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O papel do stress oxidativo na regulação dos transportadores de aminoácidos renais na hipertensão e no envelhecimento

Oxidative stress on the regulation of renal amino acid transporters in hypertension and aging

Vanda Carina Ribeiro Pinto

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Pinto V, Amaral J, Silva E, Simão S, Cabral JM, Afonso J, Serrão MP, Gomes P, Pinho MJ, Soares-da-Silva P (2011) Age-related changes in the renal dopaminergic system and expression of renal amino acid transporters in WKY and SHR rats. Mechanisms of Ageing and Development 132 (6-7):298-304

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Index

INTRODUCTION

Hypertension and the kidney

Hypertension, or high blood pressure, is a major contributor to cardiovascular disease. It has been recently reported that approximately 25% of the adult population worldwide suffer from hypertension (Kearney et al. 2005). As many as 90-95% of all cases of hypertension are classified as essential (primary) hypertension with unknown cause of the disease (Chobanian et al. 2003). The remaining 5-10% of cases (secondary hypertension) are caused by known factors including endocrine disorders, kidney diseases and tumors (Taler 2008). This severe condition is associated with a significant increase in the risk for progression to heart failure, arrhythmias or sudden death (Levy et al. 1990; Lorell et al. 2000).

The causes that underlie hypertension are complex, because both genetic and environmental factors participate in the pathogenesis of this disease (Moore et al. 2002; Coy 2005; Marteau et al. 2005; Kunes et al. 2006). It has been estimated, however, that 30–50% of essential hypertension is heritable. Approximately 30–35% of subjects with normal blood pressure are salt-sensitive; in hypertensive patients, this percentage is as high as 50–70% (Burnier et al. 2006; Haddy 2006). Because the kidney is important in the long-term regulation of blood pressure and is the major organ involved in the regulation of sodium homoeostasis, many studies have focused on the abnormal renal handling of salt in the pathogenesis of hypertension. Moreover, several studies have shown that human essential hypertension is associated with increased sodium transport in the renal

proximal tubule (Ortiz et al. 2001; Hussain et al. 2003). Several rodent models of genetic hypertension have been established for experimental investigation of hypertension genetics and pathophysiology, including spontaneously hypertensive rats of the Okamoto-Aoki strain (SHR), Dahl salt-sensitive rats, Milan hypertensive rats, and Prague hypertensive rats (Grisk et al. 2001). In these rats arterial hypertension can be transferred with a renal graft from the hypertensive strain to normotensive recipients. Furthermore, renal grafts from the respective normotensive control strains lowered arterial pressure in these genetically hypertensive rat strains (Bianchi et al. 1974; Dahl et al. 1974; Heller et al. 1993; Grisk et al. 2001).

SHR have been the most frequently used experimental animal models in research on genetics and pathology of arterial hypertension (Grisk et al. 2001). By the age of 3–6 weeks, the pressure natriuresis and diuresis relationship in these animals has already shifted to elevated arterial pressure levels. Renal afferent arteriolar resistance and tubular sodium reabsorption is increased when compared with normotensive animals (Roman 1987). These abnormalities in renal function are consistent with an involvement of renal mechanisms in the pathophysiology of hypertension in SHR.

Renal systems controlling sodium homeostasis

The renal renin-angiotensin-aldosterone system (RAAS) and dopaminergic system control renal electrolyte balance through various receptor-mediated pathways with counter-regulatory interactions. In order to conserve sodium during low sodium intake,

the RAAS is upregulated to produce angiotensin II (Ang II). Stimulation of the principal membrane bound cell surface receptor for Ang II, the AT_1R, leads to sodium reabsorption. On the other hand, during high sodium intake the local renal production of dopamine is increased, leading to inhibition of sodium reabsorption (Felder et al. 2006).

The renin-angiotensin-aldosterone system

The RAAS plays an important role in regulating blood volume and systemic vascular resistance, which together influence cardiac output and arterial pressure (Covic et al. 2009). Renin, which is primarily released by the kidneys, stimulates the formation of angiotensin in blood and tissues, which in turn stimulates the release of aldosterone from the adrenal cortex (Harrison-Bernard 2009). The release of renin is stimulated by sympathetic nerve activation (acting via β_1-adrenoceptors), renal artery hypotension (caused by systemic hypotension or renal artery stenosis) and decreased sodium delivery to the distal tubules of the kidney (Paul et al. 2006; Covic et al. 2009). When renin is released into the blood, it acts upon a circulating substrate, angiotensinogen, that undergoes proteolytic cleavage to form angiotensin I. Angiotensin converting enzyme forms Ang II, which constricts resistance vessels (via Ang II AT_1 receptors) thereby increasing systemic vascular resistance and arterial pressure (Paul et al. 2006; Harrison-Bernard 2009). Ang II also acts on the adrenal cortex to release aldosterone, which in turn acts on the kidneys to increase sodium and fluid retention; stimulates the release of vasopressin from the posterior pituitary, which increases fluid retention by the kidneys;

facilitates noradrenaline release from sympathetic nerve endings and inhibits noradrenaline reuptake by nerve endings, thereby enhancing sympathetic adrenergic function and resulting in cardiac and vascular hypertrophy (Paul et al. 2006; Harrison-Bernard 2009).

Aldosterone plays a pivotal role in electrolyte and fluid homeostasis and thus in the control of blood pressure. The classical view of aldosterone action is that it targets epithelia of the distal colon and renal nephron to stimulate sodium reabsorption and potassium excretion (Laragh et al. 1964; Ngarmukos et al. 2001). The classical actions of aldosterone are mediated by the mineralocorticoid receptor (MR) that translocates to the nucleus upon ligand binding (**Figure 1**). The steroid-bound receptor modulates gene expression by functioning as a transcription factor (Yang et al. 2009; Ackermann et al. 2010). The classical actions of aldosterone include increasing the expression and activity of the αENaC, Na^+,K^+-ATPase α_1-subunit and sodium-hydrogen exchanger (NHE) (Loffing et al. 2001; Fuller et al. 2005; Drumm et al. 2006; Pinto et al. 2008). However, other intrinsic proteins localized to the apical membrane of epithelia in the intestine and kidney are now also recognized as final effectors.

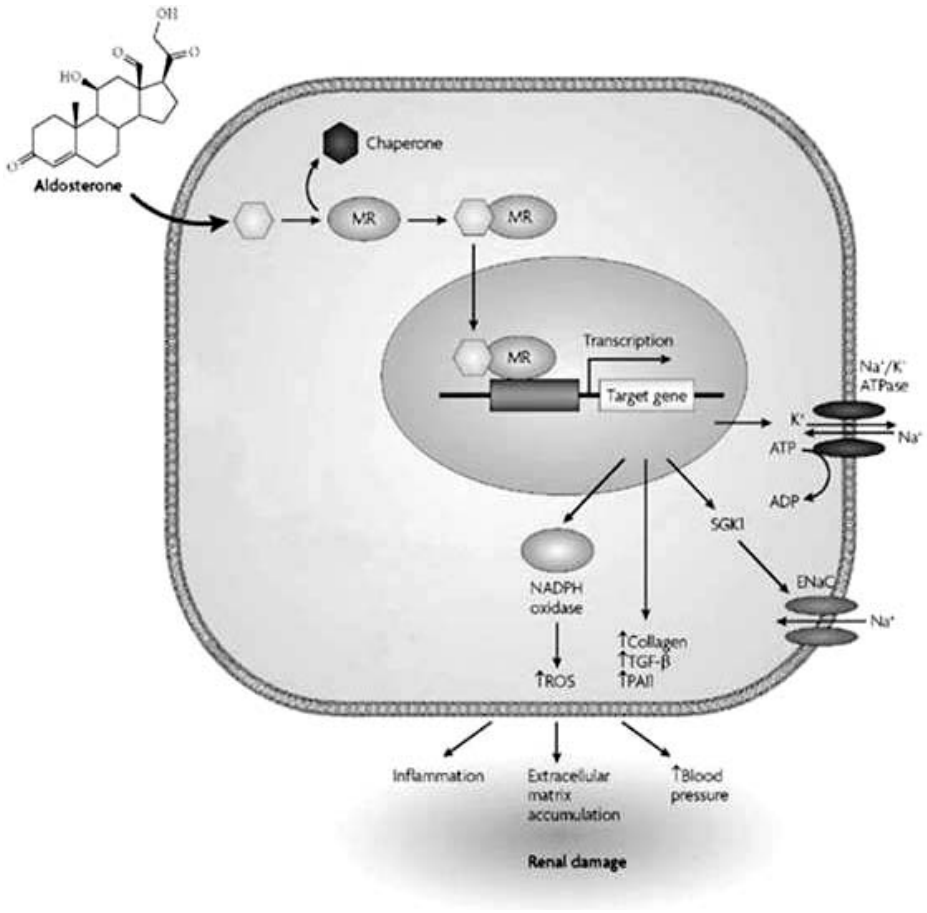

Figure 1–Activation of the MR by aldosterone results in its dissociation from molecular chaperones, translocation into the nucleus and binding to hormone-response elements in the regulatory region of target gene promoters to enhance expression. Aldosterone enhances the gene expression of profibrotic molecules and stimulates inflammation through the generation of ROS by increasing the expression of NADPH oxidase. Abbreviations: MR, mineralocorticoid receptor; ROS, reactive oxygen species; SGK1, serine/threonine-protein kinase-1; TGF-β, transforming growth factor-β; NADPH oxidase, nicotinamide adenine dinucleotide phosphate-oxidase; PAI1, plasminogen activator inhibitor-1 [Adapted from (Perico et al. 2008)].

Aldosterone increases activity of the luminal sodium-hydrogen exchanger type 3 (NHE3) in the proximal portion of the colon and the apical thiazide-sensitive sodium-chloride cotransporter (NCC) in the distal renal tubule (Cho et al. 1998; Kim et al. 1998).

Inappropriate aldosterone secretion leads to hypertension in the case of hyperaldosteronism, or to hypotension in the case of hypoaldosteronism (Epstein 2001).

The renal dopaminergic system

Intrarenal dopamine plays a central role in the regulation of sodium metabolism (Aperia 2000). Several clinical observations as well as studies on animals with various forms of genetic hypertension suggest that defects in the renal dopaminergic system may contribute to the development of hypertension, by causing salt retention (Hussain et al. 2003).

In the mammalian kidney, dopamine is primarily produced in the proximal tubule (Soares-da-Silva et al. 1998; Aperia 2000; Gomes et al. 2008). The dopamine precursor L-dihydroxyphenylalanine (L-DOPA) is filtered at the glomerulus and is taken up by the proximal tubule and converted to dopamine by aromatic L-amino acid decarboxylase (L-AADC), which is highly expressed in the proximal tubule (Soares-da-Silva et al. 1991). The regulation of this non-neuronal dopaminergic system depends mainly on the availability of L-DOPA, on its decarboxylation into dopamine and on cell outward amine transfer mechanisms (Soares-da-Silva et al. 1991; Pestana et al. 1994). In the kidney, dopamine is metabolized predominantly by catechol-*O*-methyl-transferase (COMT) and monoamine oxidase (MAO) to 3,4-dihydroxyphenylacetic acid (DOPAC), and to homovallinic acid (Soares-da-Silva et al. 1991; Pestana et al. 1994). Studies have shown the overexpression of sodium-independent and pH-sensitive amino acid transporter LAT2 (SLC7A8) in the SHR

kidney (Pinho et al. 2003), which might contribute to enhanced L-DOPA uptake in the proximal tubule and increased dopamine production, as an attempt to overcome the defect in D_1 receptor function. In agreement with these findings, immortalized renal proximal tubular epithelial cells from the SHR also overexpressed LAT2. However, only 25% of L-DOPA uptake in SHR cells occurred through LAT2; 50% of L-DOPA uptake occurred through LAT1 (SLC7A5) and the remaining 25% through sodium-dependent transport systems (Pinho et al. 2004).

The effects of dopamine, in mammals, are mediated by five dopamine receptor subtypes. These five receptor subtypes differ in their primary structures and show distinct affinities for dopamine receptor agonists and antagonists (Zeng et al. 2007). The D_1-like receptors are composed of the D_1 and D_5 receptor subtypes (D_{1A} and D_{1B}, for rodent homologues). The D_1-like receptors couple to the stimulatory G-proteins G_s and G_q, and activate adenylate cyclase activity to increase cytosolic cAMP levels (**Figure 2**) (Hussain et al. 2003). The D_2-like receptors are composed of the D_2, D_3 and D_4 receptor subtypes, which couple to the inhibitory G-proteins G_i and G_o and modulate ion channel activity and/or inhibit adenylate cyclase activity. All of the dopamine receptor subtypes have been shown to regulate, directly or indirectly, sodium transport in the proximal and distal nephron and blood pressure (Hussain et al. 2003; Zeng et al. 2004). The mechanisms through which renal dopamine is thought to produce natriuresis involve the activation of D_1-like receptors that inhibit the activity of both apical (eg, NHE exchange and chloride-bicarbonate exchange and Na-P cotransport) and basolateral (Na^+,K^+-ATPase and $NaHCO_3$

cotransport) transporters (Aperia et al. 1987; Felder et al. 1990; Lokhandwala et al. 1991; Jose et al. 1992).

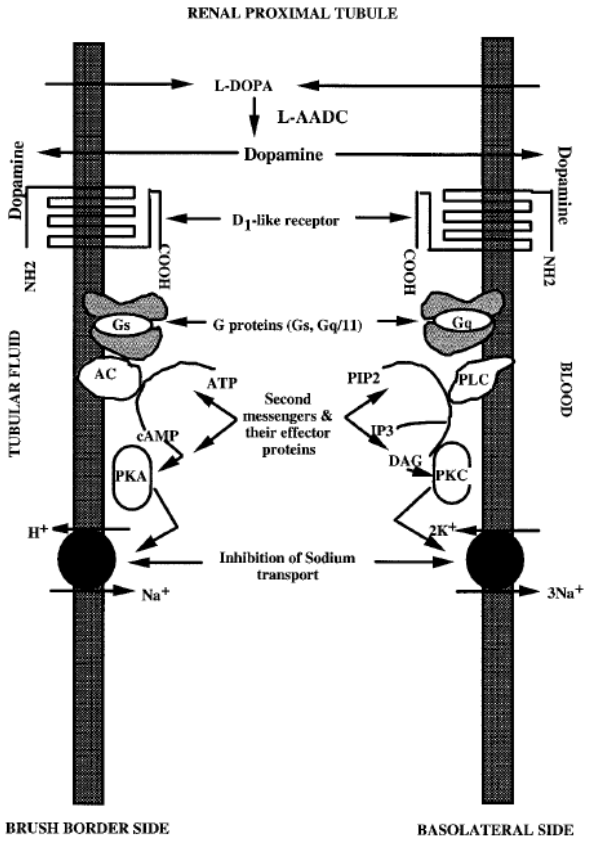

Figure 2–Hypothetical scheme of dopamine synthesis and D_1-like receptor signaling pathway that causes inhibition of sodium transporters in proximal tubles of rat kidney. Abbreviations: L-AADC, L-aromatic amino acid decarboxylase; AC, adenylate cyclase; PLC, phospholipase C; PKA, protein kinase A; PKC, protein kinase C; PIP2, phosphatidylinositol bisphosphate; IP3, inositol trisphosphate; L-DOPA, L-dihydroxyphenylalanine; DAG, diacylglycerol. [Adapted from (Hussain et al. 2003)].

The SHR is a genetic model of hypertension characterized by the resistance to the natriuretic effect of dopamine and D_1-like receptor agonists, as a result of a defective

transduction of the D_1 receptor signal in renal proximal tubules (Sanada et al. 1999; Jose et al. 2010; Zeng et al. 2011). It has been suggested that increased oxidative stress in renal proximal tubules of the SHR could be a mechanism responsible for defective dopamine D_1 receptor/G-protein coupling (White et al. 1998).

Oxidative stress: relation to aging and hypertension

Oxidative stress is defined as an excess in the levels of oxidants over antioxidants within a biological system, and the direct consequence of this is a shift in the redox state of the biological compartment towards one that is more oxidizing. Reactive oxygen species (ROS), which include radical species such as superoxide anions and hydroxyl radicals, and non-radical oxidants such as hydrogen peroxide (H_2O_2), are particularly important effectors of cellular redox status. In the organism they can be produced by xanthine oxidase, nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), mitrochondrial oxidative phosphorylation, lypoxygenase, cytochrome P450 mono-oxygenase and heme-oxygenase. ROS can result in cell damage by reacting with various cellular constituents, including membrane lipids, proteins, and DNA (Wei et al. 2002). It is widely accepted that oxidative stress is closely linked to aging (Harman 1998; Kujoth et al. 2007) and to a variety of pathological processes, including cancer, diabetes and cardiovascular and renal diseases (Spector 2000; Makino et al. 2003; Touyz et al. 2004). Presently, ROS are known to be normal products of cell metabolism and recognized for playing a dual role as both harmful and beneficial species to the organism (Valko et al.

2006; Valko et al. 2007). For example, ROS play a major physiological role in several aspects of intracellular signaling and regulation. Cells are capable of generating ROS endogenously and constitutively which are utilized in the induction and maintenance of signal transduction pathways involved in cell growth and differentiation (Droge 2002).

There is an increase in the incidence of hypertension as well as an increase in the generation of ROS and inflammation with age. There is evidence that ROS can influence vascular reactivity either directly or through intermediate pathways such as reduction of nitric oxide (NO) availability or by oxidation of arachidonic acid with the generation of vasoactive lipid mediators (Schnackenberg et al. 1998). Thus oxidative stress may account for endothelial dysfunction, but it is unknown whether this abnormality is a primary event or a consequence of increased blood pressure. Furthermore, studies have indicated that an increase in oxidative stress within the renal medulla selectively reduces medullary blood flow resulting in chronic hypertension (Makino et al. 2002). Attempts to counteract the hypertensive effect of ROS have led to the use of exogenous administration of antioxidants thought to improve the vascular function and reduce the blood pressure in animal models (Chen et al. 2001; Hoagland et al. 2003) and in human hypertension (Duffy et al. 2001; Boshtam et al. 2002).

The opposing effects of angiotensin and dopamine on the redox status

Accumulating evidence suggests that Ang II stimulates intracellular formation of ROS such as the superoxide anion and H_2O_2 (Sachse et al. 2007). Ang II activates several

subunits of the membrane-bound NADPH oxidase and also increases ROS formation in the mitochondria (Sachse et al. 2007). Some of these effects may be induced by aldosterone and not directly by Ang II. For example, aldosterone has been shown to increase superoxide production and to induce cardiovascular injury in mineralocorticoid-induced hypertensive animals. These effects were blocked by the administration of MR antagonists, as well as by antioxidants and/or NADPH oxidase inhibitors (Beswick et al. 2001; Schiffrin 2006). Moreover, in cultured rat aortic endothelial cells, aldosterone was shown to induce superoxide generation via MR activation of NADPH oxidase (Iwashima et al. 2008). Podocyte injury and proteinuria are enhanced in rats infused with aldosterone. An antioxidant, TEMPOL, and eplerenone, a MR antagonist, significantly reduced oxidative stress markers and prevented podocyte damage and proteinuria (Nagase et al. 2008). Several reports have indicated that aldosterone which causes serine/threonine-protein kinase-1 (SGK1) upregulation, increases ROS generation and podocyte injury (**Figure 1**) (Nishiyama et al. 2004; Nagase et al. 2006). Aldosterone-induced ROS production can activate extracellular signal–regulated kinase 1/2 (ERK 1/2), c-Jun N-terminal kinase (JNK), and big mitogen-activated protein kinase (BMK1) in rat renal cortex and cultured mesangial cells (Nishiyama et al. 2004; Nishiyama et al. 2005).

The renal dopaminergic system can counteract the pro-oxidant effects of the RAAS (Yasunari et al. 2000; Gildea 2009). Recently, it was shown that age-related hypertension in rats may be reversed through the use of exercise which reduces ROS and inflammation in the kidneys from old rats, while simultaneously increasing the abundance of the D_1

receptor (Asghar et al. 2007). Moreover, both D_2 (Armando et al. 2007) and D_5 receptor subtype (Yang et al. 2006) knock-out mice have increased ROS production. D_1-like receptors were found to decrease NADPH oxidase activity and ROS production, mediated via PKA- and PKC-dependent mechanisms in human kidney cells (Yu et al. 2011). In addition, Han et al reported that D_1-like receptors regulate NADPH oxidase activity through the redistribution of NOX2 and subunits ($p22^{phox}$ and Rac1) in membrane microdomains and intracellular vesicles (Han et al. 2008).

In summary, although the cell signaling pathways of the dopamine receptor family are complex and interconnected, they work together to maintain normal blood pressure at least in part by inhibiting RAAS activity and ROS production. The availability of dopamine to activate its specific receptors is determined by factors affecting renal synthesis, mainly the amounts of L-DOPA and sodium delivered to the kidney and the degree of degradation of the amine (Soares-da-Silva et al. 1993). Moreover, the activity of the amino acid transporters that promote L-DOPA uptake in renal epithelial cells is thought to rate-limit the synthesis of renal dopamine.

Epithelial amino acid transporters

Amino acid transporters are responsible for the uptake of amino acids derived from diet in the small intestine, the release into the blood, and subsequent uptake of amino acids from the blood into tissues such as liver or skeletal muscle or the reabsorption of amino acids from the urine along the kidney nephron. In the central

nervous system, amino acid transporters regulate the transport of amino acids across the blood-brain barrier or are involved in the reuptake of neurotransmitter amino acids such as glycine, aspartate, or glutamate from the synaptic cleft and are important for the metabolic coupling of astrocytes and neurons. Other amino acid transporters are involved in basic cellular functions such as cell volume regulation, the synthesis of glutathione (GSH), the provision of amino acids for protein synthesis and energy metabolism.

In the kidney nephron, the proximal tubule is the major site of nutrient reabsorption. About 95–99% of all amino acids are reabsorbed in the proximal convoluted tubule and proximal straight tubule. All kidney cells express some amino acid transporters that are involved mostly in house-keeping functions. Some kidney cells also require additional uptake of amino acids, which are used as precursors for the synthesis of paracrine and/or endocrine substances such as NO (Verrey et al. 2009). As reviewed by Broer (Broer 2008), five transport activities in kidney and intestine were proposed: 1) the "neutral system" transporting all neutral amino acids; 2) the "basic system" transporting cationic amino acids together with cystine; 3) the "acidic system" transporting glutamate and aspartate; 4) the "iminoglycine system" transporting proline, hydroxyproline, and glycine; and 5) the β-amino acid system. The epithelial amino acid transport systems and their mediators are summarized in **Table 1**.

Table 1–Epithelial amino acid transport systems and their mediators (Broer 2008).

System	cDNA	SLC	Amino Acid Substrates	Analogs	Affinity	Mechanism	Ions	Expression*
A	SNAT2	SLC38A2	G,P,A,S,C,Q,N,H,M	MeAIB	Medium	S	Na^+	Ub
	SNAT4	SLC38A4	G,A,S,C,Q,N,M,AA^+	MeAIB	Medium	S	Na^+	K
ASC	ASCT1	SLC1A4	A,S,C	Cysteic acid	High	A	Na^+	K
	ASCT2	SLC1A5	A,S,C,T,Q		High	A	Na^+	K,I (AM)
asc	4F2 hc/asc1	SLC3A2/SLC7A10	G,A,S,C,T	D-AA^0	High	A		K
B^0	B^0AT1	SLC6A19	AA^0	BCH	Low	S	Na^+	K,I (AM)
	B^0AT2	SLC6A15	P,L,V,I,M	BCH	High	S	Na^+	K
$B^{0,+}$	$ATB^{0,+}$	SLC6A14	AA^0, AA^+, β-Ala	BCH	High	S	Na^+, Cl^-	I (AM)
$b^{0,+}$	rBAT/$b^{0,+}$AT	SLC3A1/SLC7A9	R,K,O,cystine		High	A		K,I (AM)
β	TauT	SLC6A6	Tau, β-Ala		High	S	Na^+, Cl^-	K (AM,BM)
Gly	XT2	SLC6A18	G		NR	NR	NR	K (AM)
IMINO	IMINO	SLC6A20	P, HO-P	MeAIB	Medium	S	Na^+, Cl^-	K,I (AM)
L	4F2hc/LAT1	SLC3A2/SLC7A5	H,M,L,I,V,F,Y,W	BCH	High	A		
	4F2hc/LAT2	SLC3A2/SLC7A8	AA^0 except P	BCH	Medium	A		K,I (BM)
	LAT3	SLC43A1	L,I,M,F	BCH	Low	U		K
	LAT4	SLC43A2	L,I,M,F	BCH	Low	U		
N	SNAT3	SLC38A3	Q,N,H		Low	S	Na^+(S), H^+(A)	K (BM)
	SNAT5	SLC38A5	Q,N,H,S,G		Low	S	Na^+(S), H^+(A)	K
PAT (Imino acid)	PAT1	SLC36A1	P,G,A GABA, β-Ala	MeAIB	Low	S	H^+	K,I (AM)
	PAT2	SLC36A2	P,G,A	MeAIB	Medium	S	H^+	K
T	TAT1	SLC16A10	F,Y,W		Low	U		K,I (BM)
X^-_{AG}	EAAT2	SLC1A2	E,D	D-Asp	High	S	Na^+,H^+(S), K^+(A)	K (BM)
	EAAT3	SLC1A1	E,D	D-Asp	High	S	Na^+,H^+(S), K^+(A)	K,I (AM)
x^-_c	4F2 hc/xCT	SLC3A2/SLC7A11	E, cystine$^-$		High	A		Ub
y^+	CAT-1	SLC7A1	R,K,O,H		Medium	U		Ub
y^+L	4F2hc/y^+LAT1	SLC3A2/SLC7A7	K,R,Q,H,M,L		High	A	Na^+(S-AA^0)	K,I (BM)
	4F2hc/y^+LAT2	SLC3A2/SLC7A6	K,R,Q,H,M,L,A,C		High	A	Na^+(S-AA^0)	K,I (BM)

NR, not reported; A, antiport; AA^0, neutral amino acids; AA^+, cationic amino acids; U, uniport; S, symport; S-AA^0, symport together with neutral amino acids; K, kidney; I, intestine; AM, apical membrane; BM, basolateral membrane; Ub, ubiquitous. Amino acids are given in one-letter codes. O, ornithine; HO-P, hydroxyproline. Affinity: high, <100 µM; medium, 100 µM to 1 mM; low, >1 mM. * Expression in epithelial cells of kidney and intestine.

Luminal amino acid transporters of the proximal tubule

Neutral amino acids represent more than 80% of the free plasma amino acids and are all transported by the luminal B^0AT1 transporter (SLC6A19) (**Figure 3**), though with different apparent affinities (Broer et al. 2004; Verrey et al. 2009). This neutral amino acid cotransporter with broad selectivity is expressed in the luminal brush border membrane of the early segments of the kidney proximal tubule and similarly along the small intestine

(Romeo et al. 2006). Expressed in *Xenopus* oocytes, the B^0AT1 cDNA induces a sodium-dependent, chloride-independent uptake of neutral amino acids with an affinity for L-leucine uptake (Broer et al. 2004). Its defect has been shown to cause Hartnup disorder, an autosomal recessive condition which is characterized by a urinary loss of neutral amino acids (Kleta et al. 2004). Another member of the same SLC6 amino acid transporter cluster, SLC6A20 in human, has been identified as the molecular correlate of system IMINO, mediating the cotransport of L-proline (Kowalczuk et al. 2005; Takanaga et al. 2005). This transporter called SIT1 or $IMINO^B$ was shown to localize to the brush border membrane of the proximal tubule in mice by immunofluorescence (Romeo et al. 2006). As expected for system IMINO, it also transports hydroxy-L-proline and betaine but does not transport glycine. SIT1/$IMINO^B$ appears to require, as B^0AT1 and B^0AT3, the association with collectrin for its surface expression in kidney. This is suggested by the low brush border membrane expression of SIT1/$IMINO^B$ in collectrin knock-out mice and by the large urinary L-proline loss in these animals (Malakauskas et al. 2007). Collectrin (Tmem27) is a relatively short type I transmembrane protein (25 kDa) that is approximately 40% identical with the membrane anchor region of the RAS enzyme ACE2 (Danilczyk et al. 2006; Malakauskas et al. 2007).

