ca^{2+} uptake (Ellard et al. 2007). One study done on macrophages has demonstrated that STC-1 upregulates UCP-2 (Wang et al. 2009b) which is required for uniport activation (Trenker et al. 2007). The accumulation of Ca^{2+} within mitochondria would tend to lower cytosolic Ca^{2+} concentrations. Since high cytosolic Ca^{2+} is toxic to cells, this effect could also be cytoprotective in nature (Zhang et al. 2000).

In summary, the functional effects of STC-1 during dehydration are possibly connected to the uncoupling of oxidative phosphorylation, which result in ROS reduction, an increase in cellular NO and Ca^{2+} accumulation within the mitochondria. As in the case of other cell types, the above changes can also be cytoprotective to nephron cells. In addition to this, through ROS and NO, STC-1 may be regulating major kidney functions that are altered during dehydration: namely, sodium and water reabsorption. As such, renal STC-1 could have a role in regulating both the composition and volume of the extracellular fluid. Finally, as STC-1 targeting appears to be unchanged, though increased, during dehydration, the ligand could prove to have a similar role in the normal hydrated state as it does during dehydration.

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