Cationic amino acids and the disulfide-linked L-cysteine dimmer enter the epithelial cells via the cystinuria transporter ($b^{0,+}AT$) (**Figure 3**). This transporter is made of a catalytic subunit belonging to the SLC7 family and a disulfide linked accessory subunit referred to as heavy chain and called rBAT (SLC3A1) (Broer 2008). Similarly to the

basolateral heterodimeric exchangers of the SLC7/SLC3 family, it functions as an obligatory antiporter and specifically exchanges its cationic substrates or L-cystine against intracellular neutral amino acids. $b^{0,1}$AT mRNA is expressed in kidney and small intestine, and, to a smaller extent, in heart, liver, placenta, and lung. rBAT protein was found in the apical membrane of the renal proximal tubule, increasing from the S1 to the S3 segment and in the microvilli of the small intestine. On the other hand, although $b^{0,1}$AT protein is also expressed in the apical membrane of the proximal tubule, expression levels decrease from the S1 to the S3 segment (Wagner et al. 2001).

The high-affinity transporter for anionic amino acids, referred to as EAAT3 (SLC1A1) (**Figure 3**), is expressed in the intestine and kidney, as well as in the brain (Kanai et al. 1992; Hediger 1999). It is localized in the proximal tubule brush border membrane with an axial gradient: low amounts in S1 and highest levels in the later segments S2 and S3 (Hediger 1999). This transporter has been shown to cotransport its substrates with three sodium ions and one proton in exchange for one potassium ion (Zerangue et al. 1996). Unlike the SLC6 and SLC7 transporters no SLC1 associated transmembrane protein has been identified.

ASCT2 (SLC1A5) is a sodium -dependent exchanger of neutral amino acids that belongs to the SLC1 family and has been shown to be expressed at the mRNA level in kidney. ASCT transporters are sodium-dependent obligatory exchangers of amino acids (in particular alanine, serine, cysteine, and threonine) that are structurally related to the EAAT transporters. ASCT2 belongs to a restricted group of transporters that share

specificity for glutamine, since glutamine is the major precursor of urinary ammonia, thus playing a key role in acid-base homeostasis (Utsunomiya-Tate et al. 1996; Avissar et al. 2001).

Basolateral amino acid transporters of the proximal tubule

The best characterized basolateral transporters of the proximal tubule amino acid reabsorption machinery function as obligatory exchangers (System L and system y^+L) (**Figure 3**) and thus do not perform net basolateral amino acids export (Verrey et al. 2005). System L conveys the sodium-independent transport of large branched and aromatic neutral amino acids in almost all types of cells (Wagner et al. 2001). The first isoform of system L, LAT1 (SLC7A5), preferentially mediates the sodium-independent transport of large neutral amino acids such as leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, methionine and histidine and is a major route for providing tumour cells with branched and aromatic amino acids (Kanai et al. 1998). LAT1 is widely expressed in nonepithelial cells such as brain, spleen, thymus, testis, skin, liver, placenta, skeletal muscle, and stomach and has a high affinity for amino acid substrates (Kanai et al. 1998; Prasad et al. 1999). Recent studies have demonstrated that LAT1 is a major L-type amino acid transporter in a variety of cancer cells including hepatic, oral, breast, bladder and colon (Storey et al. 2005). Although the transport of leucine by LAT1 in pig LLC-PK1 renal cells has been previously described (Soares-da-Silva et al. 2004), LAT1 has a very limited tissue distribution in the kidney (Pinho et al. 2007). The heavy subunit 4F2hc brings LAT1 to the plasma membrane. In the absence of 4F2hc, LAT1 is found in intracellular

compartments, whereas 4F2hc can reach the plasma membrane independently (Nakamura et al. 1999). Moreover, 4F2hc interacts with several light chains to form system L (with LAT1 and LAT2), system y^+L (with y^+LAT1 and y^+LAT2), system X_c^- (with xCT), or system asc (with asc1) (Wagner et al. 2001).

LAT2 (SLC7A8) is highly expressed in polarized epithelia (Segawa et al. 1999), suggesting an important role in transepithelial amino acid transport, but it has a lower affinity for amino acid substrates than LAT1 (Segawa et al. 1999; Wagner et al. 2001). LAT2 is a major sodium-independent amino acid transporter and its functionality is dependent on the abundance of 4F2hc. The heterodimerization of LAT2 with 4F2hc is a prerequisite for the transporter to reach the cell surface. Moreover, 4F2hc may regulate the functional surface expression of the transporter (Nakamura et al. 1999; Pineda et al. 1999). Expression of LAT2 and 4F2hc induces amino acid transport with characteristics of system L, namely, sodium-independent transport of neutral amino acids sensitive to classic system L inhibitors (Pineda et al. 1999; Segawa et al. 1999). LAT2 also transports small neutral amino acids such as L-alanine, L-glycine, L-cysteine, and L-serine and glutamine, all of which are poor substrates for LAT1 (Pineda et al. 1999; Segawa et al. 1999; Wagner et al. 2001).

System y^+L was first functionally described in erythrocytes (Deves et al. 1992). However, its presence has also been revealed in placenta, liver, small intestine and kidney (Desjeux et al. 1980; Rajantie et al. 1981; Eleno et al. 1994; Furesz et al. 1997). It has an axial distribution along the kidney proximal tubule and small intestine similar to that of

LAT2-4F2hc (Bauch et al. 2003; Dave et al. 2004). y^+LAT1 (SLC7A7) plays an important role for the transepithelial transport of its cationic substrates which it transports out of the cells in exchange for extracellular neutral amino acids and sodium (Torrents et al. 1998; Pfeiffer et al. 1999). The functional importance of this electroneutral exchange for transcellular cationic amino acid transport is demonstrated by the disease, lysinuric protein intolerance, that is caused by mutations of the y^+LAT1 gene which is characterized by the urinary loss of L-arginine, L-ornithine, and L-lysine and by a poor intestinal absorption of these amino acids (Borsani et al. 1999; Torrents et al. 1999). This leads to low plasma concentrations of these amino acids and to an impaired function of the urea cycle and hyperammonemia (Tanner et al. 2007).

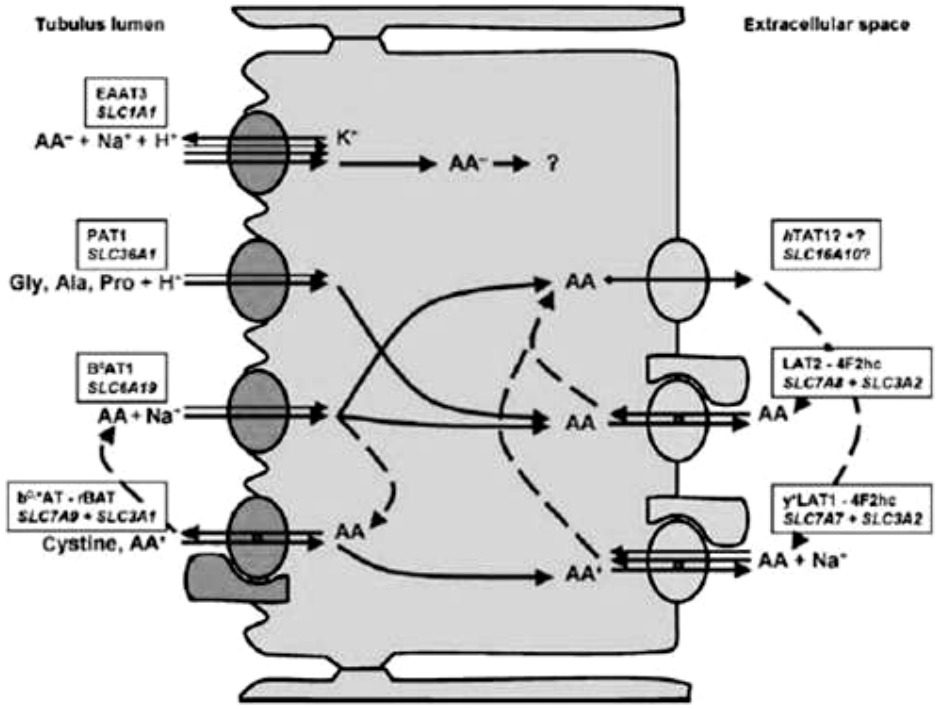

Figure 3–Luminal and basolateral transporters belonging to the amino acid absorption/reabsorption machinery. Names of transporters and of the corresponding genes are indicated in rectangles. Recycling pathways are shown by dotted lines. AA, neutral amino acids; AA+, cationic amino acids; AA– anionic amino acids [Adapted from (Verrey et al. 2005)].

Modulation of amino acid transporters by oxidative stress

Although the mechanisms responsible for the regulation of amino acid transporters are not fully understood, several factors have been shown to regulate their expression and/or activity. These factors include osmotic shock, growth factors, peptide hormones and protein phosphorylation (Fleck et al. 2003). Several evidence points towards the possible regulation of amino acid transport by oxidative stress. Oxidative stress has been found to stimulate the activity of NMDA receptors (Agostinho et al. 1995) and the calcium-independent, carrier-mediated release of glutamate and [^{3}H]-D-aspartate

(Rego et al. 1996) from cultured retina cells. On the other hand, oxidative stress conditions induced by ascorbate/Fe^{2+} reduces significantly the activity of the excitatory amino acid (EAA) transporter, mainly by protein oxidation (Agostinho et al. 1997).

The cystine/glutamate exchange system (X_c^-) is another amino acid transport system that is regulated by oxidative stress. System X_c^- transports one molecule of cystine, the oxidized form of cysteine, into cells and thereby releases one molecule of glutamate into the extracellular space. Cystine, a disulfide formed between two cysteine molecules, is the predominant form in the extracellular space, whereas cysteine is the prevailing form in cells due to highly reducing conditions. These amino acids are important not only as precursors for protein and antioxidant glutathione (GSH) biosynthesis, but also for the maintenance of physiological redox conditions inside/outside of the cell (Conrad et al. 2011). One of most important features of system X_c^- is that its activity is highly inducible by various stimuli, including electrophilic agents like diethyl maleate, cystine deprivation in the culture medium, oxidized low density lipoprotein and hydrogen peroxide (Conrad et al. 2011).

Peroxynitrite generated from the reaction of nitric oxide with superoxide anion leads to alterations in cell signaling and function via the modification of cellular lipids and protein thiols (Beckman 1996). In bovine aortic endothelial cells, 3-morpholinosydnonimine (SIN-1), a donor of nitric oxide and peroxynitrite, caused an increase in L-cystine transport and intracellular glutathione (GSH) levels. Induction of L-cystine transport in both aortic endothelial and smooth muscle cells in response to SIN-1

was abolished by inhibitors of protein and RNA synthesis. It has been suggested that under conditions of overproduction of NO or peroxynitrite in inflammation, endothelial cells can adapt by increasing system X_c^- transport activity and their GSH levels to counteract NO-mediated changes in cellular thiols (Li et al. 1999).

More recently, exposure of Caco-2 cells to the S-nitrosothiol type nitric oxide donor, SNAP, was found to increase sodium-dependent alanine uptake mediated by ASCT2 (Uchiyama et al. 2005). Peroxynitrite stimulated sodium-dependent alanine transport, and the NADPH oxidase inhibitor DPI, and superoxide dismutase, partially inhibited SNAP induced sodium-dependent alanine transport which suggested that NO-related radicals as well as NO itself might be responsible for stimulating ASCT2 (Uchiyama et al. 2005).

AIM

The broad objective of this thesis is to gain further insight into the role of oxidative stress on the renal expression and regulation of the amino acid transporters potentially involved in the uptake of L-DOPA in hypertension and aging.

The following questions were raised:

CHAPTER I: Is the function and expression of sodium-dependent ASCT2 altered in hypertension? Does intracellular H_2O_2 play a role in the regulation of ASCT2 in immortalized renal proximal tubular epithelial (PTE) cells from WKY and SHR? Can H_2O_2 modulate the activity of other transporters, such as NHE in PTE cells?

CHAPTER II: What are the effects of aging on the renal oxidative stress status in WKY and SHR?

CHAPTER III: How does aging affect the renal dopaminergic system and the regulation of LAT1, LAT2, 4F2hc, and ASCT2 in WKY and SHR?

CHAPTER IV: Do age-related changes in the renal expression of amino acid transporters potentially involved in L-DOPA uptake parallel changes in the activation of the renal aldosterone/MR system?

CHAPTER I - H_2O_2 and the regulation of renal ASCT2 in hypertension

Pinho MJ, ***Pinto V****, Serrão MP, Jose PA, Soares-da-Silva P (2007) Underexpression of the* Na^+*-dependent neutral amino acid transporter ASCT2 in the spontaneously hypertensive rat kidney. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 293 (1):R538-547*

Pinto V*, Pinho MJ, Jose PA, Soares-da-Silva P (2010) Role of* H_2O_2 *on the kinetics of low-affinity high-capacity* Na^+*-dependent alanine transport in SHR proximal tubular epithelial cells. Biochemical and Biophysical Research Communications 398 (3):553-558*

Pinto V*, Pinho MJ, Hopfer U, Jose PA, Soares-da-Silva P (2008) Oxidative stress and the genomic regulation of aldosterone-stimulated NHE1 activity in SHR renal proximal tubular cells. Molecular and Cellular Biochemistry 310 (1-2):191-201*

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Underexpression of the Na^+-dependent neutral amino acid transporter ASCT2 in the spontaneously hypertensive rat kidney

Maria João Pinho,[1] Vanda Pinto,[1] Maria Paula Serrão,[1] Pedro A. Jose,[2] and Patrício Soares-da-Silva[1]

[1]*Institute of Pharmacology and Therapeutics, Faculty of Medicine, Porto, Portugal;*
and [2]*Department of Pediatrics, Georgetown University, Washington, District of Columbia*

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Pinho MJ, Pinto V, Serrão MP, Jose PA, Soares-da-Silva P. Underexpression of the Na^+-dependent neutral amino acid transporter ASCT2 in the spontaneously hypertensive rat kidney. *Am J Physiol Regul Integr Comp Physiol* 293: R538–R547, 2007. First published May 2, 2007; doi:10.1152/ajpregu.00906.2006.—This study examined the inward transport of L-[^{14}C]alanine, an ASCT2 preferential substrate, in monolayers of immortalized renal proximal tubular epithelial (PTE) cells from Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats. The expression of ASCT2 in WKY and SHR PTE cells and kidney cortices from WKY and SHR was also evaluated. L-[^{14}C]alanine uptake was highly dependent on extracellular Na^+. Replacement of NaCl by LiCl or choline chloride abolished transport activity in SHR and WKY PTE cells. In the presence of the system L inhibitor BCH, Na^+-dependent L-alanine uptake in WKY and SHR PTE cells was inhibited by alanine, serine, and cysteine, which is consistent with amino acid transport through ASCT2. The saturable component of Na^+-dependent L-alanine transport under V_{max} conditions in SHR PTE cells was one-half of that in WKY PTE cells, with similar K_m values. Differences in magnitude of Na^+-dependent L-alanine uptake through ASCT2 between WKY and SHR PTE cells correlated positively with differences in ASCT2 protein expression, this being more abundant in WKY PTE cells. Abundance of ASCT2 transcript and protein in kidney cortices of SHR rats was also lower than that in normotensive WKY rats. In conclusion, immortalized SHR and WKY PTE cells take up L-alanine mainly through a high-affinity Na^+-dependent amino acid transporter, with functional features of ASCT2 transport. The activity and expression of the ASCT2 transporter were considerably lower in the SHR cells.

L-alanine transport; hypertension; alanine-serine-cysteine-threonine transporter-2

TRANSPORT OF NEUTRAL amino acids across membranes of mammalian cells proceeds through a variety of different transport systems (reviewed in Refs. 4, 9, and 18). At the level of the kidney and small intestine epithelia, distinct transporters are located in the apical and basolateral membranes to ensure the vectorial transport of amino acids across the epithelial cells (13). Recently, several amino acid transporters have been identified and shown to play a role in the cellular uptake and/or basolateral extrusion of neutral amino acids. Indeed, it has been proposed (13) that neutral amino acids are absorbed from the luminal fluid via Na^+-dependent systems, like the proline transporter IMINO/SIT (SLC6A20) (20, 37), the neutral amino acid exchanger ASCT2 (SLC1A5) (16), or the broad specific neutral amino acid transporter B^0AT1 (SLC6A19), whose molecular structure has been identified recently (7). The exit path for neutral amino acids to the blood stream is supposed to proceed through the system L. In kidney and small intestine epithelial cells, type-2 L-amino acid transport (LAT2), together with 4F2hc, was found to be present in basolateral membrane, which is well suited for the exit path of intracellular amino acids (28, 32). System y^+L, most likely y^+LAT1 (38, 45), located at the basolateral membrane, is expected to mediate the obligatory exchange of intracellular basic amino acids against extracellular neutral amino acids, cotransported with sodium ions (27). Because these two transport systems function as obligatory exchangers, they cannot contribute to the net transepithelial transport of amino acids but are thought to play a role in extending the transport selectivity of putative parallel functioning by unidirectional transporters.

System ASC transport activity is ubiquitous and characterized by its preference for small neutral amino acids including alanine, serine, and cysteine. The system ASC of neutral amino acid transporters (SLC1A4 and SLC1A5) belongs to the solute carrier family-1 (SLC1), which also includes the high-affinity glutamate transporters (13, 14, 40, 46). Human ATB^0 was identified by RT-PCR and enzymatic restriction analysis in the human proximal tubule cell line HKPT (17) and corresponds to rodent ASCT2. The two ASC transporters exhibit distinct substrate selectivity. SLC1A4 encodes the Na^+-dependent amino acid transporter ASCT1, which accepts L-alanine, L-serine, L-theonine, and L-cysteine in a stereospecific manner. ASCT2, the second isoform of the ASC transport system, is encoded by SLC1A5. In the kidney and intestine, ASCT2 is present in the brush-border membranes of the proximal tubule cells and enterocytes, respectively (3). In addition to the typical system ASC substrates, it also accepts L-glutamine and L-asparagine at higher affinity as well as methionine, leucine, and glycine with lower affinity. Both ASCT1 and ASCT2 mediate the Na^+-dependent obligatory exchange of substrate amino acids (5, 38, 46).

We previously reported that overexpression of Na^+-independent LAT2 in the spontaneously hypertensive rat (SHR) kidney is organ specific and precedes the onset of hypertension. This overexpression is accompanied by an enhanced ability to take up L-3,4-dihydroxyphenylalanine (L-DOPA) (29). These observations formed the basis for the hypothesis that overexpression of renal LAT2 leads to enhanced renal production of dopamine in the SHR in an attempt to compensate for the decreased dopamine-mediated natriuresis generally observed in this genetic model of hypertension. Furthermore, we have demonstrated that immortalized renal proximal tubular epithelial (PTE) cells from Wistar-Kyoto rats (WKY) and

Address for reprint requests and other correspondence: P. Soares-da-Silva, Institute of Pharmacology and Therapeutics, Faculty of Medicine, 4200 Porto, Portugal (e-mail: pss@med.up.pt).

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SHR transport L-DOPA quite efficiently through the apical cell border, in a Na^+-independent manner (30). LAT2 was almost exclusively responsible for L-DOPA transport in WKY cells, whereas in SHR cells, 25% of L-DOPA uptake was through a Na^+-dependent system, 25% through LAT2, and the remaining 50% through LAT1. Differences in L-DOPA handling between SHR and WKY cells may result from the overexpression of LAT1 and LAT2 transporters in the former (30).

In an attempt to understand better differences in the handling of L-amino acids in hypertension, the present study examined the function and expression of ASCT2. The inward transport of L-[^{14}C]alanine, an ASCT2 preferential substrate, was evaluated in monolayers of immortalized renal PTE cells from the SHR and its normotensive control, WKY. The quantification of ASCT2 mRNA and ASCT2 protein was performed in immortalized renal PTE cells and kidney cortices from WKY and SHR.

METHODS AND MATERIALS

Cell culture. Immortalized renal PTE cells from WKY and SHR (44) were maintained in a humidified atmosphere of 5% CO_2-95% air at 37°C. SHR and WKY PTE cells were grown in DMEM Nutrient Mixture-Ham's F-12 (Sigma, St. Louis, MO) supplemented with 100 U/ml penicillin G, 0.25 μg/ml amphotericin B, 100 μg/ml streptomycin (Sigma), 5% fetal bovine serum (Sigma), and 25 mM HEPES (Sigma). For subculturing, the cells were dissociated with 0.10% trypsin-EDTA, split 1:4, and subcultured in Costar flasks with 75- or 162-cm^2 growth areas (Costar, Badhoevedorp, The Netherlands). The cell medium was changed every 2 days, and the cells reached confluence after 3–5 days of incubation. For 24 h before each experiment, the cells were maintained in fetal bovine serum-free medium. Experiments were generally performed 2–3 days after cells reached confluence and 6–8 days after the initial seeding; each squared centimeter contained ~80–100 μg of cell protein.

Uptake of L-amino acids. Flux measurements in immortalized renal PTE cells from the WKY and SHR were performed as previously described (30). Briefly, on the day of the experiment, growth medium was aspirated, and the cell monolayers were preincubated for 15 min in Hanks' medium at 37°C. The Hanks' medium had the following composition (in mM): NaCl 137, KCl 5, $MgSO_4$ 0.8, Na_2HPO_4 0.33, KH_2PO_4 0.44, $CaCl_2$ 0.25, $MgCl_2$ 1.0, Tris·HCl 0.15, and sodium butyrate 1.0, pH = 7.4. Uptake was initiated by the addition of 1 ml of Hanks' medium with a given concentration of the substrate. Time course studies were performed in experiments in which cells were incubated with 0.25 μM L-[^{14}C]alanine for 1, 3, 6, 12, 30, and 60 min. Saturation experiments were performed in cells incubated for 6 min with 0.25 μM radiolabeled amino acid in the absence and in the presence of increasing concentrations of the unlabeled substrate. To achieve Na^+-free and Cl^--free conditions, NaCl was replaced by LiCl or sodium gluconate (NaGlu). In experiments performed to determine the Na^+ dependence of transport, sodium chloride was replaced by an equimolar concentration of choline chloride. To determine whether L-[^{14}C]alanine transport is an electrogenic process, cells were depolarized by the addition of 50 mM KCl or NH_4Cl (5); in these experiments, 100 mM sucrose added to Hanks' balancing for the increased osmolarity represented the control situation. In inhibition studies, test substances were applied from the apical side and were present during the incubation period only. During preincubation and incubation, the cells were continuously shaken and maintained at 37°C. Uptake was terminated by the rapid removal of uptake solution by means of a vacuum pump connected to a Pasteur pipette followed by a rapid wash with cold Hanks' medium. Subsequently, cells were solubilized by 0.1% vol/vol Triton X-100 (dissolved in 5 mM Tris·HCl, pH 7.4), and radioactivity was measured by liquid scintillation counting.

Immunoblotting. Cell monolayers and renal cortical membranes from the WKY and SHR were washed with PBS and then lysed in RIPA buffer containing 150 mM NaCl, 50 mM Tris·HCl, pH 7.4, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 2 μg/ml leupeptin, and 2 μg/ml aprotinin. Protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin as standard. Cell lysates were boiled in sample buffer (35 mM Tris·HCl, pH 6.8, 4% SDS, 9.3% dithiothreitol, 0.01% bromophenol blue, 30% glycerol) at 95°C for 5 min. Samples containing 60 μg of protein were separated by SDS-PAGE with 10% polyacrylamide gel and then electroblotted onto nitrocellulose membranes (Bio-Rad). Blots were blocked for 1 h with 5% nonfat dry milk in PBS (10 mmol/l PBS) at room temperature with constant shaking. Blots were then incubated with anti-ASCT2 polyclonal antibody (1:800; Chemicon International) in 5% nonfat dry milk in PBS-T (0.01% Tween 20-PBS) overnight at 4°C. The immunoblots were subsequently washed and incubated with fluorescently labeled goat anti-rabbit (1:10,000; IRDye 800, Rockland) or the fluorescently labeled goat anti-mouse secondary antibody (1:5,000; AlexaFluor 680, Molecular Probes) for 60 min at room temperature and protected from light. The membrane was washed and imaged by scanning at both 700 and 800 nm with an Odyssey Infrared Imaging System (LI-COR Biosciences).

RT-PCR. One microgram of total RNA was reverse transcribed to cDNA with SuperScript First Strand Synthesis System for RT-PCR (Invitrogen) according to manufacturer's instructions. The reverse transcription was performed at 50°C and with the use of 5 μg/μl random hexamers. The ASCT2 cDNA was amplified by PCR using the following set of rat-specific primers: forward 5′-GCC TGA TCG GAG GTG CAG CC-3′ and reverse 5′-CGG GTA AAG AGG AAG TAG ATG-3′, corresponding to nucleotides 334 and 983 of the rat cDNA (GenBank accession no. AJ132846). The $B^{0,+}$ cDNA was amplified by PCR using the following set of primers: forward 5′-AAC AGT ATT GGG ATA AAG TGA-3′ and reverse 5′-TAA TGG CAT CAG AGT AAC AG-3′, corresponding to nucleotides 755 and 1136 of the rat $B^{0,+}$ mRNA sequence (GenBank accession no. NM_001037544). PCR was performed with Platinum TaqPCRx DNA Polymerase (Invitrogen). Amplification conditions were as follows: hot start of 2 min at 94°C; 30 cycles of denaturing (94°C for 30s), annealing (55°C for 30 s), and extension (72°C for 45 s); and a final extension of 7 min at 72°C. The PCR products were separated by electrophoresis in a 2% agarose gel and visualized under UV light in the presence of ethidium bromide.

Real-time PCR quantification of rat ASCT2. Kidney cortices from WKY and SHR (4 and 12 wk of age) and immortalized renal PTE WKY and SHR cells were homogenized (Diax, Heidolph) in Trizol reagent (75 mg/ml; Invitrogen), and total RNA was extracted according to the manufacturer's instructions. All animal interventions were performed in accordance with the European Directive no. 86/609 and the rules of the *Guide for the Care and Use of Laboratory Animals*, 7th ed, Washington, DC. Institue for Laboratory Animal Research (ILAR), 1996. The RNA preparation was further treated with DNase (Ambion), to eliminate potential genomic DNA contamination. Reverse transcription was performed with SuperScript First Strand System for RT-PCR (Invitrogen), using 5 μg/μl random hexamers as primers at 50°C, according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA in a total volume of 20 μl. Standards for ASCT2 and GAPDH were obtained by conventional PCR amplification, using Platinum TaqPCRx DNA Polymerase (Life Technologies) and the following rat-specific primers: rASCT2 forward primer 5′-CGT CCT CAC TCT TGC CAT CAT-3′ and reverse primer 5′-CCA AAA GCA TCA CCC TCC AC-3′ (nucleotide positions 1298 and 1427 in rat ASCT2 sequence NM_175758); rGAPDH forward primer 5′-GGC ATC GTG GAA GGG CTC ATG AC-3′ and reverse primer 5′-ATG CCA GTG AGC TTC CCG TTC AGC-3′

(nucleotide positions 1348 and 1512 in rat GAPDH sequence M17701). PCR products were gel purified with Qiaex II (Qiagen), quantified by spectrophotometry at 260 nm, and further diluted accordingly in serial steps. All PCR fragments were cloned and sequenced. Real-time PCR was carried out using a LightCycler (Roche, Mannheim, Germany). Each RT-PCR reaction mixture (50 μl) included reverse transcription products corresponding to 50 ng of total RNA or standard DNA, 1× SYBR Green I master mix (LightCycler FastStart DNA Master^{PLUS} SYBR Green I, Roche), and 0.5 μM each forward and reverse primer, mentioned above. Cycling conditions were as follows: denaturation (95°C for 1 min), amplification and quantification (95°C for 10 s, 60°C for ASCT2 and 62°C for GAPDH for 10 s, and 72°C for 5 s, with a single fluorescence measurement at the end of the 72°C for 5 s segment) repeated 35 times, a melting curve program (65–95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement), and a cooling step to 40°C. Amplification specificity was checked using melting curves, following the manufacturer's instructions. In addition, PCR products were separated by electrophoresis in a 2% TBE agarose gel to confirm that correct band sizes were obtained. Target mRNAs were quantified by measuring the threshold cycle (when fluorescence is statistically significantly above background) and reading against a calibration curve. Results were analyzed with LightCycler Software v.3.5 (Roche Applied Science, Mannheim, Germany) using the second derivate maximum method. The relative amount of each mRNA was normalized to the housekeeping gene (GAPDH) mRNA. Each sample was tested in duplicate.

Drugs. L- and D-Amino acids, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), and *N*-(methylamino)-isobutyric acid were purchased from Sigma Chemical (St. Louis, MO). L-[^{14}C]alanine (specific activity 152 mCi/mmol) was purchased from Amersham Pharmacia Biotech (Little Chalfont, UK).

Data analysis. K_m and maximum velocity (V_{max}) values for the uptake of L-[^{14}C]alanine were determined from a competitive uptake inhibition protocol (10) and calculated from nonlinear regression analysis using the GraphPad Prism statistics software package (21). For calculation of the IC_{50}, the parameters of the equation for one-site inhibition were fitted to the experimental data (21). Arithmetic means are given with SE. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons. A P value < 0.05 was assumed to denote a significant difference.

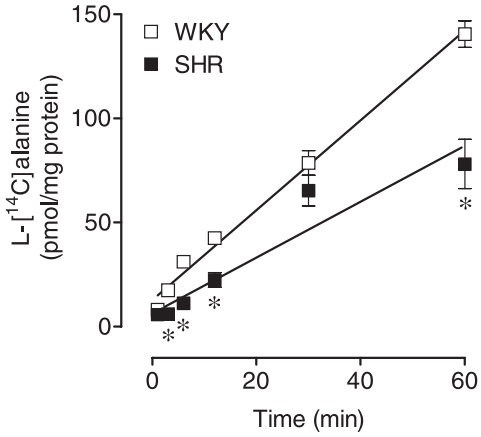

Fig. 1. Time course of L-[^{14}C]alanine transport in immortalized Wistar-Kyoto (WKY) and spontaneously hypertensive rat (SHR) proximal tubular epithelial (PTE) cells. Values represent means of 4–6 experiments per group, and error bars show SE. Significantly different from values for WKY PTE cells (*$P < 0.05$).

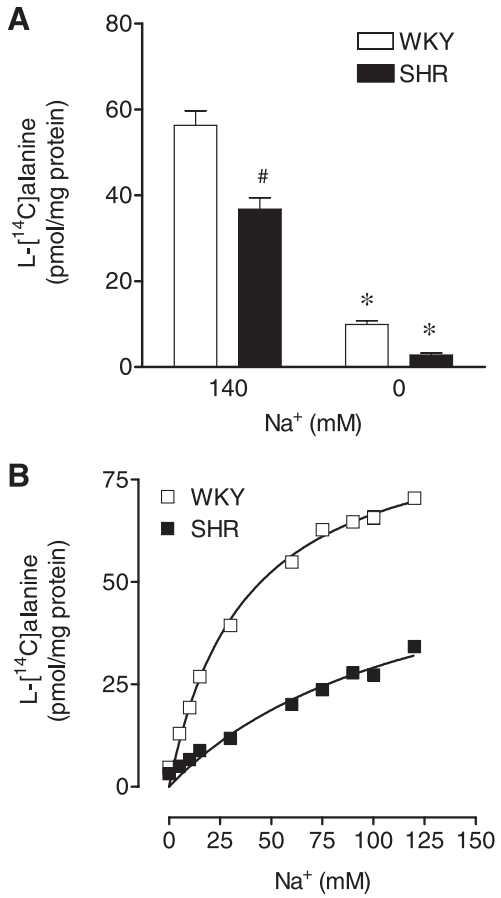

Fig. 2. *A*: effect of sodium chloride replacement by an equimolar concentration of choline chloride on 0.25 μM [L-^{14}C]alanine transport. Columns represent the mean of 4 experiments per group; vertical lines show SE. *B*: [L-^{14}C]alanine transport as a function of extracellular sodium concentration in immortalized WKY and SHR PTE cells. Symbols represent means of 4 experiments per group; error bars show SE. Significantly different from corresponding control values (*$P < 0.05$) and corresponding values for WKY PTE cells (#$P < 0.05$).

RESULTS

Inward transfer of L-alanine. To determine the initial rates of uptake, SHR and WKY PTE cells were incubated with a nonsaturating (0.25 μM) concentration of L-[^{14}C]alanine for 1, 3, 6, 12, 30, and 60 min. In both types of cells, uptake of nonsaturating concentration of L-[^{14}C]alanine was linear with time up to 60 min of incubation (Fig. 1). As depicted in Fig. 1, the initial rate for L-[^{14}C]alanine uptake was significantly lower in SHR than in WKY PTE cells.

Since transfer of neutral amino acids across the plasma membrane can be mediated by both Na^+-dependent and Na^+-independent transport systems, a set of experiments was performed replacing NaCl with an equimolar concentration of choline chloride to determine a potential Na^+ dependence of L-[^{14}C]alanine apical uptake. Na^+ removal from the uptake solution almost completely abolished transport activity in both SHR and WKY PTE cells (Fig. 2*A*). Complete Na^+ activation curves are shown in Fig. 2*B*. Linearization of data according to the Hill equation yielded a Hill coefficient of 0.98 and 1.0 for SHR and WKY PTE cells, respectively. A more detailed analysis of the Na^+-dependent L-[^{14}C]alanine uptake using

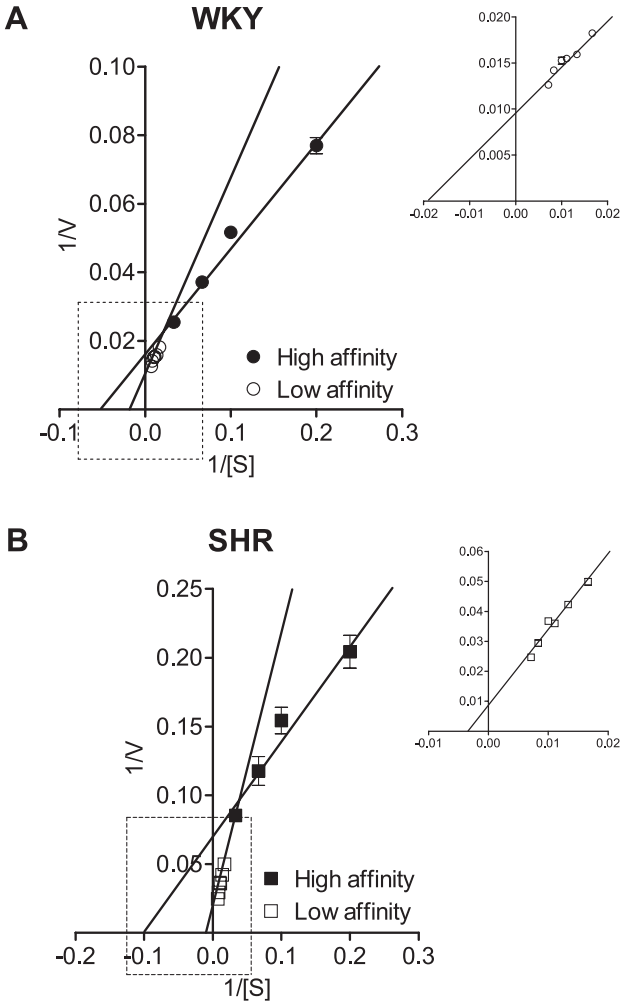

Fig. 3. Lineweaver-Burk reciprocal plots of velocity (V) and substrate concentrations ([S]) are presented for L-[^{14}C]alanine transport as a function of extracellular sodium concentration in immortalized WKY (*A*) and SHR (*B*) PTE cells. Lines were derived from a weighted least squares analysis of the data points. *Inset*: Lineweaver-Burk reciprocal plots of velocity and substrate for the low-affinity component.

Lineweaver-Burk plots revealed the presence of high- and low-affinity uptake processes in both WKY and SHR PTE cells (Fig. 3, *A* and *B*). The kinetic parameters (K_m and V_{max}) for the high- and low-affinity Na^+-dependent L-[^{14}C]alanine uptake in WKY and SHR PTE cells are given in Table 1. The effect of BCH on high- and low-affinity Na^+-dependent L-[^{14}C]alanine uptake was also evaluated. In the WKY PTE cells, BCH induced a decrease in the affinity for Na^+ without changes in V_{max} values, in both low- and high-affinity components, thus behaving as a competitive inhibitor (Table 1). In the SHR PTE cells, the effects of BCH on K_m and V_{max} for the high- and low-affinity components of Na^+-dependent L-[^{14}C]alanine uptake did not attain a statistical significance (Table 1).

The replacement of NaCl by LiCl reduced the transport activity by ~90% in both SHR and WKY PTE cells (Fig. 4*A*). Replacing NaCl with NaGlu produced a significant reduction in L-alanine uptake in both WKY and SHR PTE cells, this being more marked in the latter (Fig. 4*A*). The addition of 50 mM KCl, but not of 50 mM NH_4Cl, to the uptake solution, a manipulation that reduces cell membrane potential, resulted in a slight but statistically significant ($P < 0.05$) reduction (~13% decrease) in L-[^{14}C]alanine uptake (Fig. 4*B*), suggesting that alanine uptake occurs mainly through nonelectrogenic transporters.

In the presence of extracellular 140 mM Na^+, the system A inhibitor *N*-(methylamino)-isobutyric acid (MeAIB) had no inhibitory effect on L-alanine accumulation in both types of cells (WKY, 91 ± 8%, and SHR, 115 ± 7% of control).

Subsequent experiments were designed to determine the apparent kinetics of L-alanine transporters under Na^+ V_{max} experimental conditions (extracellular 140 mM Na^+). Cells were incubated for 6 min in the absence or presence of increasing concentrations of unlabeled substrate (3–3,000 μM). The effect of BCH on the kinetic parameters was also investigated. In both types of cells, the accumulation of L-[^{14}C]alanine was found to be concentration dependent and a saturable process (Fig. 5). The apparent kinetic parameters of L-[^{14}C]alanine uptake, determined by nonlinear analyses of the inhibition curves, are given in Table 2. The transport capacity of the saturable component of L-[^{14}C]alanine transport in SHR was lower than that for WKY PTE cells, at all substrate concentrations with similar K_m values (Table 2). Experiments were also conducted in the presence of BCH to reduce the contribution of L-type amino acid transport. In the presence of BCH, K_m values for L-[^{14}C]alanine uptake in SHR PTE cells were markedly increased without changes in V_{max}, whereas K_m values in WKY PTE cells changed only slightly (Table 2).

Substrate selectivity of L-alanine uptake was investigated by inhibition experiments in which the accumulation of 0.25 μM L-[^{14}C]alanine was measured in the presence of 1 mM unlabeled amino acids and selective analogs (Fig. 6*A*). In both cell lines, L-alanine uptake was markedly (>80%) inhibited by L-isomers of neutral amino acids, such as alanine, serine, threonine, and cysteine, which is consistent with amino acid transport through ASCT2. Nevertheless, the profile of inhibition differs considerably in the case of other neutral and aromatic amino acids, such as leucine, isoleucine, phenylalanine, methionine, tyrosine, and histidine, which, in WKY PTE cells, produced moderate inhibition (10–50%). By contrast, in SHR PTE cells, neutral and aromatic amino acids produced a high degree of inhibition (75–90%) on L-[^{14}C]alanine uptake. The basic amino acids lysine and arginine also reduced the accumulation of L-[^{14}C]alanine in SHR PTE cells. In WKY

Table 1. *Na^+-K_m and V_{max} values for uptake of L-[^{14}C]alanine in immortalized WKY and SHR PTE cells*

	WKY		SHR	
	Na^+-K_m	V_{max}	Na^+-K_m	V_{max}
Vehicle				
High affinity	18±1	59±2	9±1	15±1
Low affinity	55±4†	105±3†	182±14†	111±23†
BCH, 3 mM				
High affinity	29±3*	58±2	23±8	14±4
Low affinity	155±12*†	118±5†	105±33†	58±18†

Values are means ± SE of 6 experiments/group; Na^+-K_m (mM). V_{max}, maximum velocity (pmol·mg protein^{-1}·6 min^{-1}); WKY, Wistar Kyoto rats; SHR, spontaneously hypertensive rats; PTE cells, proximal tubular epithelial cells; BCH, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid. *Significantly different from corresponding control values ($P < 0.05$); †significantly different from corresponding values for the high-affinity state ($P < 0.05$).

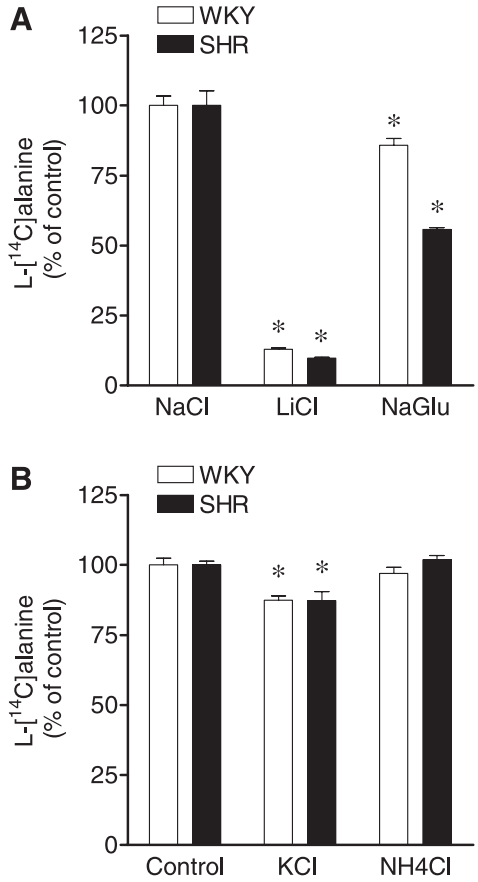

Fig. 4. Ion dependence of L-[^{14}C]alanine transport in immortalized WKY and SHR PTE cells. *A*: replacement of NaCl with equimolar concentrations of lithium chloride and sodium gluconate. *B*: addition of 50 mM KCl or 50 mM NH_4Cl; the control medium contained 100 mM sucrose to maintain osmolarity. Columns represent the mean of 4–6 experiments per group; error bars show SE. Significantly different from corresponding control values (*$P < 0.05$).

PTE cells, L-alanine uptake was also inhibited by glutamine and asparagine.

To prove that the uptake of L-[^{14}C]alanine occurs through system ASC, substrate specificity was examined in the presence of BCH 3 mM. As shown in Fig. 6*B*, the SHR cell profile of inhibition changed considerably, becoming less sensitive to glycine, isoleucine, phenylalanine, methionine, tyrosine, and histidine (0–35% inhibition) as well as to cationic amino acids. By contrast, glutamine was able to reduce significantly L-[^{14}C]alanine uptake in SHR PTE cells. WKY PTE cells were less affected by the presence of 3 mM BCH in the uptake solution.

These results indicate that L-alanine transport in SHR and WKY PTE cells is largely promoted through a high-affinity, Na^+-dependent, Li^+-sensitive amino acid transporter, insensitive to amino acid analog MeAIB, with specificity for alanine, serine, and cysteine. These are features of ASC-like activity. As evidenced by V_{max} values, the Na^+-dependent and Li^+-sensitive transport of L-[^{14}C]alanine was lower in SHR than in WKY PTE cells. However, in SHR PTE cells, L-alanine transport was sensitive to BCH as well as to basic amino acids, and the absence of Cl^- in the media inhibited the uptake. These observations suggest that, in SHR PTE cells, the Na^+-dependent component might be the combination of system $B^{0,+}$ and ASCT2.

Expression of ASCT2. The presence of ASCT2 protein in SHR and WKY PTE cells was studied by means of immunoblotting using an antibody raised against ASCT2. As shown in Fig. 7, antibodies against ASCT2 recognized the presence of the protein in immortalized WKY and SHR PTE cells and in renal cortical membranes from WKY and SHR. The abundance of ASCT2 (corrected for β-actin) was lower in SHR than in WKY PTE cells (Fig. 7*A*). The reduced ASCT2 protein expression in SHR PTE cells correlates positively with the lower transport capacity observed in SHR PTE cells compared with WKY PTE cells. The reduced ASCT2 protein expression in the SHR was also observed in renal cortical membranes in both 4- and 12-wk-old rats (Fig. 7, *B* and *C*).

ASCT2 transcript abundance. Detection of ASCT2 transcript in immortalized PTE cells and kidney cortices from WKY and SHR at 12 wk of age was performed by conventional RT-PCR, using rat-specific primers. As depicted in Fig. 8*A*, all samples amplified the expected 650-bp fragment. Transcript abundance of ASCT2 was measured by quantitative real-time PCR in immortalized PTE cells and kidney cortices from WKY and SHR. The expression of the ASCT2 transcript was normalized to that of the housekeeping gene GAPDH, which was identical in WKY and SHR. Data are presented as

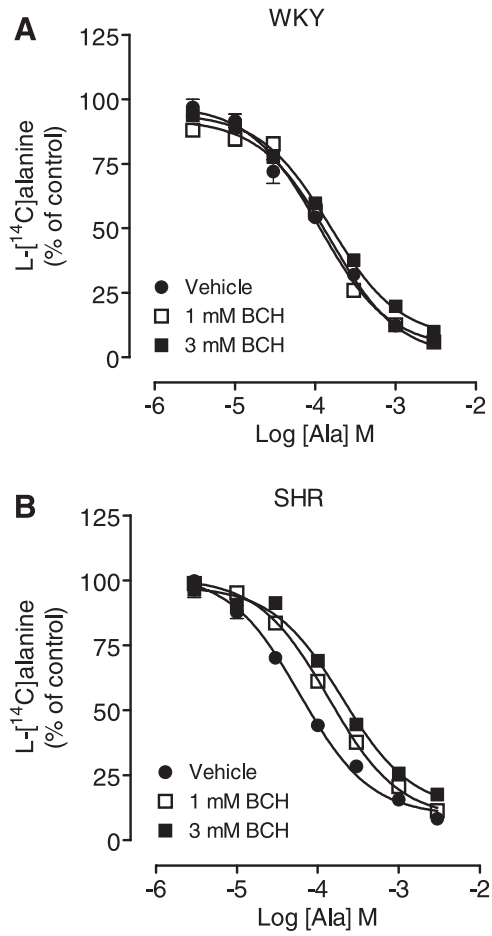

Fig. 5. Effect of increasing concentrations of L-alanine ([Ala]; 3, 10, 30, 100, 300, 1,000 and 3,000 μM) on the uptake of L-[^{14}C]alanine (0.25 μM) in WKY (*A*) and SHR (*B*) PTE cells in the absence and in the presence of 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH; 1 and 3 mM). Symbols represent means of 4–8 experiments per group; vertical lines show SE.

Table 2. *Kinetic parameters for uptake of L-[^{14}C]alanine in immortalized WKY and SHR PTE cells*

	WKY		SHR	
	K_m	V_{max}	K_m	V_{max}
L-Alanine	110±11	32,554±2,766	60±3*	13,816±628*
L-Alanine +1 mM BCH	152±13†	32,862±2,573	141±20†	18,657±2,861*
L-Alanine +3 mM BCH	152±8†	29,979±1,491	208±16†*	15,961±1,492*

Values are means ± SE of 16 experiments/group; K_m (μM); V_{max} (pmol·mg protein^{-1}·6 min^{-1}). Significantly different from WKY cells (*$P < 0.05$) and without BCH (†$P < 0.05$).

the ratio of ASCT2 to GAPDH. The mRNA expression of ASCT2 was lower ($P < 0.05$) in WKY than in SHR PTE cells (Fig. 8*B*), which is not consistent with higher ASCT2 protein in WKY than in SHR PTE cells. Posttranscriptional events may be responsible for the low expression of ASCT2 protein in SHR cells, as previously reported by Tailor et al. (36). As depicted in Fig. 8*C*, the expression of ASCT2 transcript was markedly lower in SHR than in WKY rat kidney cortices, at both 4 and 12 wk of age, which correlated well with differences in ASCT2 protein expression between WKY and SHR. ASCT2 mRNA decreased with age in both WKY and SHR.

Detection of $B^{0,+}$ transcript. To explore the possible involvement of system $B^{0,+}$, a Na^+- and Cl^--dependent amino acid transporter sensitive to BCH as well as neutral and basic amino acids (35, 41), conventional RT-PCR was performed in immortalized PTE cells and kidney cortices from WKY and SHR. A specific primer set, designed based on the rat $B^{0,+}$ sequence NM_00103744, was used. As shown in Fig. 9, the expected 400-bp fragment corresponding to $B^{0,+}$ was present only in the SHR immortalized PTE cells.

DISCUSSION

The present study shows that renal WKY and SHR PTE cells take up L-[^{14}C]alanine mainly through the high-affinity Na^+-dependent amino acid transporter system ASCT2. The SHR PTE cells were found to be endowed with lower expression level and function of ASCT2. Furthermore, findings described here in immortalized WKY and SHR PTE cells are consistent with that occurring in vivo in WKY and SHR. In fact, the abundance of ASCT2 transcript and protein in kidney cortices was also markedly lower in SHR than in normotensive WKY.

Although the expression of ASCT2 in renal epithelial cells has been reported previously (3, 11, 15), it was only recently that functional evidence for ASC-like activity in the apical membrane of kidney epithelial cells was observed. Oppedisano and co-workers observed transport activity with characteristics of ASCT2 (22, 23) in liposomes obtained from rat renal apical plasma membranes. The immortalized renal PTE cells from WKY and SHR are well-established models in our laboratories (25, 26, 29, 30) that have been used to evaluate both the diversity and the regulation of amino acid transport systems (29). In the present study, L-alanine was used to assess the presence of ASCT2, one of the transport systems responsible for the Na^+-dependent inward transfer of this amino acid.

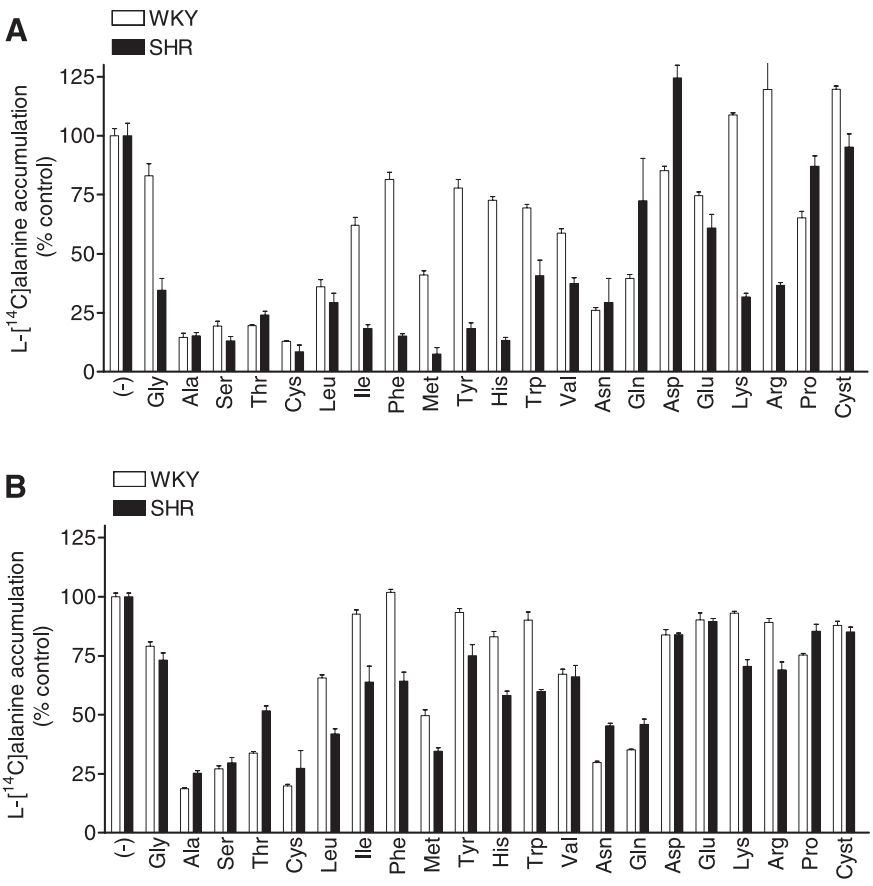

Fig. 6. L-Amino acid inhibition of the uptake of 0.25 μM L-[^{14}C]alanine in immortalized WKY and SHR PTE cells in the presence of 140 mM NaCl (*A*) and 140 mM NaCl with 3 mM BCH (*B*). Columns represent the mean of 4–6 experiments per group; error bars show SE.

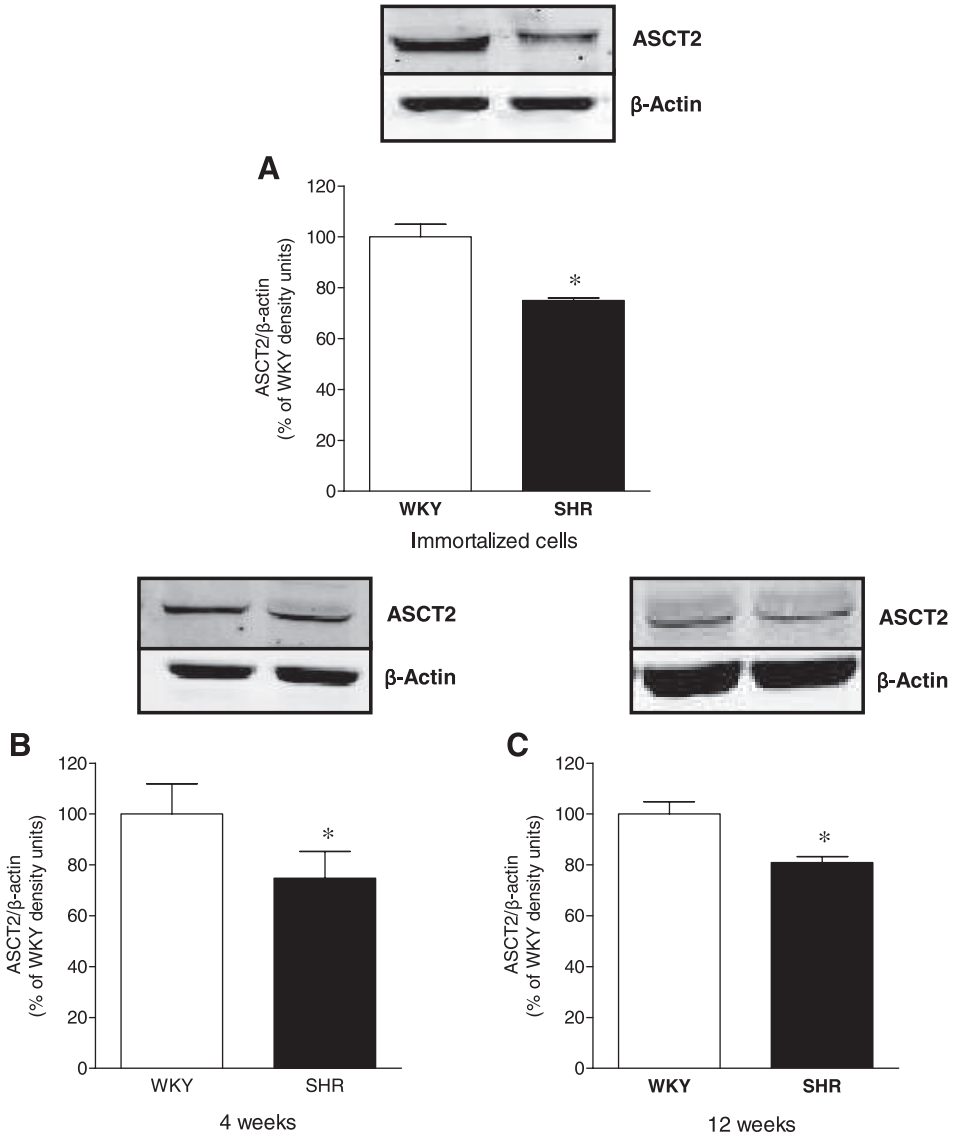

Fig. 7. Immunoblot analysis of ASCT2 amino acid transporter in immortalized WKY and SHR PTE cells (*A*) and in kidney cortices from WKY and SHR [at 4 wk (*B*) and 12 wk of age (*C*)]. Each lane contains an equal amount of protein (60 μg). Western blot analysis was repeated 3 times. Significantly different from corresponding values for WKY PTE cells (*$P <$ 0.05).

However, transport systems B^0 and ASCT2 are most likely to be responsible for the Na^+-dependent uptake of alanine in kidney brush border membranes. WKY and SHR PTE cells transport quite efficiently L-alanine through the apical cell border, and several findings suggest that this uptake process is a facilitated mechanism and proceeds through ASCT2. Most of L-[^{14}C]alanine entered the cells in a Na^+-dependent manner, and only a minor component of L-[^{14}C]alanine uptake (~10–15%) was found not to require extracellular Na^+. The Na^+ activation Hill coefficient of unity indicates a 1:1 Na^+-to-alanine activation stoichiometry for secondary active transport in both cell lines. However, in-depth analysis of this process revealed the presence of high- and low-affinity states for the Na^+-dependent L-[^{14}C]alanine uptake in both cell lines. Although this could be interpreted as the presence of two transporter entities, it is likely that this is not the case. In fact, at low extracellular Na^+ concentrations, the Na^+-dependent L-[^{14}C]alanine uptake in both WKY and SHR PTE cells is a high-affinity, low-capacity process, and increases in extracellular Na^+ reduced the affinity for the substrate but increased the capacity to take up L-[^{14}C]alanine. Another finding that supports this suggestion is that BCH decreases the affinity of the transporter but does not affect the Na^+-dependent L-[^{14}C]alanine uptake, this being particularly evident for WKY PTE cells. It is also likely that differences in the Na^+-dependent L-[^{14}C]alanine uptake between WKY and SHR cells may be related to the presence of two different Na^+-dependent L-[^{14}C]alanine transporters in the latter, as discussed below.

The L-[^{14}C]alanine uptake was unaffected by MeAIB, suggesting that inward transfer in WKY and SHR PTE cells was not promoted by the system A. BCH only reduced L-[^{14}C]alanine uptake ~25%; this low level of sensitivity to BCH supports the view that L-alanine transport is mediated by an ASC-like transporter. Furthermore, L-[^{14}C]alanine uptake was also found to be markedly inhibited by Li^+. Small neutral amino acids such as alanine, serine, and cysteine significantly inhibited the uptake of L-[^{14}C]alanine in both cell lines. System B^0 is largely electrogenic, with high affinity for phenylalanine (7). The uptake of L-[^{14}C]alanine in WKY and SHR PTE cells was largely nonelectrogenic. In the presence of BCH (to

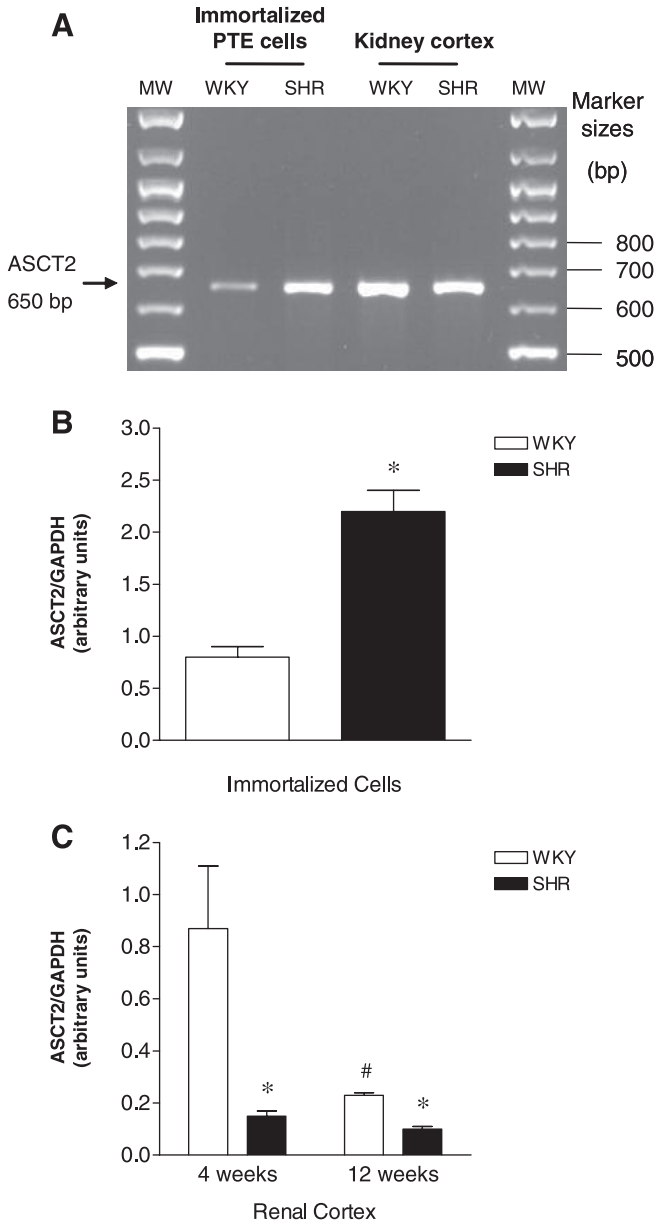

Fig. 8. *A*: RT-PCR detection of ASCT2 in total RNA extracted from WKY and SHR PTE cells and kidney cortices from WKY and SHR (12 wk of age). MW, GeneRuler DNA Ladder Mix (MBI, Fermentas). *B* and *C*: abundance of ASCT2 transcript in WKY and SHR PTE cells (*B*) and in kidney cortices from WKY and SHR (4 and 12 wk of age) (*C*). Results are expressed as ratio to GAPDH, as determined by quantitative real-time PCR. Arithmetical mean values were used to calculate the percentage of control levels. Data are means ± SE. *Significantly different from WKY ($P < 0.05$) and significantly different from 4 wk of age ($P < 0.05$).

exclude the role of system L), L-[^{14}C]alanine uptake in SHR and WKY PTE cells was less sensitive to phenylalanine than to alanine. Taken together, these results indicate that the Na^+-dependent alanine uptake in SHR and WKY cells proceeds mainly through the Na^+-dependent neutral amino acid ASCT2 transporter and not by systems B^0 and A. This suggestion agrees with data in previous reports that characterized the Na^+-dependent alanine uptake in renal epithelial cells (19, 34).

In SHR PTE cells, the ability of the transporter to take up L-[^{14}C]alanine appeared to be lower than that observed in WKY PTE cells. As shown in time course experiments, the initial rate of L-[^{14}C]alanine uptake in SHR PTE cells was already two times lower than that in WKY PTE cells. In addition, the saturable component of Na^+-dependent L-[^{14}C]alanine transport under V_{max} conditions in SHR was one-half that in WKY PTE cells. In the presence of BCH, the system L inhibitor, K_m values for transport increased and became similar in both cell lines. The K_m values reported here are in close agreement with those described previously for human ASCT2 expressed in *Xenopus*oocytes (~169.7 μM) (24) and for the pig kidney epithelial cell line LLC-PK_1 (380 μM) (19). Furthermore, differences in the magnitude of Na^+-dependent L-[^{14}C]alanine uptake through ASCT2 between WKY and SHR PTE cells correlated positively with differences in the expression of ASCT2 protein, this being more abundant in WKY than in SHR PTE cells. The discrepancy between mRNA concentration and protein expression observed in SHR PTE cells might be related to posttranscriptional events. Several levels of nuclear posttranscriptional events can be regulated, such as the control of splicing efficiency, precursor RNA stability, polyadenylation, or RNA transport (2). Whether this overproduction in SHR involves a *cis*- or *trans*-regulatory mechanism or whether any labile protein factor affected the regulation is unknown. Studies to elucidate the molecular mechanism of mASCT2 overproduction in SHR PTE cells are required.

Different routes for alanine uptake are present in SHR PTE cells. SHR cells, but not WKY cells, were also found to take up L-[^{14}C]alanine in a Cl^--dependent manner (~45% of L-alanine uptake) that, in the absence of BCH, was sensitive to inhibition by leucine, isoleucine, phenylalanine, methionine, tyrosine, and histidine and to the cationic amino acids lysine and arginine. The Na^+- and Cl^--dependent L-[^{14}C]alanine transporter most likely involved corresponds to system $B^{0,+}$, a transporter sensitive to BCH, neutral and basic amino acids (35, 41). Thus the major Na^+-dependent L-alanine transporter in WKY cells is ASCT2, contributing to ~85% of the total L-alanine uptake. By contrast, in SHR cells Na^+-dependent component may result of ASCT2 (~55%) and system $B^{0,+}$ (~45%). A minor contribution to L-alanine uptake by Na^+-independent transporters is also observed in both cell lines.

To determine whether the findings obtained in immortalized WKY and SHR PTE cells might reflect the in vivo situation, a set of experiments was conducted in renal cortices from SHR

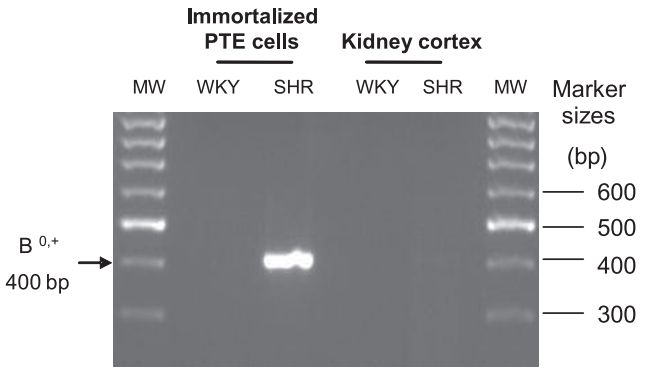

Fig. 9. RT-PCR detection of system $B^{0,+}$ in total RNA extracted from WKY and SHR PTE cells and kidney cortices from WKY and SHR (12 wk of age). MW, GeneRuler DNA Ladder Mix (MBI, Fermentas).

and WKY rats of 4 and 12 wk of age. An ASCT2-specific fragment was detected in the mRNA samples from the rat kidney, suggesting that this transporter was also expressed in renal cortices. The quantitative real-time PCR experiments correlated positively with data from immunoblots, which indicated that the expression of ASCT2 in renal cortices from SHR was lower than that in the WKY. This suggests that immortalized SHR and WKY PTE cells constitute a good experimental model for the study of ASCT2.

Neutral amino acid transporter ASCT2 displays substrate-induced Na^+ antiport activity; therefore, as an obligatory exchanger, it cannot mediate net amino acid uptake (6). Thus the role of ASCT2 in proximal tubule homeostasis is that of a mechanism for the delivery of glutamine for ammoniagenesis (4, 8) and for the removal of other small neutral amino acids from the extracellular space, maintaining their low extracellular levels. ASCT2 belongs to a restricted group of transporters that share specificity for glutamine, since glutamine is the major precursor of urinary ammonia, thus playing a key role in acid-base homeostasis. Interestingly, however, glutamine was higher in muscle and plasma of SHR at 6 wk of age and thereafter (12). These differences, because they occurred most strikingly in SHR during the prehypertensive state, were suggested to be related to the development of hypertension (12). However, ammonium urinary excretion was identical in WKY and SHR (31). The possibility that the SHR uses less glutamine in renal ammoniagenesis because of underexpression of ASCT2 needs to be evaluated.

Another interesting observation is that ASCT2 has been shown to be regulated by nitric oxide (NO) in the human intestinal cell line Caco-2 (39). NO is inactivated by reaction with superoxide (O_2^-) to produce peroxynitrite. In the kidney of SHR the regulation of renal oxygen consumption by NO is impaired (1), due to the increased superoxide production observed in this model of hypertension (33, 42, 43). NO availability in the kidney is decreased in SHR, resulting in increased oxygen consumption. By lowering intrarenal oxygen levels, reduced NO may contribute to susceptibility to renal injury (1). Taken together, these observations and those described in the present study suggest that, in SHR, oxidative stress might be downregulating ASCT2 by decreasing intrarenal NO availability. Therefore, the modulation of renal ASCT2 transporter in hypertension is worthy of further attention.

In conclusion, immortalized SHR and WKY PTE cells take up L-alanine mainly through a high-affinity Na^+-dependent amino acid transporter, with functional features of ASCT2 transport. The activity and expression of the ASCT2 transporter were considerably lower in the SHR cells. As a compensatory mechanism, in SHR cells, L-alanine is also transported by other amino acid transport systems, namely $B^{0,+}$, that account for ~45% of total Na^+-dependent L-[^{14}C]alanine uptake. Finally, findings obtained in immortalized cells match those in vivo: ASCT2 is underexpressed at the kidney cortex level in the SHR.

GRANTS

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Role of H_2O_2 on the kinetics of low-affinity high-capacity Na^+-dependent alanine transport in SHR proximal tubular epithelial cells

Vanda Pinto [a], Maria João Pinho [a], Pedro. A. Jose [b], Patrício Soares-da-Silva [a,*]

[a] Institute of Pharmacology and Therapeutics, Faculty of Medicine, University of Porto, 4200-319 Porto, Portugal
[b] Center for Molecular Physiology Research, Children's National Medical Center, Department of Pediatrics, George Washington School of Medicine and Health Sciences, Washington, DC, USA

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ABSTRACT

The presence of high and low sodium affinity states for the Na^+-dependent [^{14}C]-L-alanine uptake in immortalized renal proximal tubular epithelial (PTE) cells was previously reported (Am. J. Physiol. 293 (2007) R538–R547). This study evaluated the role of H_2O_2 on the Na^+-dependent [^{14}C]-L-alanine uptake of ASCT2 in immortalized renal PTE cells from Wistar Kyoto rat (WKY) and spontaneously hypertensive rat (SHR). Na^+ dependence of [^{14}C]-L-alanine uptake was investigated replacing NaCl with an equimolar concentration of choline chloride in vehicle- and apocynin-treated cells. Na^+ removal from the uptake solution abolished transport activity in both WKY and SHR PTE cells. Decreases in H_2O_2 levels in the extracellular medium significantly reduced Na^+-K_m and V_{max} values of the low-affinity high-capacity component in SHR PTE cells, with no effect on the high-affinity low-capacity state of the Na^+-dependent [^{14}C]-L-alanine uptake. After removal of apocynin from the culture medium, H_2O_2 levels returned to basal values within 1 to 3 h in both WKY and SHR PTE cells and these were found stable for the next 24 h. Under these experimental conditions, the Na^+-K_m and V_{max} of the high-affinity low-capacity state were unaffected and the low-affinity high-capacity component remained significantly decreased 1 day but not 4 days after apocynin removal. In conclusion, H_2O_2 in excess is required for the presence of a low-affinity high-capacity component for the Na^+-dependent [^{14}C]-L-alanine uptake in SHR PTE cells only. It is suggested that Na^+ binding in renal ASCT2 may be regulated by ROS in SHR PTE cells.

1. Introduction

Renal absorption of amino acids is accomplished by at least three different types of Na^+-dependent transporters for neutral amino acids [1]: the proline transporter IMINO/SIT (SLC6A20), the broad specific neutral amino acid transporter B^0AT1 (SLC6A19) and the neutral amino acid exchanger ASCT2 (SLC1A5). ASCT2 is present in the brush-border membranes of the proximal tubule cells and enterocytes in the kidney and intestine, respectively [2]. In addition to its preference for small neutral amino acids (alanine, serine, and cysteine), it also accepts L-glutamine and L-asparagine at higher affinity, as well as methionine, leucine and glycine with lower affinity [3,4]. Additionally, because of its specificity for glutamine, the major precursor of urinary ammonia, ASCT2 plays and important role in acid–base homeostasis [5]. Recently it has been shown that the activity and expression of the glutamine/amino acid transporter (ASCT2) were significantly lower in SHR PTE cells, which matched the *in vivo* findings; ASCT2 was under-expressed at the kidney cortex level in the SHR [6].

Several lines of evidence point towards the possible regulation of amino acid transport by oxidative stress [7–10]. Studies by our group have show that immortalized renal PTE SHR cells generate more hydrogen peroxide (H_2O_2) than WKY cells [11]. In SHR cells, the NADPH oxidase inhibitor apocynin reduced their increased ability to generate H_2O_2 and reverted differences in receptor-mediated events between WKY and SHR cells [11–13]. Differences between WKY and SHR PTE cells in their sensitivity to angiotensin II correlate with the higher H_2O_2 generation that accompanies an enhanced expression of glycosylated and nonglycosylated AT_1 receptor forms in lipid rafts [12]. Likewise, the increased generation of H_2O_2 was found responsible for the amplification of the response downstream to alpha$_2$-adrenoceptor activation in SHR PTE cells [13] as well as the aldosterone-induced stimulation of NHE1 activity [11].

Although crystallographic studies of Na^+-coupled secondary transporters have greatly advanced the understanding of the structural principles that underlie transporter function [14–16], the mechanisms by which these transporters couple substrate and sodium transport are still largely unknown. We have previously

* Corresponding author. Address: Institute of Pharmacology and Therapeutics, Faculty of Medicine, 4200 Porto, Portugal. Fax: +351 22 5513643.
E-mail addresses: pss@med.up.pt, psoaresdasilva@netcabo.pt (P. Soares-da-Silva).

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shown that WKY and SHR PTE cells transport L-alanine efficiently through the apical cell border and several findings suggested this uptake process is a facilitated mechanism that proceeds through ASCT2. The Na^+ activation Hill coefficient of unity indicates a 1:1 Na^+/alanine activation stoichiometry for secondary active transport in both cell lines [6]. At low extracellular Na^+ concentrations, the Na^+-dependent [^{14}C]-L-alanine uptake in both WKY and SHR PTE cells is a high-affinity low-capacity process. Increments in extracellular Na^+, reduced the affinity for the substrate, but increased the capacity to take up [^{14}C]-L-alanine [6].

The aim of this study was, therefore, to determine the role of H_2O_2 on the kinetic parameters for Na^+-dependent [^{14}C]-L-alanine uptake and on ASCT2 expression in immortalized renal PTE cells from WKY and SHR.

2. Methods and materials

2.1. Cell culture

Immortalized renal PTE cells from the WKY and SHR [17] were maintained as previously described [6]. The cell medium was changed every 2 days, and the cells reached confluence after 3–5 days of incubation. For 24 h prior to each experiment, the cells were maintained in fetal bovine serum-free medium. Experiments were generally performed 2–3 days after cells reached confluence and 6–8 days after the initial seeding; each cm^2 contained about 80–100 μg of cell protein.

2.2. Uptake of L-amino acids

Flux measurements in immortalized renal PTE cells from the WKY and SHR were performed as previously described [18]. All experiments were carried out in the presence of BCH (3 mm), to minimize the contribution of L-type amino acid transports. Saturation experiments were performed in cells incubated for 6 min with 0.25 μM radiolabeled amino acid. In experiments performed to determine the Na^+-dependence of transport, sodium chloride was replaced by an equimolar concentration of choline chloride. Radioactivity was measured by liquid scintillation counting.

2.3. Measurement of H_2O_2

H_2O_2 was measured fluorometrically using the Amplex™ Red Hydrogen Peroxide Assay Kit (Molecular Probes, Inc., Eugene, OR), as described previously [11].

2.4. Immunoblotting

Western blot was performed as previously described [6]. Blots were incubated anti-ASCT2 polyclonal antibody (1:800; Chemicon International) or mouse anti-β-Actin (Santa Cruz Biotechnology). After incubation with fluorescently-labeled goat anti-rabbit (1:10,000; IRDye™ 800, Rockland) or the fluorescently-labeled goat anti-mouse secondary antibody (1:5000; AlexaFluor 680, Molecular Probes) the membrane was washed and imaged by scanning at both 700 and 800 nm, with an Odyssey Infrared Imaging System (Li-COR Biosciences).

2.5. Drugs

L- and D-Amino acids, 2-aminobicyclo (2,2,1)-heptane-2-carboxylic acid (BCH) and apocynin were purchased from Sigma Chemical Company, St. Louis, Mo, USA. [^{14}C]-L-alanine (specific activity 152 mCi/mmol) was purchased from Amersham Pharmacia Biotech (Little Chalfont, UK).

2.6. Data analysis

K_m and V_{max} values for the uptake of [^{14}C]-L-alanine, were determined from a competitive uptake inhibition protocol [19], and calculated from non-linear regression analysis using the GraphPad Prism statistics software package [20]. For calculation of the IC_{50} the parameters of the equation for one site inhibition were fitted to the experimental data [20]. Arithmetic means are given with S.E.M. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Newman–Keuls test for multiple comparisons. A P value <0.05 was assumed to denote a significant difference.

3. Results

3.1. H_2O_2 and the sodium kinetic parameters for the high and low-affinity Na^+-dependent [^{14}C]-L-alanine uptake

The effect of H_2O_2 availability on the Na^+ dependence of [^{14}C]-L-alanine uptake was investigated replacing NaCl with an equimolar concentration of choline chloride in the absence and presence of apocynin. Na^+ removal from the uptake solution almost completely abolished transport activity in both WKY and SHR PTE cells (Fig. 1). The sodium concentrations and alanine uptake data were transformed using the Eadie–Hofstee equation. The low- and high-affinity components in both WKY and SHR PTE cells were identified and subsequently plotted as a double reciprocal Lineweaver–Burk plot as previously reported [6]. The sodium kinetic parameters (Na^+-K_m and V_{max}) for the high- and low-affinity Na^+-dependent [^{14}C]-L-alanine uptake in WKY and SHR PTE cells are given in Table 1. Treatment with apocynin (100 μM) for 4 days decreased H_2O_2 levels [11], but had no effect on the high- and low-affinity states of Na^+-dependent [^{14}C]-L-alanine uptake in WKY PTE cells (Fig. 2A and B) or on the high-affinity state of SHR PTE cells (Fig. 2C). However, apocynin significantly decreased the low-affinity high-capacity state (Fig. 2D). Treatment with apocynin (100 μM) for 4 days significantly reduced Na^+-K_m and V_{max} values of the low-affinity high-capacity component of Na^+-dependent [^{14}C]-L-alanine uptake (from 361 ± 7 to 48 ± 6 mM, and 137 ± 2 to 40 ± 2 pmol/mg/6 min, respectively) in SHR PTE cells only. The high-affinity low-capacity sodium K_m and V_{max} values of ASCT2 were unaffected by apocynin treatment.

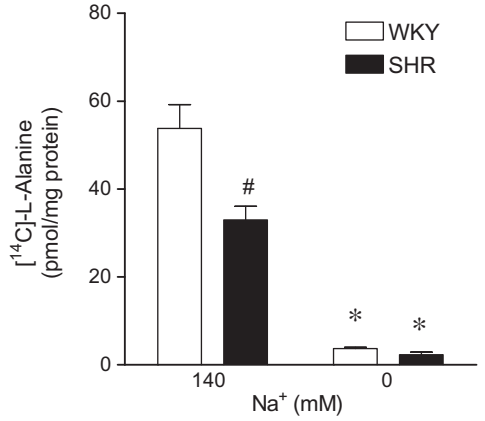

Fig. 1. Effect of sodium chloride replacement by an equimolar concentration of choline chloride on 0.25 μM [^{14}C]-L-alanine transport. Cells were incubated for 6 min with 0.25 μM [^{14}C]-L-alanine in the presence of BCH (3 mM). Significantly different from corresponding control values ($^*P < 0.05$) and corresponding values for WKY PTE cells ($^{\#}P < 0.05$). Symbols represent the mean of four experiments per group; error bars show S.E.M.

Table 1
Sodium K_m (mM) and V_{max} (pmol/mg protein/6 min) values for uptake of [^{14}C]-L-alanine in immortalized WKY and SHR PTE cells in the presence of BCH (3 mM).

		WKY		SHR	
		Na^+-K_m	V_{max}	Na^+-K_m	V_{max}
Vehicle	High affinity	16 ± 1	53 ± 2	21 ± 5	26 ± 3
	Low affinity	111 ± 1[#]	101 ± 1[#]	361 ± 7[#]	137 ± 2[#]
Apocynin (100 μM)	High affinity	15 ± 6	47 ± 7	29 ± 4	32 ± 2
	Low affinity	138 ± 15[#]	104 ± 7[#]	48 ± 6[*,#]	40 ± 2[*,#]

Values are means ± S.E.M. of eight experiments per group.
[*] Significantly different from corresponding vehicle values ($P < 0.05$).
[#] Significantly different from corresponding values for the high-affinity state ($P < 0.05$).

3.2. Reversibility of apocynin effects on the sodium kinetic parameters for the high and low-affinity Na^+-dependent [^{14}C]-L-alanine uptake

To evaluate the nature of the relationship between H_2O_2 availability and the effects of apocynin on the low-affinity high-capacity Na^+-dependent [^{14}C]-L-alanine uptake, it was decided to determine whether these effects could be reversed after return of H_2O_2 production to basal values. H_2O_2 levels were measured before and after (1, 3, 6 and 24 h) apocynin treatment for 4 days in WKY and SHR cells. Treatment with apocynin reduced H_2O_2 levels in the extracellular medium by ~20% in WKY PTE cells and ~30% in SHR PTE cells (Fig. 4A and B). H_2O_2 levels returned to control values 1 and 3 h after apocynin removal in both WKY and SHR PTE cells, respectively. Thereafter, H_2O_2 levels were kept stable up to 24 h after apocynin withdrawal (Fig. 4A and B). Therefore, in this new set of experiments the Na^+-dependent [^{14}C]-L-alanine uptake was evaluated 1 and 4 days after apocynin removal in SHR PTE cells treated for 4 days with the antioxidant. Despite apocynin removal and the return of H_2O_2 levels to control values, Lineweaver–Burk plots show that the high-affinity low-capacity Na^+-dependent [^{14}C]-L-alanine uptake was not affected in apocynin-treated cells (Fig. 3A), whereas the low-affinity high-capacity component of Na^+-dependent [^{14}C]-L-alanine uptake was markedly reduced in apocynin-treated cells (Fig. 3B). However, 4 days after apocynin removal (Fig. 3C and D) the low-affinity high-capacity Na^+-dependent [^{14}C]-L-alanine uptake was restored (Fig. 3D). Sodium kinetic parameters for the high- and low-affinity Na^+-dependent [^{14}C]-L-alanine uptake were determined and results show that 1 day after H_2O_2 levels were restored, sodium K_m and V_{max} values of the low-affinity high-capacity component were still reduced, but not 4 days later (Table 2), indicating that the effect of apocynin is not long lasting and that H_2O_2 in excess is required to maintain normal sodium K_m and V_{max} values in SHR PTE cells.

3.3. Expression of ASCT2

The presence of ASCT2 protein in WKY and SHR PTE cells was studied by means of immunoblotting using an antibody raised

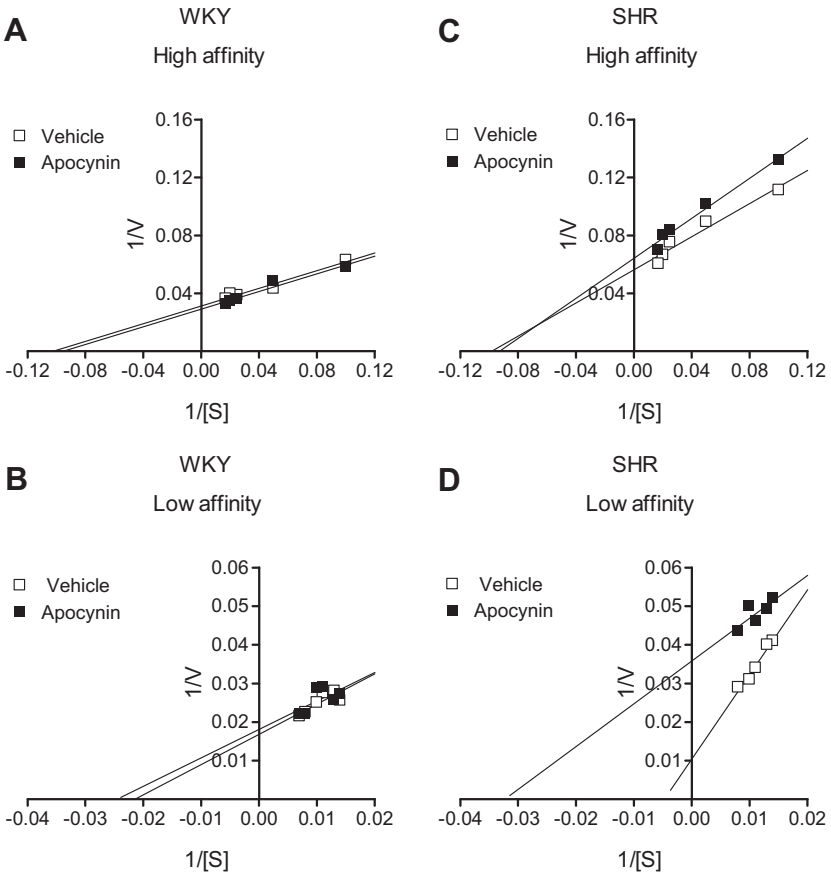

Fig. 2. Lineweaver–Burk reciprocal plots of velocity and substrate concentrations for [^{14}C]-L-alanine transport as a function of extracellular sodium concentration in immortalized (A and B) WKY PTE cells, (C and D) SHR PTE cells. Lineweaver–Burk reciprocal plots of velocity and substrate for the (A and C) high and (B and D) low-affinity components in the presence of BCH (3 mM). Points: mean of two observations within an experiment, representative of 6 separate experiments. Lines were derived from a weighted least-squares analysis of the data points.

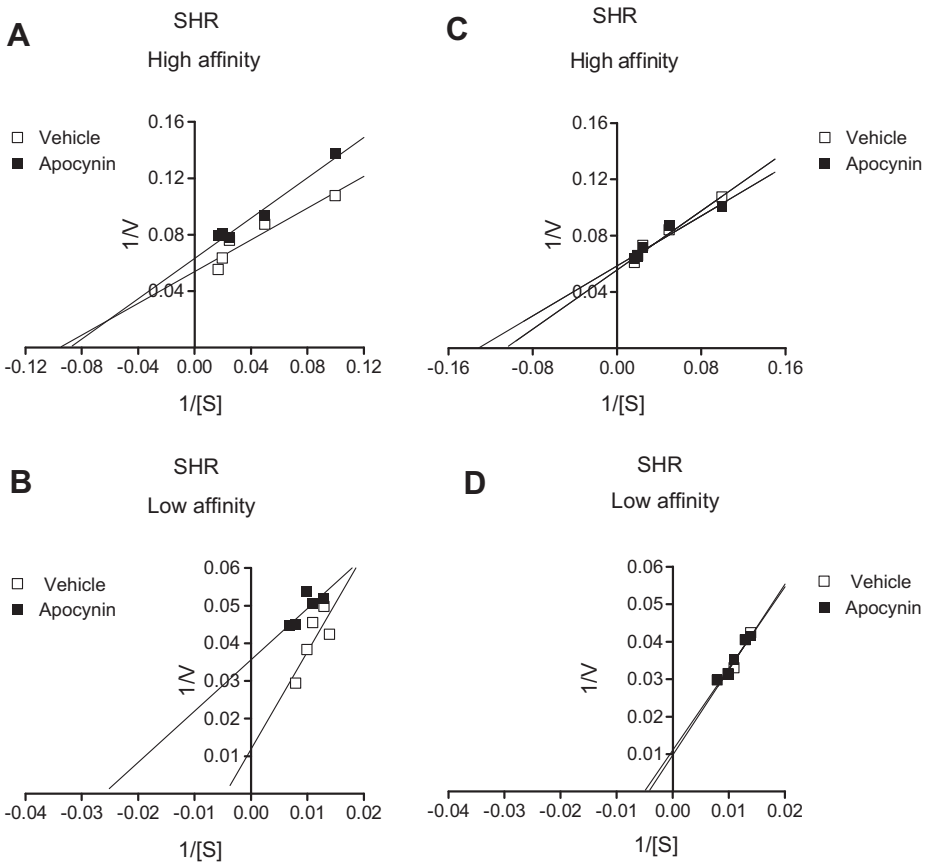

Fig. 3. Lineweaver–Burk reciprocal plots of velocity and substrate concentrations for [^{14}C]-L-alanine transport as a function of extracellular sodium concentration in immortalized SHR PTE cells after treatment with apocynin (100 μM) for 4 days and without apocynin for 1 and 4 days before each experiment. Lineweaver–Burk reciprocal plots of velocity and substrate for the (A and C) high and (B and D) low-affinity components in the presence of BCH (3 mM). Points: mean of two observations within an experiment, representative of six separate experiments. Lines were derived from a weighted least-squares analysis of the data points.

against ASCT2. The anti-ASCT2 antibody recognized the presence of the protein in immortalized WKY and SHR PTE cell lines. The abundance of ASCT2 (corrected for β-actin) in SHR PTE cells was lower than in WKY PTE cells (Fig. 4C), which correlates positively with the lower transport capacity observed in SHR PTE cells when compared to WKY PTE cells. Treatment with apocynin (100 μM) for 4 days had no effect on ASCT2 expression in both cell lines (Fig. 4C).

4. Discussion

In the present study inhibition of H_2O_2 production by apocynin during cell growth was shown to significantly reduce Na^+-K_m and V_{max} values of the low-affinity high-capacity component of Na^+-dependent [^{14}C]-L-alanine uptake in immortalized SHR PTE cells.

The evaluation of several amino acid transport systems using immortalized renal PTE cells from WKY and SHR has been well documented in our group [6,18,21,22]. Recent studies indicate that WKY and SHR PTE cells take up [^{14}C]-L-alanine mainly through the high-affinity Na^+-dependent amino acid transporter system ASCT2. Moreover, SHR PTE cells were found to have a lower expression level and function of ASCT2 [6]. The System A and System N transporters of the SLC38 family each mediate Na^+-dependent transport of small, zwitterionic (net neutral) amino acids and exhibit marked inhibition at low extracellular pH [23]. The uptake of L-alanine in the SHR cells was not sensitive to pH. In the presence of extracellular 140 mM Na^+, system A inhibitor *N*-(methyamino)-isobutyric acid (MeAIB), had no inhibitory effect on the L-alanine accumulation in both types of cells (WKY, 97 ± 8% and SHR, 115 ± 7% of control) [6]. This suggests that the inward transfer of L-alanine in WKY and SHR cells is not promoted by SNAT's (SLC38). On the other hand, system B (SLC6) is highly electrogenic, with high affinity for phenylalanine [24]. Contrasting, L-alanine uptake in the WKY and SHR cells was significantly inhibited by small amino acids, such as alanine, serine and cysteine. Besides, the uptake of L-alanine in WKY and SHR cells was largely non-electrogenic [6].

Since the imbalance between NO and reactive ROS production is an important factor in the development of hypertension [25,26], we hypothesised whether oxidative stress could have an effect on ASCT2 regulation and function. The effect of decreased availability of H_2O_2 on [^{14}C]-L-alanine uptake was determined using apocynin. Apocynin has antioxidant properties and reduces ROS production *in vivo* and *in vitro* [27]. It inhibits the membrane recruitment of regulatory cytosolic NADPH oxidase subunits such as $p47^{phox}$, $p67^{phox}$, and rac-1 by direct interaction with $p47^{phox}$ [28]. Previous studies have shown that SHR PTE cells are endowed with an increased capacity to generate H_2O_2 when compared with WKY PTE cells and treatment with apocynin for 4 days reduces significantly the rate of H_2O_2 production in WKY and SHR PTE cells [11]. The experiments described here were designed to evaluate the effect of H_2O_2 availability on the sodium high- and low-affinity states for the Na^+-dependent [^{14}C]-L-alanine uptake in WKY and SHR PTE cells. As previously described [6], Lineweaver–Burk plots from data obtained in the present study also revealed the presence of high- and low-affinity states for the Na^+-dependent [^{14}C]-L-alanine uptake processes in both cell lines. At low extracellular Na^+ concentrations, the Na^+-dependent [^{14}C]-L-alanine uptake in both WKY and SHR PTE cells is a high-affinity low-capacity process

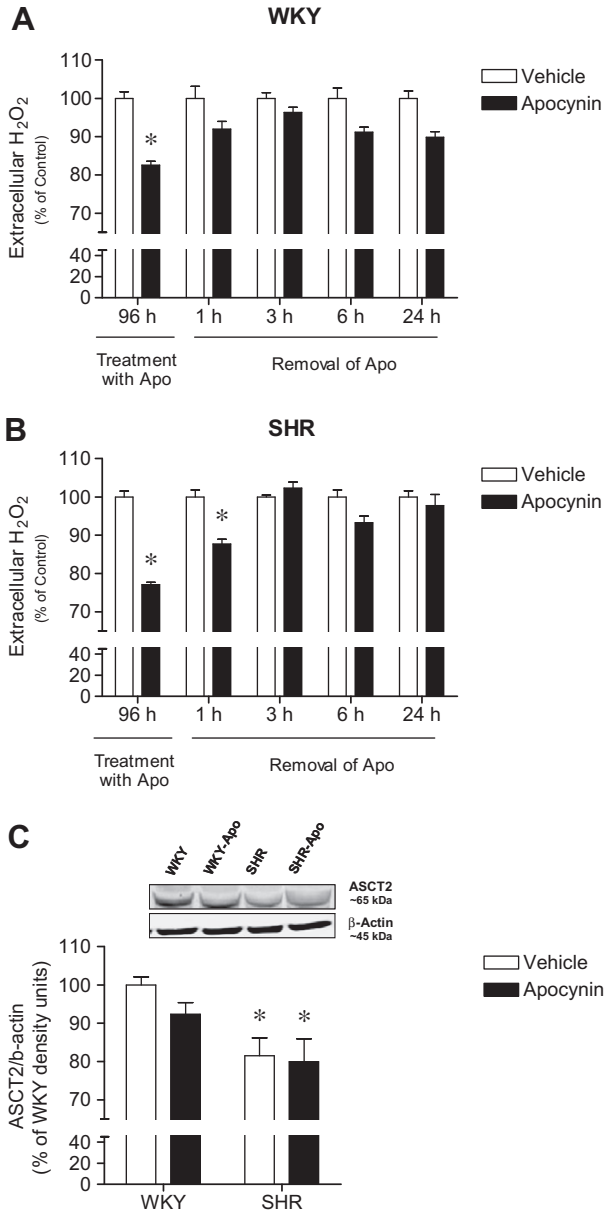

Fig. 4. Extracellular H_2O_2 in A) WKY and B) SHR PTE cells in the presence of apocynin (4 days after seeding) and after 1, 3, 6 and 24 h following apocynin removal. Each column represents the mean of six experiments per group; vertical lines indicate SEM. Significantly different from values for control (*$P < 0.05$). (C) ASCT2 protein expression in immortalized WKY and SHR PTE cells and effect of apocynin (100 μM) for 4 days on ASCT2 protein expression. Each lane contains equal amount of protein (60 μg), Western blotting was repeated 6×. β-Actin was used as a control. Significantly different from corresponding control values (*$P < 0.05$).

and increases in extracellular Na^+ reduced the affinity for the substrate, but increased the capacity to take up [^{14}C]-L-alanine. Apocynin treatment during cell growth (4 days) had no effect on the high- and low-affinity states of Na^+-dependent [^{14}C]-L-alanine uptake in WKY PTE cells or on the high-affinity state of SHR PTE cells. However, the reduction in H_2O_2 levels significantly decreased the Na^+-K_m and V_{max} of the low-affinity high-capacity component of [^{14}C]-L-alanine uptake in SHR PTE cells. These results suggest that when H_2O_2 levels are reduced after apocynin treatment the Na^+-dependent [^{14}C]-L-alanine uptake by ASCT2 in SHR PTE cells functions predominantly as a high-affinity low-capacity transporter. We have previously found (data not shown) that treatment with

Table 2
Sodium K_m (mM) and V_{max} (pmol/mg protein/6 min) values in the presence of BCH (3 mM) for uptake of [^{14}C]-L-alanine in immortalized SHR PTE cells after treatment with apocynin 100 μM for 4 days and subsequent removal for 1 and 4 days.

		1 day		4 days	
		Na^+-K_m	V_{max}	Na^+-K_m	V_{max}
Vehicle	High affinity	16 ± 3	23 ± 2	14 ± 2	28 ± 1
	Low affinity	218 ± 26[#]	96 ± 8[#]	274 ± 8[#]	105 ± 2[#]
Apocynin (100 μM)	High affinity	16 ± 1	19 ± 1	18 ± 5	24 ± 3
	Low affinity	55 ± 8[*,#]	27 ± 2[*,#]	320 ± 38[#]	107 ± 10[#]

Values are means ± S.E.M. of eight experiments per group.
[*] Significantly different from corresponding vehicle values ($P < 0.05$).
[#] Significantly different from corresponding values for the high-affinity state ($P < 0.05$).

a non toxic concentration (100 μM) of exogenous H_2O_2 (for 21 min or 1 day) failed to change the Na^+-K_m and V_{max} values for the Na^+-dependent [^{14}C]-L-alanine uptake, possibly due to the rapid decomposition of H_2O_2 by catalase.

There have been recent crystallographic advances relating to Na^+-coupled transporters specifically about the coupling of substrates to ions, the conformational state of the transporter at different stages of the transport cycle and how the substrate and ion pathway is alternately opened and closed, or gated, to maintain a tightly coupled transport mechanism [29–31]. Sodium ions have been shown to be 'gate-keepers' of aspartate transporter GltPh and other members of this Na^+-coupled transporter family, including ASCT2, which has a similar structural fold [16]. In the present study ROS have been shown to be important in modulating sodium coupling in SHR PTE cells. The mechanism by which this occurs is still not known. However, it is possible that oxidative stress may have an effect on the conformations of ASCT2 in SHR PTE cells as they proceed through the transport cycle, which may result in differential sodium binding and unbinding. ROS may also have an effect on steric, chemical and electrical properties of the sodium binding site. Nevertheless, ROS has shown to regulate other amino acid transport systems in different cell models. Oxidative stress was shown to stimulate NMDA receptor activity [8,32] and the Ca^{2+}-independent carrier-mediated release of glutamate and aspartate from cultured retina cells [33,34]. On the other hand, reuptake of glutamate in astrocytes, a critical mechanism involved in the maintenance of physiological excitatory amino acid neurotransmission, is inhibited by ROS [35].

The results show that the apocynin effects were long lasting but reversible. The reversal process is not immediate and an adaption period is needed in order to restore basal Na^+-K_m and V_{max} values. As previously suggested, H_2O_2 may affect steric, chemical and electrical properties of the sodium binding site as well as the conformational state of the transporter. Though H_2O_2 production is recovered within 1 day after apocynin removal the apocynin-associated changes of the Na^+-dependent [^{14}C]-L-alanine uptake take longer (at least 4 days) to return to its initial state of activity. Apocynin was also found not to alter ASCT2 protein expression in either WKY or SHR PTE cells. ASCT2 expression was found to be lower in SHR than WKY PTE cells as previously described [6].

5. Conclusions

It is concluded that ROS production during growth of the cell monolayer is essential for maintaining a low-affinity high-capacity component of Na^+-dependent [^{14}C]-L-alanine uptake in immortalized SHR PTE cells. Finally, data gathered here suggest that the

modulation of Na^+ binding to ASCT2 is dependent on increased hydrogen peroxide production exclusively in SHR PTE cells.

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Oxidative stress and the genomic regulation of aldosterone-stimulated NHE1 activity in SHR renal proximal tubular cells

Vanda Pinto · Maria João Pinho · Ulrich Hopfer · Pedro A. Jose · Patrício Soares-da-Silva

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Abstract This study evaluated the effects of aldosterone upon Na^+/H^+ exchange (NHE) activity in immortalized proximal tubular epithelial (PTE) cells from the spontaneously hypertensive rat (SHR) and the normotensive controls (Wistar Kyoto rat; WKY). Increases in NHE activity after exposure to aldosterone occurred in time- and concentration-dependent manner in SHR PTE cells, but not in WKY PTE cells. The aldosterone-induced increases in NHE activity were prevented by spironolactone, but not by the glucocorticoid receptor antagonist Ru 38486. The presence of the mineralocorticoid receptor transcript was confirmed by PCR and NHE1, NHE2, and NHE3 proteins were detected by immunoblot analysis. Cariporide and EIPA, but not S3226, inhibited the aldosterone-induced increase in NHE activity, indicating that NHE1 is the most likely involved NHE isoform. Pretreatment of SHR PTE cells with actinomycin D attenuated the aldosterone-induced increases in NHE activity. The SHR PTE cells had an increased rate of H_2O_2 production when compared with WKY PTE cells. Treatment of cells with apocynin, a NADPH oxidase inhibitor, markedly reduced the rate of H_2O_2 production. The aldosterone-induced increase in NHE activity SHR PTE cells was completely prevented by apocynin. In conclusion, the aldosterone-induced stimulation of NHE1 activity is a genomic event unique in SHR PTE cells, which involves the activation of the mineralocorticoid receptor, but ultimately requires the availability of H_2O_2 in excess.

Keywords Na^+/H^+ exchanger · NHE1 · Aldosterone · H_2O_2 · Hypertension · SHR · WKY

V. Pinto · M. J. Pinho · P. Soares-da-Silva (✉)
Faculty of Medicine, Institute of Pharmacology and Therapeutics, Porto 4200, Portugal
e-mail: psoaresdasilva@netcabo.pt

U. Hopfer
Department of Physiology and Biophysics, Case Western Reserve School of Medicine, Cleveland, OH, USA

P. A. Jose
Department of Pediatrics, Georgetown University, Washington, DC, USA

Introduction

Aldosterone is a steroid hormone secreted by the adrenal cortex. This mineralocorticoid is the final endocrine signal in the rennin–angiotensin–aldosterone system that is responsible for Na^+ reabsorption and K^+ secretion in the kidney and colon [1]. Consequently, it plays a pivotal role in the control of blood volume and thus blood pressure. The classical final effectors of aldosterone action are the apical ENaC, the basolateral Na^+–K^+–ATPase and the Na^+/H^+ exchanger 3 (NHE3) located in the proximal tubule [2].

Aldosterone was shown to have short- and long-term effects on the Na^+-K^+-ATPase and on the ENaC. The transcriptional effects on these ion transporters occur after 4 h [3]. Aldosterone enters the cell by diffusion through the cellular membrane and once in the cytoplasm it binds to an intracellular receptor, the mineralocorticoid receptor (MR). As has been shown, in the collecting duct, long-term effects are dependent on the activation of the serum-and-glucocorticoid-regulated kinase (SGK) which occurs after aldosterone binds to the MR [4]. Other studies have also shown that aldosterone also stimulates epidermal growth factor receptor (EGFR) expression in the kidney [5].

Steroid hormones, however, have the ability to act through non-genomic mechanisms as well as genomic. The

non-genomic action is characterized by a rapid onset (within seconds to minutes) after aldosterone administration and by the lack of sensitivity to transcription and translation inhibitors [6]. In Madin–Darby canine kidney (MDCK) cells aldosterone makes use of the EGFR-ERK1/2 cascade to bring out its rapid effects [7, 8].

Recent studies have shown that aldosterone stimulates surface expression of NHE3 in rat renal proximal tubular brush borders [9]. Furthermore, aldosterone stimulates not only the surface expression but also the activity of NHE3 in human primary renal proximal tubular epithelial (PTE) cells [10]. The NHE is a protein found in many mammalian cell types responsible for intracellular pH and cell volume regulation by exchanging protons localized in the cell for sodium ions. Seven isoforms of NHE have already been identified and cloned [11]. NHE1, 2 and 3 have been localized in the kidney [11, 12] and in MDCK-C11 cells. Aldosterone is thought to activate NHE1 activity through non-genomic stimulation [8, 13]; however, information concerning the long-term effect of aldosterone on NHE1 is scarce.

Abnormal aldosterone signaling or hyperactivity of its final effectors has been associated to steroid hypertension in humans [14]. Hypertension has also been linked to oxidative stress. Oxidative stress has been observed in a number of models of hypertension including in the spontaneous hypertensive rat (SHR) [15] and in the mineralocorticoid hypertensive rat [16]. Studies have shown that aldosterone directly induces reactive oxygen species (ROS) generation through the activation of NADPH oxidase in the salt-loaded, aldosterone-infused hypertensive rats [17], as well as in rat mensagial cells [18]. In mineralocorticoid hypertensive rats prolonged antioxidant administration normalizes superoxide accumulation and attenuates hypertension [19].

The aim of this study was to investigate the effects of aldosterone on NHE activity in immortalized renal PTE cells from the SHR and its normotensive control, the Wistar–Kyoto rat (WKY), and to determine whether oxidative stress is involved in aldosterone actions on NHE activity.

Materials and methods

Cell culture

Immortalized renal PTE cells from 4- to 8 week-old WKY and SHR animals [20] were maintained in a humidified atmosphere of 5% CO_2−95% air at 37°C. WKY and SHR PTE cells were grown in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (Sigma Chemical Company, St. Louis, MO, USA) supplemented with 100 U/ml penicillin G, 0.25 μg/ml amphotericin B, 100 μg/ml streptomycin (Sigma), 4 μg/ml dexamethasone (Sigma), 5 μg/ml transferrin (Sigma), 5 μg/ml insulin (Sigma), 5 ng/ml selenium (Sigma), 10 ng/ml epidermal growth factor (Sigma), 5% fetal bovine serum (Sigma) and 25 mM N-2-hydroxyethylpiperazine-N′-2-ethanosulfonic acid (HEPES; Sigma). For subculturing, the cells were dissociated with 0.10% trypsin-EDTA, split 1:4 and subcultured in Costar plates with 21-cm^2 growth areas (Costar, Badhoevedorp, The Netherlands). For pH_i measurement experiments, cells were grown in 96 well plates (Costar). On the day of each experiment, the cells were maintained in fetal bovine serum-free medium for 2 h. Experiments were generally performed 1–2 days after cells reached confluence and 4–5 days after the initial seeding; each cm^2 contained about 50 μg of cell protein.

NHE activity

NHE activity was assayed as the initial rate of intracellular pH (pH_i) recovery after an acid load imposed by 20 mM NH_4Cl followed by removal of Na^+ from the Krebs' modified buffer solution (in mM: NaCl 140, KCl 5.4, $CaCl_2$ 2.8, $MgSO_4$ 1.2, NaH_2PO_4 0.3, HEPES 10, glucose 5, and pH 7.4) in the absence of CO_2/HCO_3. In these experiments NaCl was replaced by an equimolar concentration of tetramethylammonium chloride (TMA). In intracellular pH measurement experiments, WKY and SHR PTE cells were grown in 96 well plates. The cell culture medium was aspirated and the cell monolayers were incubated for 30 min with 10 μM BCECF/AM, the membrane-permeant acetoxymethyl ester derivative of 2′,7′-bis (carboxyethyl)-5,6-carboxyfluorescein (BCECF) at 37°C in 5% CO_2–95% air atmosphere. Cells were placed in the sample compartment of a dual-scanning microplate spectrofluorometer (Spectramax Gemini XS, Molecular Devices, Sunnyvale, USA), and fluorescence was measured every 17 s alternating between 440 and 490 nm excitation at 535 nm emission, with a cutoff filter of 530 nm. The ratio of intracellular BCECF fluorescence at 490 and 440 nm was converted to pH_i values by comparison with values from an intracellular calibration curve using the nigericin (10 μM) and high-K^+ method [21, 22].

Intracellular buffering capacity was determined from the pH_i response to the removal of NH_3/NH_4^+ using the following formula: intracellular buffering capacity (βI)=$\Lambda[NH_4^+]_i/\Lambda pH_i$ where $[NH_4^+]_i$ is the intracellular concentration just before NH_3/NH_4^+ removal, calculated as $[NH_4^+]_i=[NH_4^+]_0 \times 10^{(7.4\text{-}pHi)}$ and ΛpH_i is the pH_i change on removal of NH_4^+ [23].

In experiments aimed to evaluate the sensitivity of the Na^+-dependent pH_i recovery to selective inhibitors of NHE isoforms, cells were treated with increasing concentrations of 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA) or vehicle for 0.5 h before starting the Na^+-dependent pH_i recovery.

Reverse transcription (RT)-PCR

To identify the presence of the MR, immortalized renal PTE WKY and SHR samples were examined by Reverse trancriptase-PCR analysis. Cells were homogenized (Diax, Heidolph) in Trizol Reagent (75 mg/ml; Invitrogen) and total RNA was extracted according to manufacturer's instructions. The RNA preparation was further treated with DNase (Ambion), to eliminate potential genomic DNA contamination. Reverse transcription was performed with SuperScript First Strand System for RT-PCR (Invitogen), using 5 μg/μl random hexamers as primers at 50°C, according to manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA in a total volume of 20 μl.

PCR was performed on 5 μl of cDNA using degenerate pairs of primers (Sigma Genosys) based on rattus and canis sequences (for MR DQ195096 and NP_037263) as described in Table 1. PCR reactions were carried out in 50-μl final solutions (5 μl of 10× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM of each primer, 2.5 units of Taq polymerase (Invitrogen)). Cycling conditions were as follows: (1) denaturation: 94°C, 2 min; (2) denaturation: 94°C, 30 s; annealing: 55°C (for MR) and 57°C (for 11β-HSD1), 30 s; and extension: 72°C, 30 s for 35 cycles; and (3) final extension: 72°C, 7 min.

NHE expression

In order to determine the expression of NHE1, NHE2, and NHE3 in WKY and SHR PTE cells, cells were cultured to 90% of confluence were washed twice with PBS and total cell protein extracted. Cells were lysed by brief sonication (15 s) in lysis buffer with protease inhibitors (150 mM NaCl, 50 mM Tris–HCl pH 7.4, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF aprotinin and leupeptin 2 μg/ml each) and incubated on ice for 1 h. After centrifugation (14,000 r.p.m., 30 min, 4°C), the supernatant was mixed in 6× sample buffer (0.35 M Tris–HCl, 4% SDS, 30% glycerol, 9.3% DTT, pH 6.8, 0.01% bromphenol blue) and boiled for 5 min. The proteins (30 μg) were subjected to SDS-PAGE (10% SDS-polyacrylamide gel) and electrotransfered onto nitrocellulose membranes. The transblot sheets were blocked with 5% of non-fat dry milk in Tris·HCl 25 mM pH 7.5, NaCl 150 mM and 0.1% Tween 20, overnight at 4°C. Then, the membranes were incubated with appropriately diluted antibodies: rabbit anti-NHE1, rabbit anti-NHE2 polyclonal isoform specific antibodies (Chemicon International) or the rabbit polyclonal anti-NHE3 antibody [24] and mouse anti-Actin (Santa Cruz Biotechnology). The immunoblots were subsequently washed and incubated with 0.5 μg/ml of fluorescently labeled goat anti-rabbit or goat anti-mouse secondary antibody (IRDyeTM 800 or IRDyeTM 680, Rockland Immunochemicals, Gilbertsville, PA) for 1.30 h at room temperature and protected from light. Membranes were washed and imaged by scanning at 800 or 700 nm with an Odyssey Infrared Imaging System (LI-COR Biosciences). Protein concentration was measured using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin as standard.

Measurement of H_2O_2

H_2O_2 was measured fluorometrically using the Amplex™ Red Hydrogen Peroxide Assay Kit (Molecular Probes, Inc., Eugene, OR). Amplex™ Red is a fluorogenic substrate with very low background fluorescence that reacts with H_2O_2 with a 1:1 stoichiometry to produce a highly fluorescent reagent [25]. Measurement of H_2O_2 was evaluated both directly by H_2O_2 released form the WKY and SHR monolayer cultured in 96 well plates or by H_2O_2 accumulated in the extracellular medium during 24 h after cells achieved confluence. Fluorescence intensity was measured in multiplate reader (Spectromax Gemini, Molecular Devices) at an excitation wavelength of 530 nm and emission wavelength of 590 nm at room temperature. After subtracting background fluorescence, the concentration of H_2O_2 was calculated using a resorufin-H_2O_2 standard calibration curve generated from experiments using H_2O_2 and Amplex™ Red.

Data analysis

Geometric means are given with 95% confidence limits and arithmetic means are given with SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Student's *t*-test or the Newman–Keuls test for multiple comparisons. A *P* value less than 0.05 was assumed to denote a significant difference.

Drugs

Aldosterone, apocynin, spironolactone, Ru 38486, actinomycin D, cytochalasin B, and EIPA were purchased from

Table 1 Sequences of the degenerated oligonucleotides used for RT-PCR

Target gene (Rat)	GeneBank accession no	Primer	Sequence (5′–3′)	Position	Product
MR	NP_037263	Forward	5′CCA GAT GG(A/G) GCT TT(C/T) AG3′	1006	732
		Reverse	5′GCA (A/G)TC ATT TCT TCC AGC ACA 3′	1738	

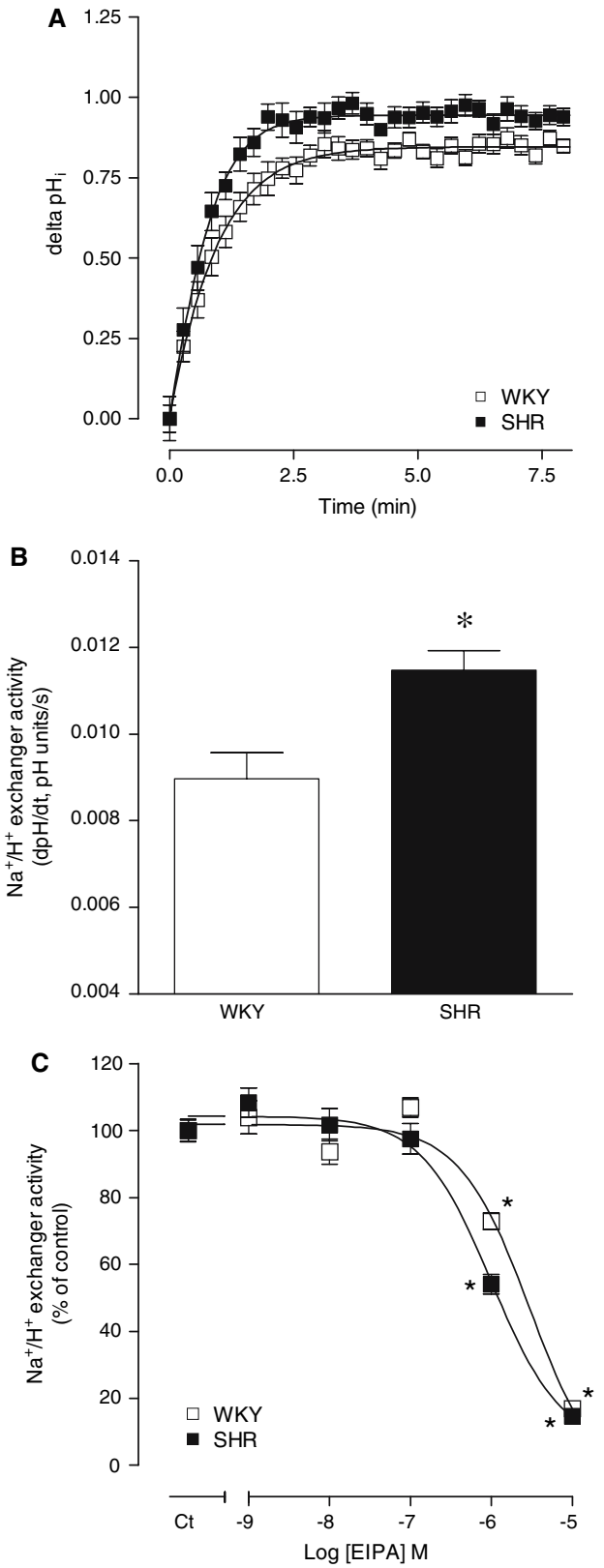


Fig. 1 Assessment of (**a**) delta intracellular pH and (**b**) NHE activity ▸ under V_{max} conditions as the initial rate of Na^+-dependent pH_i recover after an acid load imposed by exposure to NH_4Cl followed by Na^+ removal of the perfusion medium in immortalized WKY and SHR PTE cells. Influence of (**c**) EIPA (0.001–10 μM) on NHE activity in WKY and SHR PTE cells. Traces represent means of 9–14 experiments per group. Columns represent the mean of 9–15 independent determinations: vertical lines show SEM. Significantly different from values in WKY cells (*$P < 0.05$)

Sigma Chemical Company, St. Louis, MO, USA. Acetoxymethyl ester of 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM) nigericin and the Amplex™ Red Hydrogen Peroxide Assay Kit were obtained from Molecular Probes (Eugene, OR). S3226 and Cariporide were kindly provided by Dr. Jurgen Punter (Aventis Pharma).

Results

NHE activity and expression

NHE activity was assayed as the initial rate of pH_i recovery measured after an acid load imposed by 20 mM NH_4Cl followed by removal of Na^+ from the Krebs modified buffer solution, in the absence of CO_2/HCO_3. The cells were incubated in Krebs solution for 15 min, then an acid load was imposed during 5 min and subsequently the NH_4Cl was aspirated and the cells were placed into TMA solution for an additional 5 min. As shown in Fig. 1a, the Na^+-dependent recovery of pH_i in SHR cells was more pronounced than that observed in WKY cells. The pH_i recovery rates (in dpH_i/dt, pH units/s) during the linear phase of pH_i recovery after intracellular acidification in SHR were greater than in WKY cells (Fig. 1b).

The sensitivity of NHE to inhibition by EIPA, an effective NHE1 inhibitor and moderate NHE2, and NHE3 inhibitor, was evaluated in SHR and WKY immortalized PTE cells (Fig. 1c). The inhibition produced by EIPA was found to be more potent in SHR than in WKY. At the concentration of 100 nM, EIPA did not affect pH_i recovery, therefore being able to inhibit NHE1 and leave NHE2, and NHE3 unaltered.

The expression of NHE1, 2 and 3 was also evaluated in immortalized renal PTE cells from SHR and WKY. As shown in Fig. 2c, the level of expression of NHE3 in the SHR cells was greater than in WKY. However, in both cell lines NHE1 and NHE2 expression was equal (Fig. 2a and b).

Effect of aldosterone on NHE activity

Since NHE activity is an important mechanism for the maintenance and regulation of pHi in the proximal tubules we next examined the effects of aldosterone on the NHE transporter. Cells were exposed to control DMEM or aldosterone-supplemented DMEM at various concentrations (0.01, 0.1, and 1 μM) for 72 h. As shown in Fig. 3a,

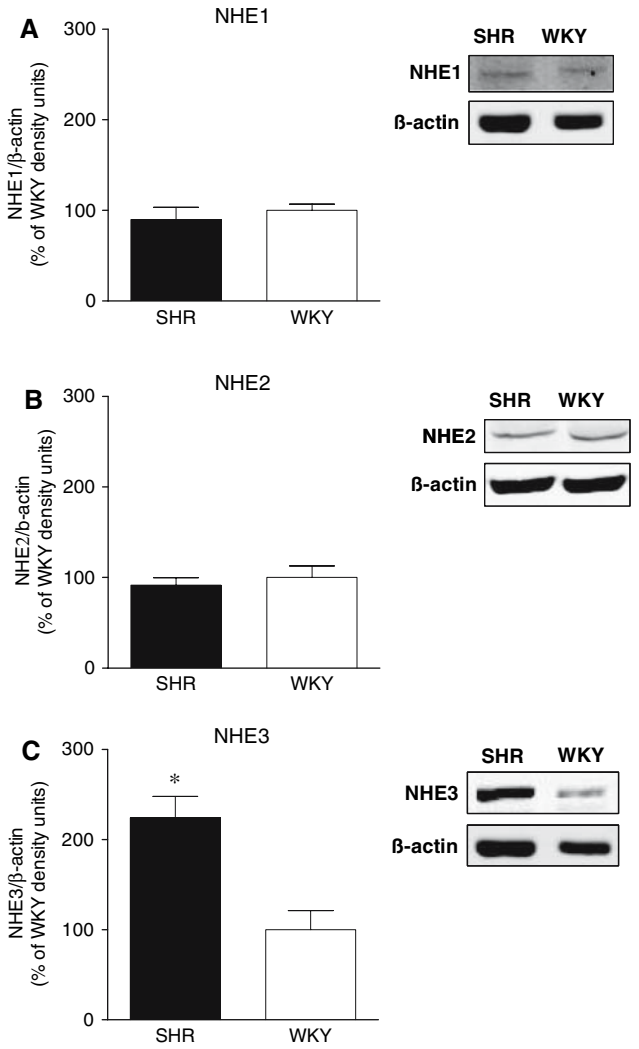


Fig. 2 Immunoblot analysis of (**a**) NHE1, (**b**) NHE2, and (**c**) NHE3 in immortalized WKY and SHR PTE cells. Each lane contains equal amount of protein (30 μg). Significantly different from corresponding values for WKY PTE cells (*$P < 0.05$)

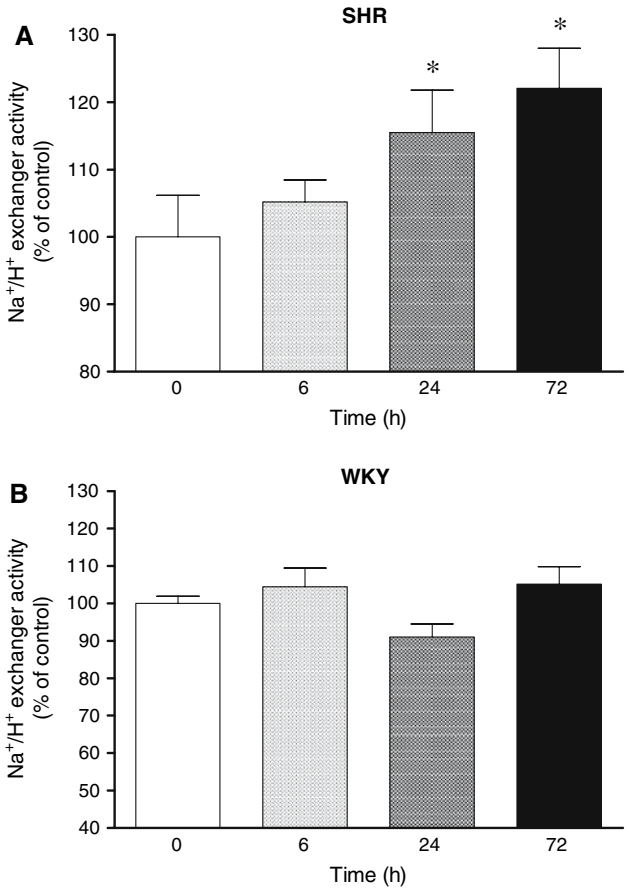


Fig. 3 Effect of aldosterone (0.01, 0.1, and 1 μM) for 72 h on NHE activity in (**a**) SHR PTE cells and (**b**) WKY PTE cells. In WKY PTE cells, the pH_i values in vehicle- and aldosterone-treated were, respectively, 7.51 ± 0.01 and 7.48 ± 0.02. In SHR PTE cells the pH_i values in vehicle- and aldosterone-treated were, respectively, 7.63 ± 0.02 and 7.66 ± 0.03. Each column represents the mean of 7–8 experiments per group; vertical lines indicate SEM. Significantly different from values for control (*$P < 0.05$)

the aldosterone-induced increase in NHE activity was a concentration-dependent effect; treatment with aldosterone 0.1 and 1 μM significantly increased NHE activity (19 ± 5 and 22 ± 6% increase, respectively) in SHR PTE cells; however, no stimulation was observed in immortalized WKY PTE cells (Fig. 3b). We next examined the time-dependent effects in the NHE activity induced by aldosterone (1 μM) in SHR and WKY PTE cells. Cells were exposed to aldosterone (1 μM) for 6, 24, and 72 h. Significant stimulatory effects on NHE activity were observed for 24 and 72 h (16 ± 6 and 21 ± 6% increase, respectively) in SHR PTE cells but not in WKY PTE cells (Fig. 4a and b).

There were no significant differences on the intrinsic buffering capacity at pH_i of 7.6 between the two conditions (vehicle, 16.02 ± 0.97 mM/H^+, pH, $n = 8$ and aldosterone (72 h) 14.09 ± 0.68 mM/H^+, pH, $n = 7$) in SHR PTE cells. Therefore, the Na^+-dependent pH_i recovery after acid load appears to be dependent on the NHE activity and the aldosterone-induced increases in the Na^+-dependent pH_i recovery after acid load may not relate changes in differences on the intrinsic buffering capacity, but rather on increases in NHE activity.

Detection of MR

To confirm the presence of the mineralocorticoid receptor, MR in immortalized WKY and SHR PTE cells, a conventional RT-PCR was performed. Degenerate primer sets, designed based on the MR canine and rattus sequences, were used. As shown in Fig. 5, the expected 732-bp fragment corresponding to MR was present in both cell lines.

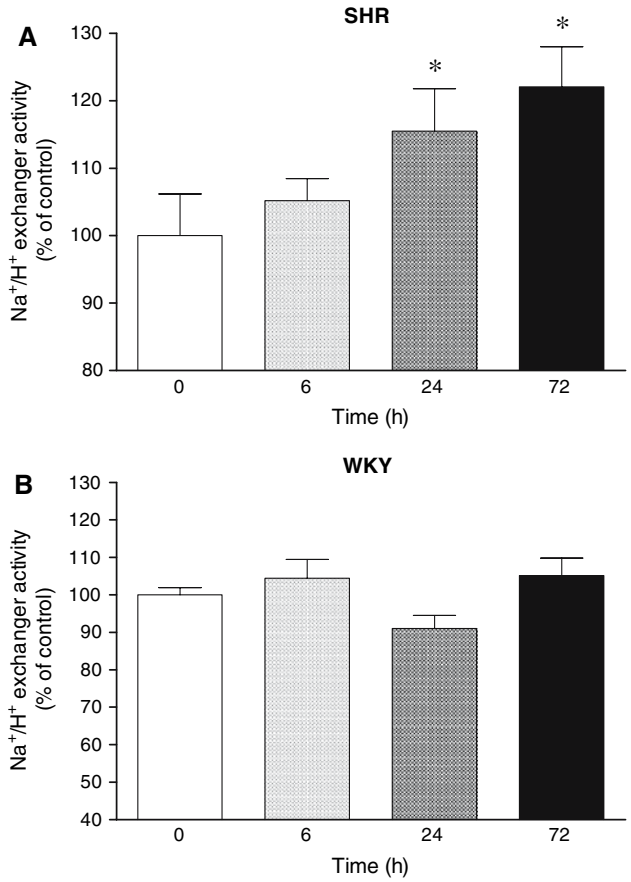


Fig. 4 Effect of aldosterone (1 μM) for 6, 24, and 72 h on NHE activity in (**a**) SHR PTE cells (**b**) and WKY PTE cells. Each column represents the mean of 7–8 experiments per group; vertical lines indicate SEM. Significantly different from values for control (*$P < 0.05$)

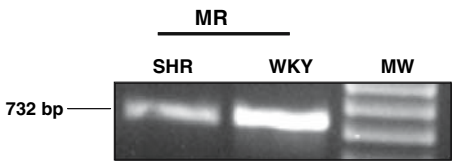


Fig. 5 PCR detection of MR in total RNA extracted from WKY and SHR PTE cells; MW—GeneRulerTM DNA Ladder Mix (MBI, Fermentas)

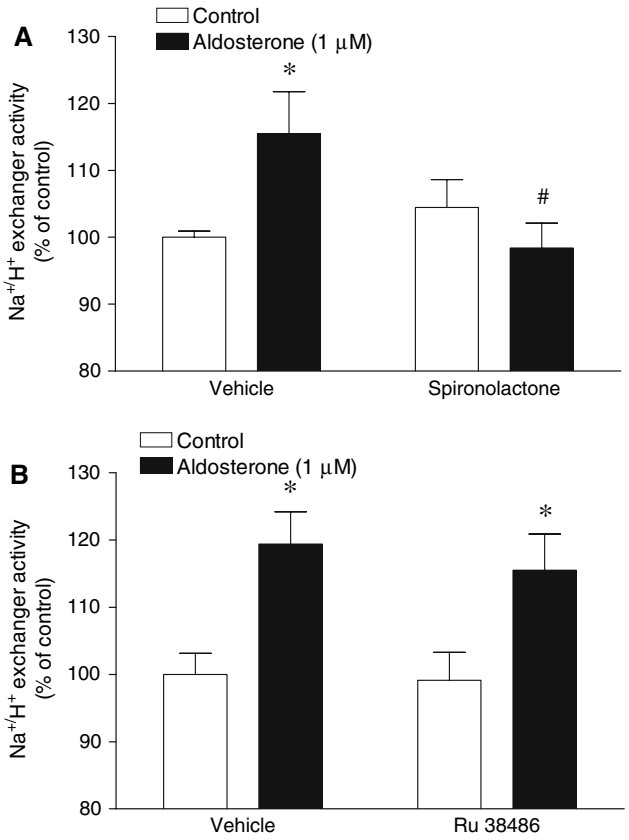


Fig. 6 Effects of the mineralocorticoid receptor antagonist sipironolactone or the glucocorticoid receptor antagonist Ru 38486 on aldosterone-induced increase in NHE activity in SHR PTE cells. (**a**) Cells were exposed to control DMEM or aldosterone (1 μM) supplemented DMEM for 24 h in the absence or presence of spironolactone (100 μM). (**b**) Cells were exposed to control DMEM or aldosterone (1 μM) supplemented DMEM for 24 h in the absence or presence of Ru 38486 (10 μM). Each column represents the mean of 7–8 experiments per group; vertical lines indicate SEM. Significantly different from values for control (*$P < 0.05$)

Effects of spironolactone and Ru 38486 on aldosterone-induced increase in NHE activity

To determine whether the stimulatory effects of aldosterone on NHE activity occurred via the MR and/or the glucocorticoid receptor (GR), SHR PTE cells were exposed to the MR antagonist spironolactone (100 μM) or the GR antagonist Ru 38486 (10 μM) for 24 h. The concentrations of spironolactone and Ru 38486 used were those previously described in the literature [26]. Results are shown in Fig. 6a for spironolactone-treated SHR PTE cells and Fig. 6b for Ru 38486-treated SHR PTE cells. Spironolactone or Ru alone caused no effect on NHE activity. The addition of Ru 38486 produced no effect on the long term stimulatory effect of aldosterone on NHE activity whereas spironolactone completely inhibited the aldosterone-induced stimulation.

Determination of the NHE transporter isoform involved in the aldosterone-induced increase in activity

In order to identify the NHE transporter isoform involved in the aldosterone-induced increase in activity SHR cells were incubated with aldosterone (1 μM) for 24 h and EIPA (100 nM), cariporide (1 μM), a selective NHE1 inhibitor or

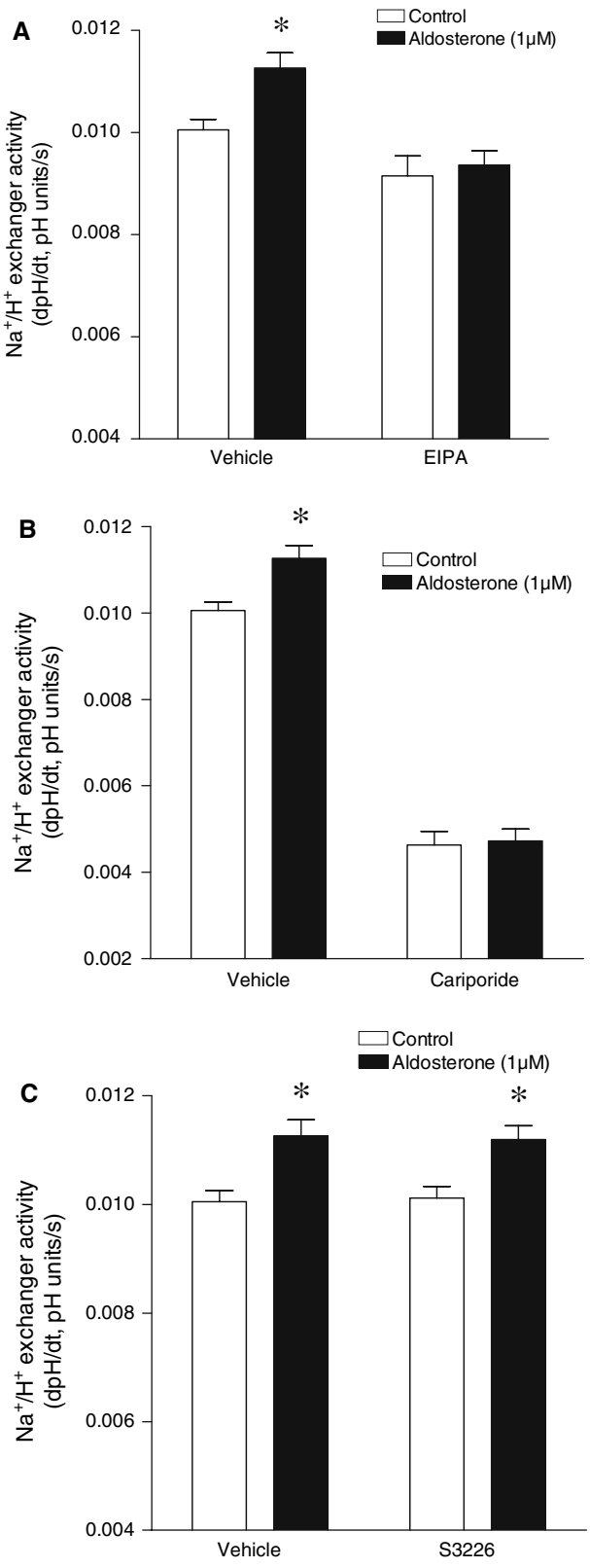


Fig. 7 Effects of (**a**) EIPA (100 nM), (**b**) Cariporide (1 μM) and (**c**) ▶ S3226 (1 μM) on NHE activity in the absence and presence of aldosterone (1 μM) for 24 h in SHR PTE cells. Each column represents the mean of 7–8 experiments per group; vertical lines indicate SEM. Significantly different from values for control (*$P < 0.05$)

S3226 (1 μM), a NHE3 inhibitor for 30 min. EIPA and cariporide completely reversed the effects of aldosterone upon the NHE activity (Fig. 7a and b), but not S3226 (Fig. 7c), indicating that NHE1 is the most likely involved exchanger isoform.

Effects of actinomycin D and cytochalasin B on aldosterone-induced increase in NHE activity

To determine whether gene transcription is required for aldosterone-induced increase in NHE activity, SHR cells were incubated with actinomycin D (400 nM), an inhibitor of gene transcription and aldosterone (1 μM) for 24 h. Recent studies demonstrate that the interaction between the cytoskeleton and the plasma membrane regulates the activity of many ion channels and transport proteins, including the NHE transporters. To determine whether the cytoskeleton is involved in the aldosterone stimulation of the NHE, SHR cells were incubated with cytochalasin B (1 μM), a disruptor of filamentous actin (F-actin). For this purpose, SHR cells were treated during 24 h with aldosterone (1 μM) and cytochalasin B for 3 h. As shown in Fig. 8, actinomycin D (Fig. 8a) and cytochalasin B (Fig. 8b) had no influence on NHE activity. However, both drugs attenuated the stimulatory effect of aldosterone upon NHE activity, though this did not attain statistical significance.

Aldosterone effect on NHE1 protein expression

Protein expression of NHE1 in SHR PTE cells treated for 24 h with aldosterone (1 μM) and aldosterone (1 μM) plus spironolactone (10 M) was evaluated by immunoblot analysis. The protein expression levels of NHE1 from aldosterone-treated cells were not significantly different from control (Fig. 9a and b).

Role of oxidative stress in the mediation of aldosterone-induced increase in NHE activity

H_2O_2 generation was measured in SHR and WKY PTE cells and the involvement of H_2O_2 in the regulation of NHE by aldosterone was determined. The SHR PTE cells revealed an increased rate of H_2O_2 formation when compared with WKY PTE cells (22.4 ± 0.9 vs. 7.9 ± 0.3 nmoles/min) (Fig. 10a). Cells were also treated with apocynin (0, 10, 30, and 100 μM) for 4 days and the rate of H_2O_2 production was determined. WKY PTE cells were less sensitive to apocynin than SHR PTE cells

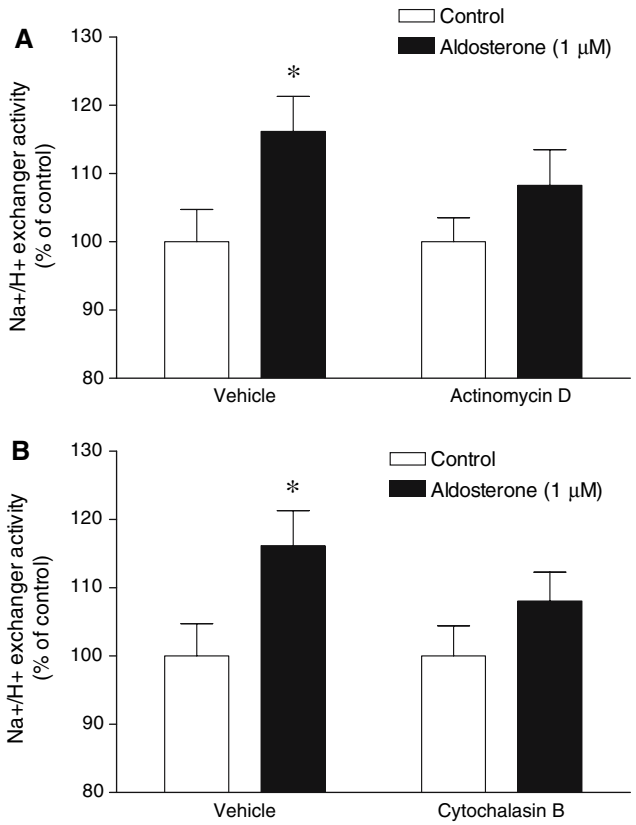


Fig. 8 Effects of (**a**) actinomycin D (400 nM) and (**b**) cytochalasin B (1 μM) on aldosterone-induced increase in NHE activity in SHR PTE cells. Cells were exposed to control DMEM or aldosterone 1 μM for 24 h in the absence or presence of actinomycin D 400 nM supplemented DMEM for 24 h. Each column represents the mean of 7–8 experiments per group; vertical lines indicate SEM. Significantly different from values for control (*$P < 0.05$)

(Fig. 10b); inhibition of H_2O_2 production in WKY PTE cells was only obtained at 100 μM apocynin, whereas in SHR PTE cells 30 μM apocynin was enough to inhibit this production in SHR PTE cells (Fig. 10c). Treatment of cells with apocynin (100 μM), an inhibitor of the NADPH oxidase complex, for 4 days reduced the rate of H_2O_2 production in WKY and SHR PTE cells, but did not change the NHE activity in both WKY (0.0065 ± 0.0002 vs. 0.0054 ± 0.0005 pH units/s) and SHR (0.0075 ± 0.0002 vs. 0.0081 ± 0.0002 pH units/s) PTE cells. However, treatment of SHR PTE cells with apocynin (100 μM) completely blocked the ability of aldosterone to increase NHE activity (Fig. 11). Treatment of cells with aldosterone (1 μM) for 24 h did not change the rate of H_2O_2 production (Fig. 12). Differences in the inhibitory effect apocynin (100 μM) on the production of H_2O_2 in the absence and the presence of aldosterone (1 μM) did not attain statistical significance (Fig. 12).

Discussion

The present study was designed to evaluate the effects of aldosterone on NHE activity in immortalized renal PTE cells from SHR and WKY and to determine the mechanisms whereby aldosterone regulates NHE activity in these cells. The aldosterone-mediated increase in NHE activity was a concentration- and time-dependent phenomenon in SHR PTE cells. The data presented here also demonstrates that the aldosterone-induced increase in NHE1 activity in immortalized renal PTE cells from SHR, but not in WKY, occurs as early as 24 h and is sustained effect for up to 72 h. The effects of aldosterone upon NHE1 activity occur through the activation of genomic mechanisms involving the stimulation of the MR, but require the availability of H_2O_2 in excess.

In order to explain the differences in sensitivity to aldosterone in WKY and SHR immortalized PTE cells, it was hypothesized that oxidative stress could be involved in such differences. In the present study, the generation of H_2O_2, a marker of oxidative stress, was evaluated in WKY and SHR PTE cells. SHR PTE cells were found to have an increased rate of H_2O_2 production. Apocynin specifically

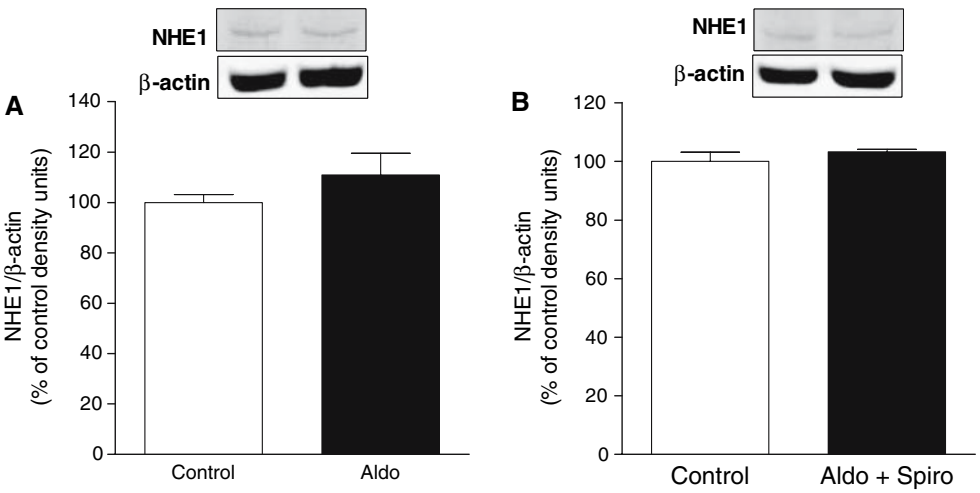
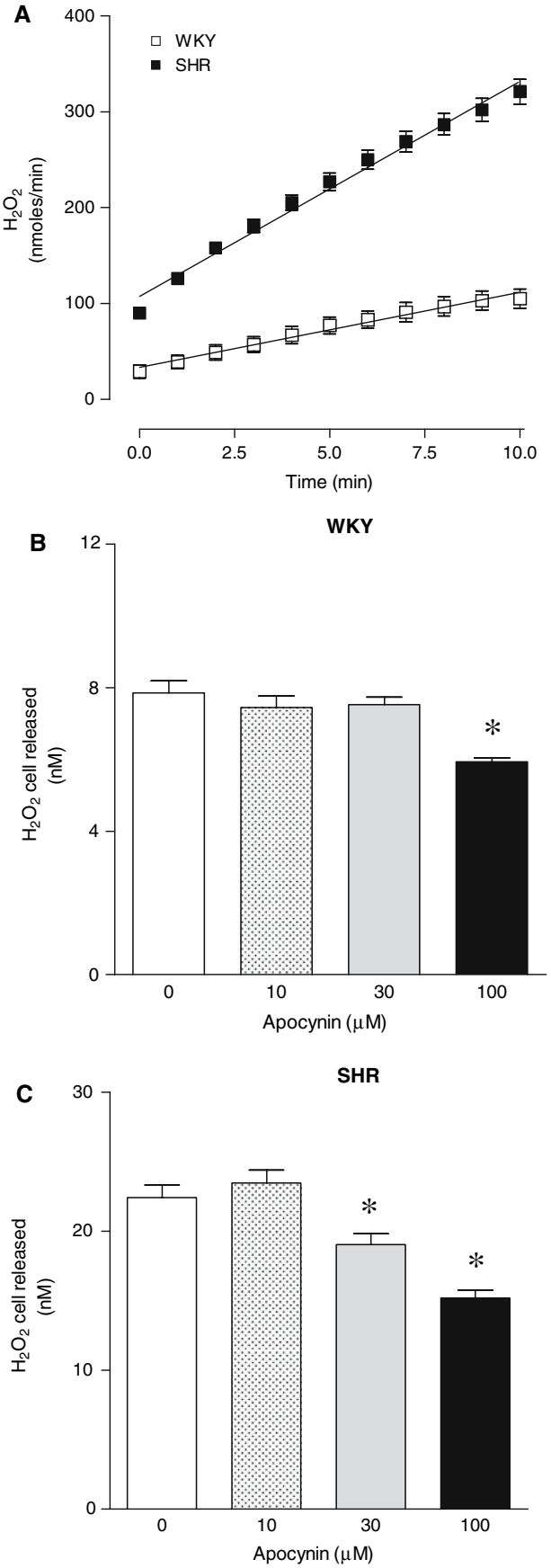


Fig. 9 Immunoblot analysis of NHE1 in immortalized SHR PTE cells and in after 24 h treatment with aldosterone (Aldo) (1 μM) or/and aldosterone and spironolactone (Aldo+Spiro). Each lane contains equal amount of protein (30 μg), western blotting was repeated 3 times



◀ **Fig. 10** (**a**) Rate of H_2O_2 (nM/min) released from WKY or SHR cell culture when cells reached confluence (4 days after of seeding). Levels of extracellular H_2O_2 (nM) in (**b**) WKY and (**c**) SHR PTE cells in control cell culture conditions or in the presence of apocynin (4 days after seeding). Each column represents the mean of 4 experiments per group; vertical lines indicate SEM. Significantly different from values for control (*$P < 0.05$)

inhibits the activity of NAD(P)H oxidase by interfering with the assembly of the cytosolic NAD(P)H oxidase components ($p40^{phox}$, $p47^{phox}$, and $p67^{phox}$) with the membranous components $gp91^{phox}$ and $p22^{phox}$ [27]. The concentration of apocynin that reduced the extracellular levels of H_2O_2 in SHR was lower (30 μM) than that in WKY (100 μM), which fits well the observation that the level oxidative stress in SHR may be higher than in WKY PTE cells. In SHR PTE cells, treatment with apocynin abolished the stimulation of aldosterone upon the NHE1 activity in SHR PTE cells. According to these results, the effects of aldosterone upon NHE1 activity in SHR PTE cells are a consequence of the oxidative stress, resulting from increases in the generation of H_2O_2. Although previous studies have indicated that aldosterone directly induces ROS generation through the activation of NADPH oxidase [17, 18], we gathered evidence that aldosterone did not increased the production of H_2O_2.

The results presented here show that aldosterone-induced increase in NHE1 activity in SHR PTE cells was partially inhibited by actinomycin D, an inhibitor of gene transcription and cytochalasin B, a disruptor of filamentous actin (F-actin). This data suggests that the long-term effect of aldosterone on NHE1 activity might be related to a genomic effect. On the other hand, it is likely that aldosterone might facilitate a "shuttle" process where NHE1 is translocated to the plasma membrane. Since the aldosterone-induced increase in NHE1 activity was prevented by apocynin, it is likely that increases in H_2O_2 production may promote the translocation of NHE1 to the plasma membrane induced by aldosterone or facilitate the intracellular events induced by the activation of the MR. These aspects are currently under evaluation in our laboratory.

Aldosterone enters the cell by diffusion through the cellular membrane and once in the cytoplasm it binds to the intracellular MR receptor. In the present study, mRNA of MR was detected in WKY and in SHR PTE cells indicating that aldosterone has the potential to bind to the MR, and activate NHE; however, only SHR PTE cells responded to aldosterone. It is clear that the mechanism responsible for the activation of NHE by aldosterone in SHR PTE cells is different from WKY PTE cells. The long term effect of aldosterone was inhibited by the MR antagonist spironolactone, which indicates that the effect of aldosterone occurs through the MR. High concentrations of aldosterone (1 μM) may activate both the MR and the GR, as reported

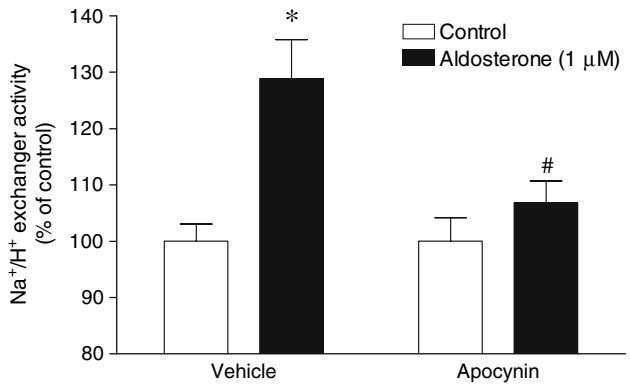


Fig. 11 Effect of apocynin treatment (4 days) on aldosterone-induced NHE activity in SHR PTE cells. Each column represents the mean of 8 experiments per group; vertical lines indicate SEM. Significantly different from values for control ($^{*}P < 0.05$) or values for apocynin alone ($^{\#}P < 0.05$)

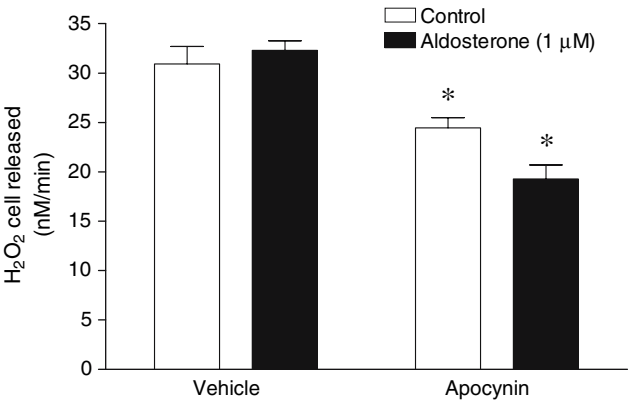


Fig. 12 Effect of aldosterone (1 μM for 24 h) on production of H_2O_2 in SHR PTE cells in the absence and the presence of apocynin (4 days). Each column represents the mean of 8 experiments per group; vertical lines indicate SEM. Significantly different from values for control ($^{*}P < 0.05$)

by Ebata et al. [26]. However, in the present study aldosterone (1 μM) most likely was devoid of effects upon the GR, as revealed by the finding that Ru 38486 did not prevent the aldosterone-induced stimulation of NHE.

WKY and SHR PTE cells were found to express all three NHE isoforms. NHE1 expression was identical in both cell lines, whereas NHE3 in SHR cells was more abundant than in WKY PTE cells, which fits well the data by Kelly et al. [28] in freshly isolated PTE cells from SHR and WKY. However, according to Pedrosa et al. [29], NHE1 in immortalized SHR PTE cells is significantly more abundant than in WKY PTE cells. Recent studies have shown that serum deprivation leads to an increase in NHE1 mRNA and protein expression in immortalized PTE cells [30], which may offer an explanation for this apparent discrepancy. It is possible that immortalized SHR PTE cells are more affected by serum deprivation than WKY PTE cells and, therefore, have a high expression of NHE1 in serum deprivation conditions. NHE2 was also detected in WKY and SHR PTE cells and its expression was identical in both cell lines. NHE2 is also expressed in other proximal tubular cell lines for example MCT and RKPC2 [30].

Previous studies in other cell types, namely vascular smooth muscle cells, have reported genomic effects of aldosterone through NHE1 [26]. In order to identify the NHE isoform involved, 100 nM EIPA was used to selectively inhibit the NHE1 isoform. This concentration of EIPA completely reversed the stimulatory effects of aldosterone upon NHE activity. To confirm this result, cariporide, a selective NHE1 inhibitor and S3226, a NHE3 inhibitor, were also used. As predicted, cariporide inhibited the stimulation produced by aldosterone, whereas S3226 was devoid of effects. In hypertension, there is evidence that NHE1, the isoform most frequently studied, exhibits increased activity in a large variety of cell types. In several examples of increased NHE1 activity, the primary change is not in NHE1 expression, but in post-translational regulation of activity. In immortalized cells from hypertensive patients, enhanced NHE1 activity appears to reflect increased V_{max} rather than increased NHE1 mRNA [31]. Similarly, vascular smooth muscle cells from SHR were found to be endowed with increased NHE1 activity as evidenced by increases in V_{max}, compared to WKY, without changes in NHE1 mRNA levels or protein abundance [32]. These observations are in agreement with the results presented here. The present study shows that total protein levels of NHE1 were not increased in the presence of aldosterone. It has also been reported that increased extracellular sodium concentration together with the prolonged treatment with aldosterone may contribute to the increase in NHE1 activity. In fact, Yamamuro et al. [33] showed an apparent cardiomycocyte hypertrophy caused by long term exposure to aldosterone in the presence of elevated extracellular sodium concentration which is thought to occur via NHE1 with the involvement of the MR. The medium used in the present study was the ideal for SHR and WKY cells; however, it is possible that the aldosterone-stimulated increase of NHE1 activity in SHR PTE cells may be also effected by extracellular sodium concentration.

In conclusion, we demonstrate that aldosterone increases NHE1 activity under V_{max} conditions through the activation of the MR in immortalized SHR PTE cells, but not in WKY PTE cells. This activation occurs through genomic mechanisms and requires the availability of H_2O_2 in excess. High blood pressure is a major clinical issue in patients afflicted with primary or secondary aldosteronism. However, the precise mechanisms of blood pressure elevation in mineralocorticoid excess are still unclear. The present findings, together with previous reports, show that enhanced NHE1 activity might have an important role in the pathogenesis of hypertension.

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ERRATUM

Erratum to: Oxidative stress and the genomic regulation of aldosterone-stimulated NHE1 activity in SHR renal proximal tubular cells

Vanda Pinto · Maria João Pinho · Ulrich Hopfer · Pedro A. Jose · Patrício Soares-da-Silva

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In the original article, Fig. 3 was not displayed correctly. Everything else in the paper remains correct.

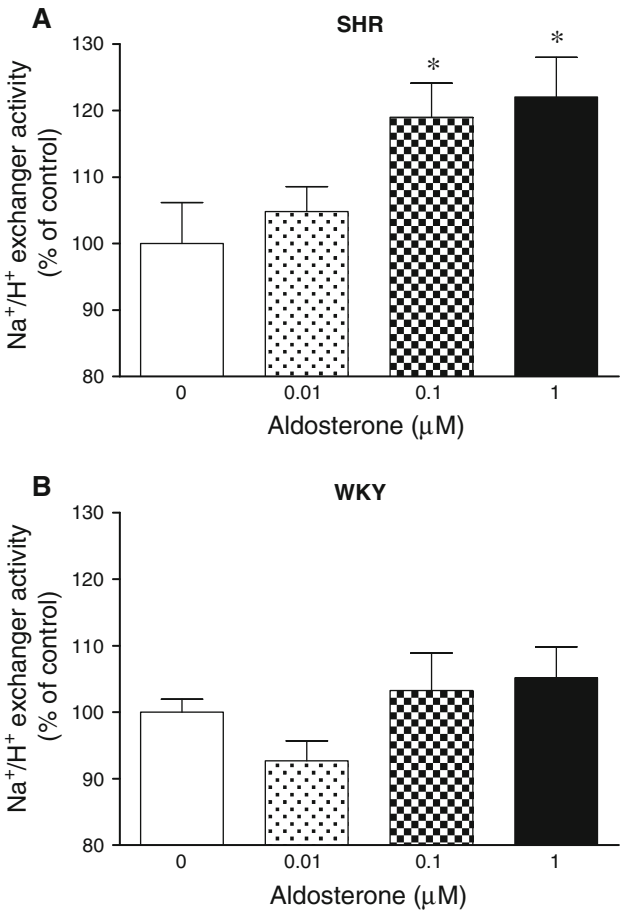

Fig. 3 Effect of aldosterone (0.01, 0.1, and 1 μM) for 72 h on NHE activity in (**a**) SHR PTE cells and (**b**) WKY PTE cells. In WKY PTE cells, the pH_i values in vehicle- and aldosterone-treated were, respectively, 7.51 ± 0.01 and 7.48 ± 0.02. In SHR PTE cells, the pH_i values in vehicle- and aldosterone-treated were, respectively, 7.63 ± 0.02 and 7.66 ± 0.03. Each *column* represents the mean of 7–8 experiments per group; *vertical lines* indicate SEM. Significantly different from values for control (*$P < 0.05$)

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V. Pinto · M. J. Pinho · P. Soares-da-Silva (✉)
Faculty of Medicine, Institute of Pharmacology and Therapeutics, Porto 4200, Portugal
e-mail: pss@med.up.pt

U. Hopfer
Department of Physiology and Biophysics, Case Western Reserve School of Medicine, Cleveland, OH, USA

P. A. Jose
Department of Pediatrics, Georgetown University, Washington, DC, USA

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CHAPTER II - The renal redox status of aged Wistar-Kyoto and spontaneously hypertensive rats

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Age-related changes in renal expression of oxidant and antioxidant enzymes and oxidative stress markers in male SHR and WKY rats

Sónia Simão, Pedro Gomes [1], Vanda Pinto, Elisabete Silva, João S. Amaral, Bruno Igreja [2], Joana Afonso, Maria Paula Serrão, Maria João Pinho, Patrício Soares-da-Silva *

Institute of Pharmacology and Therapeutics, Faculty of Medicine, University of Porto, Alameda Prof. Hernâni Monteiro, 4200-319 Porto, Portugal

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ABSTRACT

Oxidative stress has been hypothesized to play a role in aging and age-related disorders, such as hypertension. This study compared levels of oxidative stress and renal expression of oxidant and antioxidant enzymes in male normotensive Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHR) at different ages (3 and 12 months). In the renal cortex of 3-month old SHR increases in hydrogen peroxide (H_2O_2) were accompanied by augmented expression of NADPH oxidase subunit Nox4 and decreased expression of antioxidant enzymes SOD1 and SOD3. A further increase in renal H_2O_2 production and urinary TBARS was observed in 12-month old WKY and SHR as compared with 3-month old rats. Similarly, expressions of NADPH oxidase subunit $p22^{phox}$, SOD2 and SOD3 were markedly elevated with age in both strains. When compared with age-matched WKY, catalase expression was increased in 3-month old SHR, but unchanged in 12-month old SHR. Body weight increased with aging in both rat strains, but this increase was more pronounced in WKY. In conclusion, renal oxidative stress in 12-month old SHR is an exaggeration of the process already observed in the 3-month old SHR, whereas the occurrence of obesity in 12-month old normotensive rats may partially be responsible for the age-related increase in oxidative stress.

1. Introduction

Aging is a degenerative process affecting all living organisms. Among the many theories that have been put forward to explain the aging process, the free radical theory has received widespread attention in recent years. This theory postulates that endogenous oxygen radicals cause a pattern of cumulative damage that is responsible for the functional deterioration associated with aging (Harman, 1956). The current view is that oxidative stress, resulting from excessive production of reactive oxygen species (ROS) and/or inadequate antioxidant defense mechanisms, may be mechanistically linked to some aspects of aging and a multitude of age-associated disorders (Finkel and Holbrook, 2000; Kregel and Zhang, 2007). ROS can either be generated exogenously or produced intracellularly from several different sources. Enzymatic systems contributing to oxidative stress include, among others, the superoxide-generating enzyme, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Griendling et al., 2000; Li et al., 2001). However, under physiological conditions, the burden of ROS production is largely neutralized by an intricate antioxidant defense system that includes low molecular weight antioxidants like glutathione, vitamins E, C and A, the enzymatic scavengers superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) (Finkel and Holbrook, 2000; Kregel and Zhang, 2007).

The spontaneously hypertensive rat (SHR) is a genetic model of naturally developing hypertension that appears to be similar in many aspects to human essential hypertension (Trippodo and Frohlich, 1981). It is becoming increasingly recognized that hypertension in SHR is associated with enhanced oxidative stress (Touyz, 2004; Wilcox, 2002). This assumption results from multiple lines of evidence, including measurements of oxidative stress markers in plasma, urine and tissue or protein levels and activity of oxidant and antioxidant enzymes in the kidney and vascular tissues of SHR in comparison with their genetic normotensive controls, the Wistar Kyoto (WKY) rats (Biswas et al., 2008; Kerr et al., 1999; Schnackenberg and Wilcox, 1999; Ulker et al., 2003; Zhan et al., 2004a). Nevertheless, experimental studies have provided conflicting results when the aforementioned parameters were

Abbreviations: DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPx, glutathione peroxidase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LDH, lactate dehydrogenase; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; Nox, NADPH oxidase; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SHR, spontaneously hypertensive rat; SOD, superoxide dismutase; TBS, tris buffer saline; TBST, TBS tween 20; WKY, Wistar Kyoto.

* Corresponding author. Tel.: +351 225 513 642; fax: +351 225 513 643.
E-mail address: pss@med.up.pt (P. Soares-da-Silva).
[1] Present address: Department of Biochemistry, Faculty of Medicine, University of Porto, 4200-319 Porto, Portugal.
[2] Present address: Department of Research and Development, BIAL—Portela & Cª, S.A., S. Mamede do Coronado, 4745-457, Portugal.

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analyzed. For example, Zhan et al. (2004a) reported a marked increase in immunodetectable $p22^{phox}$ subunit of NADPH oxidase in the renal cortex of SHR compared with that in the WKY; by contrast, Chabrashvili et al. (2002) reported that there was no detectable difference for $p22^{phox}$. On the other hand, data on the expression of oxidant and antioxidant enzymes in the aged SHR kidney are scarce.

The aim of the present study was, therefore, to assess oxidative stress and renal expression of oxidant and antioxidant enzymes in SHR and WKY and whether or not the expression of these enzymes may change in the kidney during aging.

2. Materials and methods

2.1. Animal preparation and experimental design

Five-week old male Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHR) were obtained from Harlan-Interfauna Ibérica (Barcelona, Spain). Animals were housed under controlled conditions (12 h light/dark cycle and room temperature at 22 ± 2 °C) and had free access to tap water and standard rat chow (PANLAB, Barcelona, Spain). The animals were carefully maintained and monitored until 12 months of age. Blood pressure (systolic and diastolic) and heart rate were measured in conscious animals using a photoelectric tail–cuff detector (LE 5000, Letica, Barcelona, Spain). A minimum of 5 measures were made each time and the mean values were used for further calculations. Both body weight and blood pressure measurements were performed at 4 to 6 week intervals.

2.2. Metabolic study

Two days before the start of experiments, 3- and 12-month old rats were placed in metabolic cages (Tecniplast, Buguggiate, Italy) for a 24 h urine collection. The urine samples were collected in sterilized vials that were stored at −80 °C until assayed. After completion of this protocol, rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). The animals were then sacrificed by exsanguination using cardiac puncture and the blood collected into tubes containing K_3 EDTA for later determination of plasma biochemical parameters. Before excising the kidneys, the right ventricle of the heart was perfused with ice-cold saline (0.9% NaCl) to remove all blood from the kidneys. The kidneys were then excised, weighed, decapsulated, and the renal cortex and medulla rapidly separated by fine dissection. Tissue pieces were immediately frozen in liquid nitrogen and stored at −80 °C until use.

2.3. Plasma and urine biochemistry

The quantification of sodium was performed by an ion-selective electrode. The analysis of non-fasting plasma creatinine, urinary creatinine, urinary proteins and serum lactate dehydrogenase (LDH) was performed on the Cobas Mira Plus automated analyzer (Roche Diagnostics, Germany) using standardized procedures (ABX Diagnostics for Cobas Mira, Switzerland). Creatinine clearance was calculated using 24-h urine creatinine excretion in absolute values (ml/min).

2.4. Quantification of adiposity levels

Adiposity of all rats was evaluated by the Lee index, which is well correlated with the percentage of body mass (Li et al., 1998; Ricci et al., 2006). The Lee index is calculated through the cubic root of body weight (g) divided by the naso-anal length (mm) times 10^4.

2.5. H_2O_2 production by renal cortex

Fresh renal cortex was cut into square pieces and incubated at 37 °C in Krebs–HEPES buffer (in mM: NaCl 118, KCl 4.5, $CaCl_2$ 2.5, $MgCl_2$ 1.20, K_2HPO_4 1.2, $NaHCO_3$ 25.0, Na–HEPES 25.0, and glucose 5; pH 7.4) for 90 min. The supernatant was then used in the Amplex Red Hydrogen Peroxide Assay kit (Molecular Probes Inc., Eugene, OR) in order to detect H_2O_2 released from the tissue, as previously described (Furukawa et al., 2004). Amplex Red is a fluorogenic substrate with very low background fluorescence that reacts with H_2O_2 with a 1:1 stoichiometry to produce a highly fluorescent reagent. Fluorescence intensity was measured in a multiplate reader (Spectromax Gemini; Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 530 nm and emission wavelength of 590 nm at room temperature. After subtracting background fluorescence, the concentration of H_2O_2 (in nmol/mg tissue) was calculated using a resorufin–H_2O_2 standard calibration curve generated from experiments using H_2O_2 and Amplex Red.

2.6. Measurement of TBARS

As a marker of lipid peroxidation, we measured thiobarbituric acid reactive substances (TBARS) according to the method of Ohkawa et al. (1979), with some modifications. Briefly, urine samples were combined with 8.1% SDS for 10 min. Equal volumes of 28% TCA and 0.6% TBA were added and heated at 95 °C for 1 h. After cooling at room temperature, a mixture of chloroform/methanol (2:1) was added and centrifuged at 5000 rpm for 10 min. Supernatant absorbance was measured at 532 nm. A calibration curve was prepared with malondialdehyde (MDA) as a standard and results were expressed as μmol MDA/24 h urine volume. All samples gave results which were within the linear range of the MDA standard curve.

2.7. Western blotting

Renal cortices were lysed in RIPA buffer containing 150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 2 μg/ml leupeptin and 2 μg/ml aprotinin. Protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin as standard. Homogenates were boiled in 2x sample buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 2% 50 mM DTT, 0.1% w/v bromophenol blue) at 95 °C for 5 min. Samples containing 25–100 μg of protein were separated by SDS-PAGE with 10% polyacrylamide gel and then electro-blotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Blots were blocked for 1 h with 5% non-fat dry milk in TBS at room temperature with constant shaking. Blots were then incubated with antibodies goat polyclonal anti-Nox4 (1:400, Santa Cruz Biotechnology, Santa Cruz, CA); rabbit polyclonal anti-$p22^{phox}$ (1:800, Santa Cruz Biotechnology); rabbit polyclonal anti-SOD1 (1:2000, Santa Cruz Biotechnology); goat polyclonal anti-SOD2 (1:100, Santa Cruz Biotechnology); goat polyclonal anti-SOD3 (1:500, Santa Cruz Biotechnology); rabbit polyclonal anti-catalase (1:2000, Calbiochem, Nottingham, UK); mouse monoclonal anti-glutathione peroxidase (1:1000, Calbiochem) and mouse monoclonal anti-GAPDH (1:60,000, Santa Cruz Biotechnology) in 5% non-fat dry milk in TBS-T overnight at 4 °C. The immunoblots were subsequently washed and incubated with fluorescently labeled goat anti-rabbit (1:20,000; IRDyeTM 800, Rockland, Gilbertsville, PA), fluorescently labeled donkey anti-goat (1:20,000; IRDyeTM 800, Rockland), or the fluorescently labeled goat anti-mouse secondary antibody (1:20,000; AlexaFluor 680, Molecular Probes, Paisley, UK) for 60 min at room temperature and were protected from light. The membranes were washed and imaged by scanning at both 700 and 800 nm, with an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

2.8. Drugs

All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise stated.

2.9. Data analysis

Arithmetic means are given with standard error of the mean (SEM). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Newman–Keuls test. A *P* value less than 0.05 was assumed to denote a significant difference.

3. Results

3.1. General data

Body weight increased with age in both rat strains, but this increment was more pronounced in WKY than in SHR (70% versus 40% increase) (Table 1). In addition, body weight in WKY was significantly higher than in age-matched SHR (Table 1). Tibia length is an index of growth that remains constant after maturity. As shown in Table 1, tibia length was slightly higher in 12-month old rats, indicating that 3-month old rats were reaching full body growth. In contrast, the increase in body weight/tibia length ratio in WKY (58%) was greater than in SHR (18%), suggesting that aged WKY accumulate fat mass. Additionally, the obesity Lee index was augmented in 12-month WKY rats when compared with 3-month WKY rats, whereas no differences were observed in the SHR rats during aging. Fractional urinary excretion of sodium (FE Na^+) and creatinine clearance were considered as markers of renal function. In WKY, FE Na^+ and creatinine clearance were unaffected by age. FE Na^+ in 12-month SHR was lower than in 3-month SHR and 12-month WKY (Table 1). On the other hand, creatinine clearance was slightly higher in 12-month SHR than in 3-month SHR (Table 1). We have also evaluated urinary protein excretion and serum lactate dehydrogenase (LDH) levels as markers of renal injury. Urinary protein excretion was not affected by age in WKY. By contrast, urinary protein excretion in 12-month SHR was higher than in 3-month SHR. Urinary protein excretion in both SHR groups was also significantly higher than in age-matched WKY (Table 1). Following a similar pattern, serum LDH levels were higher in SHR than in age-matched WKY rats and no age-related changes were observed, although statistical significance was not reached (Table 1).

3.2. Blood pressure data

As expected, the systolic and diastolic blood pressures (SBP and DBP) determined by the tail–cuff method were significantly higher in both 3- and 12-month old SHR than in age-matched WKY (Fig. 1A and 1B). Moreover, a significant increase in both SBP and DBP was observed in 12-month versus 3-month SHR (Fig. 1A and 1B). Heart rate in SHR was higher than in WKY (Fig. 1 C).

Table 1
Physiological parameters in 3- and 12-month old WKY and SHR.

	WKY		SHR	
Parameter	3-months	12-months	3-months	12-months
Body weight (g)	332 ± 9	565 ± 10[a]	272 ± 5	380 ± 5[a,b]
Tibia length (cm)	4.00 ± 0.09	4.30 ± 0.02	3.30 ± 0.01	3.90 ± 0.03
Body weight/tibia length (g/cm)	83.0 ± 2.9	131.4 ± 2.5[a]	82.4 ± 1.4	97.4 ± 0.9[a,b]
Lee index	297.7 ± 3.1	308.2 ± 1.8[a]	301.8 ± 3.0	303.4 ± 1.7
FE Na^+ (%)	0.36 ± 0.04	0.40 ± 0.03	0.37 ± 0.04	0.20 ± 0.04[a,b]
Creatinine clearance (ml/min)	2.6 ± 0.3	3.4 ± 0.3	1.9 ± 0.2	2.7 ± 0.2[a]
Proteinuria (mg/24 h)	14.0 ± 0.8	13.9 ± 1.2	26.0 ± 1.6[b]	34.0 ± 3.3[a,b]
LDH (U/L)	248 ± 72	337 ± 44	436 ± 70	410 ± 93

Data are means ± SEM of 5–11 rats per group.
[a] P<0.05 compared with corresponding 3-months old rats.
[b] P<0.05 compared with corresponding WKY rats.

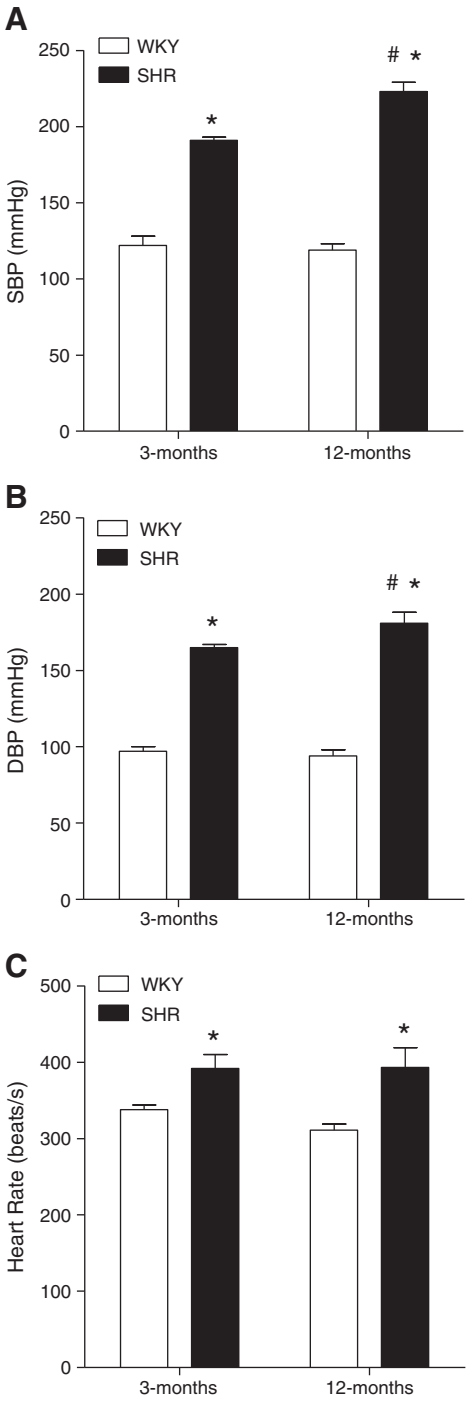

Fig. 1. Changes in systolic (A), diastolic blood pressure (B), and heart rate (C) of 3-and 12-month old WKY and SHR. Each bar represents the mean ± SEM of 6–12 rats. Significantly different from corresponding WKY values (* P<0.05) and significantly different from values in 3-month old rats (# P<0.05) using the Newman–Keuls test.

3.3. Markers of oxidative stress

To evaluate oxidative stress during aging in both WKY and SHR, we measured the production of hydrogen peroxide (H_2O_2), a hazardous ROS against tissues and cells, in renal cortex tissue samples, and assessed lipid peroxidation through the quantification of TBARS in urine samples. H_2O_2 production by the renal cortex of 3-month SHR was increased in comparison with age-matched WKY (Fig. 2A). In contrast, no changes in urinary TBARS were detected in this age group (Fig. 2B). H_2O_2 production in the renal cortex increased markedly (4–5 fold) with

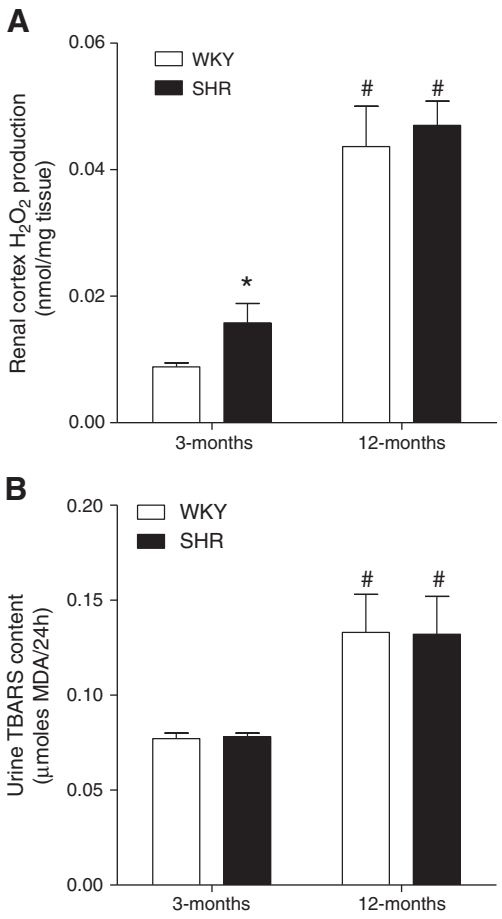

Fig. 2. Production of H_2O_2 by renal cortex of 3- and 12-month old WKY and SHR (A). Urinary levels of lipid peroxidation (TBARS) in 3- and 12-month old WKY and SHR (B). Each bar represents the mean ± SEM of 3–7 rats. Significantly different from values in 3-month old WKY (* $P<0.05$) and significantly different from corresponding values in 3-month old rats (# $P<0.05$) using the Newman–Keuls test.

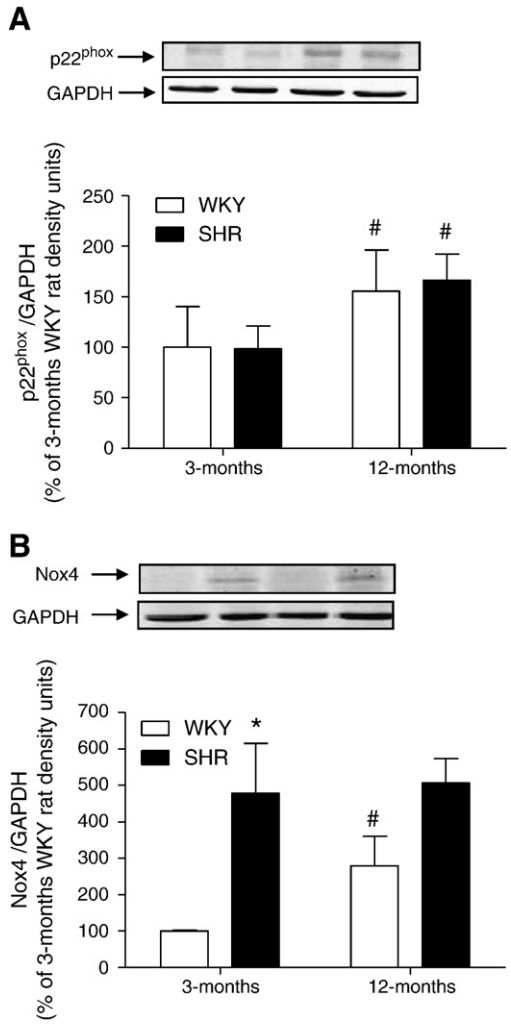

Fig. 3. Expression of the NADPH oxidase protein subunits $p22^{phox}$ (A) and Nox4 (B) in the renal cortex of 3- and 12-month old WKY and SHR. Representative immunoblots are depicted on top of the bar graphs. Values are normalized to the level of GAPDH expression in each condition and expressed as % of 3-month old WKY rats. Each bar represents the mean ± SEM of 4 independent immunoblots. Significantly different from values in 3-month old WKY (* $P<0.05$) and significantly different from corresponding values in 3-month old rats (# $P<0.05$) using the Newman–Keuls test.

age in both WKY and SHR, though no significant differences were observed between rat strains of the same age group (Fig. 2A). Similar to the results shown for oxidant production, a significant increase (2 fold) was observed in urinary TBARS in 12-month WKY and SHR when compared with 3-month rats (Fig. 2B).

3.4. Pro-oxidant enzymes

Because the NADPH oxidases are predominant sources of ROS leading to oxidative stress (Griendling et al., 2000; Li et al., 2001), the levels of two major subunits of NADPH, Nox4 and $p22^{phox}$, were evaluated by Western blotting in the renal cortex of 3- and 12-month old WKY and SHR. The abundance of Nox4 in 3-month SHR, as well as in 12-month WKY and 12-month SHR, was higher than in 3-month WKY (Fig. 3B). No significant differences in $p22^{phox}$ protein expression were found between age-matched groups of WKY and SHR strains. However, the abundance of $p22^{phox}$ in both 12-month old WKY and SHR was higher than in 3-month old WKY and SHR (Fig. 3A).

3.5. Antioxidant enzymes

Protein expression of superoxide dismutase isoforms (SOD1, SOD2 and SOD3) was determined in renal cortex of 3- and 12-month WKY and SHR (Fig. 4). Expression of SOD1 in 3-month SHR was significantly lower than in age-matched WKY (Fig. 4A). In 12-month SHR the expression of SOD1 was higher than in 3-month SHR, though no differences were detected between aged WKY and SHR (Fig. 4A). The abundance of SOD2 did not differ in 3-month WKY and SHR. In contrast, the abundance of SOD2 in 12-month WKY and SHR was higher than in the 3-month rats (Fig. 4B). SOD3 expression levels in 3-months SHR were significantly lower than in 3-month WKY. In 12-month WKY and SHR the abundance of SOD3 was higher than in the 3-month rats (Fig. 4 C). In addition, expression of catalase and GPx, the enzymes responsible for H_2O_2 catabolism, were also evaluated in the renal cortex of all groups of animals (Fig. 5). The abundance of catalase in 3-month SHR, as well as in 12-month WKY and SHR, was higher than in 3-month WKY (Fig. 5A). By contrast, GPx expression remained unchanged in all groups (Fig. 5B).

4. Discussion

The main findings of this study were that both SHR and WKY exhibited increased levels of oxidative stress in renal tissue with age and this was accompanied by similar expression profile of oxidant and antioxidant enzymes. We hypothesize that the findings reported here, which apparently conflict with the current view that hypertension is a state of oxidative stress, might arise from the fact that normotensive WKY developed obesity with aging. Common mechanisms leading to oxidative stress may, therefore, underlie hypertension and obesity.

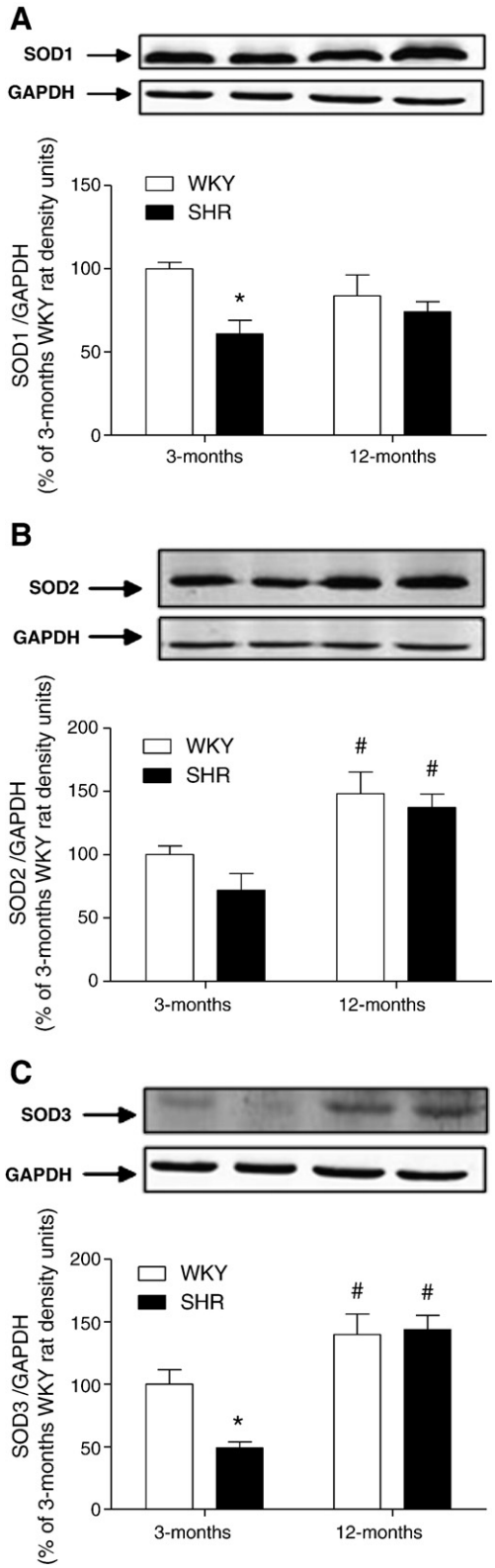

Fig. 4. Expression of SOD1 (A), SOD2 (B) and SOD3 (C) in the renal cortex of 3- and 12-month old WKY and SHR. Representative immunoblots are depicted on top of the bar graphs. Values are normalized to the level of GAPDH expression in each condition and expressed as % of 3-month old WKY rats. Each bar represents the mean ± SEM of 4 independent immunoblots. Significantly different from values in 3-month old WKY (* $P<0.05$) and significantly different from corresponding values in 3-month old rats (# $P<0.05$) using the Newman–Keuls test.

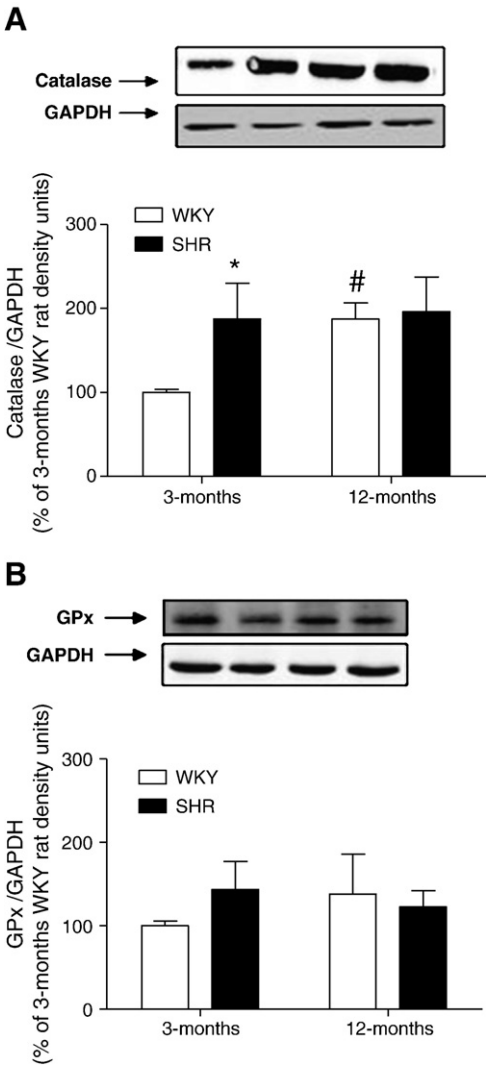

Fig. 5. Expression of catalase (A) and glutathione peroxidase (B) in the renal cortex of 3- and 12-month old WKY and SHR. Representative immunoblots are depicted on top of the bar graphs. Values are normalized to the level of GAPDH expression in each condition and expressed as % of 3-month old WKY rats. Each bar represents the mean ± SEM of 4 independent immunoblots. Significantly different from values in 3-month old WKY (* $P<0.05$) and significantly different from corresponding values in 3-months old rat (# $P<0.05$) using the Newman–Keuls test.

Oxidative stress is assumed to be an important factor in aging and in many age-related diseases (Finkel and Holbrook, 2000; Kregel and Zhang, 2007), including essential hypertension (Touyz, 2004; Wilcox, 2002). This view is based on the presence of increased production of superoxide anion and H_2O_2, reduced NO synthesis, and decreased bioavailability of antioxidants, which have been verified in experimental and human hypertension. As it concerns the SHR model, the majority of studies have been conducted in young/adult animals, ranging from 10 to 24 weeks of age. Additionally, several conflicting results have been reported in what concerns the measurement of different oxidative stress parameters in SHR and WKY. In the present study, we further confirmed the presence of oxidative stress in young SHR. The production of H_2O_2 in the renal cortex was significantly increased in 3-month old SHR although no differences were detected in urinary lipid peroxidation products. Oxidative stress in 3-month old SHR might be explained by the upregulation of NADPH oxidase subunit Nox4 and down-regulation of antioxidant SOD1 and SOD3 enzymes. Previous studies reported similar observations confirming that at the age of 10 to 12 weeks these rats develop oxidative stress (Adler and Huang, 2004; Biswas et al., 2008). Overexpression of catalase in 3-month old SHR might be an attempt to compensate for increased levels of oxidative stress.

To the best of our knowledge, no previous study has examined the effects of aging on oxidative stress in kidneys of SHR and WKY. A

recent work evaluated oxidant handling and energy metabolism in chronic kidney disease using 3- and 21–24-month old WKY, SHR and Wistar rats, although with a different approach regarding oxidative stress measurement. In this study, the expression of hemeoxygenase-1 was used as a renal marker of oxidative stress. The authors observed an increased expression of hemeoxygenase-1 in old Wistar and SHR rats as compared with the young rats (Percy et al., 2009). Furthermore, in the study of Alvarez et al., comparisons of oxidative stress levels were made during aging in hearts of Wistar and SHR rats. These authors reported increased levels of oxidative stress in aged SHR (Alvarez et al., 2008).

In the present study, we found a significant increase in H_2O_2 production by the renal cortex of 12-month old SHR. Moreover, these rats also exhibited a significant increase in urinary TBARS, a frequently used index of cell lipid peroxidation. Several reports have shown an age-associated increase in the concentrations of lipid peroxidation products (Pratico, 2002). Unexpectedly, WKY at the age of 12 months displayed similar oxidative stress levels as the age-matched SHR. It is suggested that the increased levels of H_2O_2 found in the renal tissue of aged SHR and WKY may be, at least in part, responsible for the increased urinary lipid peroxides in these rats. Altogether, the results presented here indicate that aged rats have increased levels of oxidative stress compared with young ones. In contrast, recent work demonstrated an increase in plasma (Zhan et al., 2004a) and urinary (Suzuki et al., 2008) H_2O_2 levels in 22/24-week-old SHR compared with age-matched WKY. Furthermore, the study of de Cavanagh et al. (2006) reports an increase in renal mitochondrial H_2O_2 production in 8-month old SHR compared with age-matched WKY. The discrepancy between our study and those mentioned before in terms of H_2O_2 production might be explained by the marked increase in body weight of aged WKY, even after correction for tibia length, suggesting the accumulation of fat in these rats. In fact, numerous reports have shown that oxidative stress is increased in animal models of obesity and that the adipose tissue is a major contributor to ROS production (Davi et al., 2002; Furukawa et al., 2004; Urakawa et al., 2003). Several studies demonstrated that dietary restriction is an efficient approach to confront age-related oxidative damage in different rat tissues, decreasing ROS and F_2-isoprostane levels as well as the activities of proinflammatory transcription factors (Jung et al., 2009; Opalach et al., 2010; Ward et al., 2005). It would be of interest, in future studies, to determine the importance of diet intake upon oxidative stress parameters in the aged kidney of SHR and WKY.

The NADPH oxidase complex has been described as the main source of ROS in the kidney, although other ROS-generating enzymes are also present in this organ (Paravicini and Touyz, 2008; Touyz, 2004). The NAPDH oxidase catalyzes the conversion of molecular oxygen into superoxide anion and is composed by $p22^{phox}$ and Nox2/$gp91^{phox}$ membrane subunits and the cytosolic proteins $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac (Bedard and Krause, 2007; Paravicini and Touyz, 2008). Nox4 is the most highly expressed NADPH oxidase in the kidney and does not require cytosolic subunits for its activation (Bedard and Krause, 2007). Oxidative stress in aged SHR and WKY was accompanied by increases in the abundance of $p22^{phox}$ and Nox4 proteins, which provides additional support for increased ROS production in the kidney of aged rats.

The primary lines of defense preventing biological macromolecules from ROS attack are the antioxidant enzymes. SODs are an ubiquitous family of enzymes that catalyze the conversion of superoxide anion into H_2O_2. In the subsequent step of the detoxifying cascade, H_2O_2 previously produced is converted to water and molecular oxygen by catalase or GPx, which uses reduced glutathione as the hydrogen donor. Increased levels of ROS in cells and tissues may act as a signal to enhance the activity and expression of antioxidant enzymes. According to this hypothesis, an increase in antioxidant enzymes activity and/or expression with age would be expected, this being an adaptation to help cells and tissues protect from oxidative stress. Here, we reported an increased renal expression of SOD2, SOD3 and catalase in both aged SHR and WKY. This finding may occur in response to the increased H_2O_2 production in aged rats. In addition, the increased levels of H_2O_2 present in these rats might modulate mRNA levels of antioxidant enzymes through activation of redox-sensitive transcription factors such as AP-1 and NF-κB, which can lead to the overexpression of these enzymes in aged rats. In contrast, expression of GPx remained unaltered during aging in both SHR and WKY rats, suggesting that this enzyme may be dysregulated despite the presence of oxidative stress. Some studies have shown decreases in activity and/or expression of catalase and GPx, while other studies showed the opposite trend. Fortepiani and Reckelhoff (2005) reported a decrease in the expression of both catalase and GPx in 17/19-week old SHR. In contrast, Zhan et al. (2004b) reported increases in the expression of catalase and GPx in the renal cortex of 24-week old SHR without changes in enzymatic activity. In the study of Zhan et al. (2004b), and similar to the present work, a significant increase in body weight of WKY was reported. The cause of the discrepancy between the results of Fortepiani and Reckelhoff (2005), Zhan et al., (2004b) and the present study remains at present unclear. It is possible that it rests simply on the fact that animals of different ages and body weights were used and this is an important aspect to consider. Recent studies carried out in WKY and Fischer-344 rats (21 to 24 months old) also described increases in the activity of major antioxidant enzymes with age (Judge et al., 2005; Lambertucci et al., 2007).

In conclusion, aged SHR and WKY display increased levels of oxidative stress in renal tissue with similar expression profile of oxidant and antioxidant enzymes, suggesting the existence of common mechanisms in hypertension and obesity leading to oxidative stress. It is suggested that oxidative stress levels in aged WKY might arise from increased fat mass accumulation which is operating as a confounding factor.

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CHAPTER III - Aging and the regulation of LAT1, LAT2 and ASCT2 in the spontaneously hypertensive rat and Wistar-Kyoto rat

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Age-related changes in the renal dopaminergic system and expression of renal amino acid transporters in WKY and SHR rats

Vanda Pinto, João Amaral, Elisabete Silva, Sónia Simão, José Miguel Cabral, Joana Afonso, Maria Paula Serrão, Pedro Gomes [1], Maria João Pinho, Patrício Soares-da-Silva *

Institute of Pharmacology & Therapeutics, Faulty of Medicine, University of Porto, 4200-319 Porto, Portugal

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ABSTRACT

This study examined age-related changes in renal dopaminergic activity and expression of amino acid transporters potentially involved in renal tubular uptake of L-DOPA in Wistar Kyoto (WKY) and spontaneously hypertensive rats. Aging (from 13 to 91 weeks) was accompanied by increases in systolic blood pressure (SBP) in both WKY and SHR. The sum of urinary dopamine and DOPAC and the urinary dopamine/L-DOPA ratio were increased in aged SHR but not in aged WKY. The urinary dopamine/renal delivery of L-DOPA ratio was increased in both rat strains with aging. LAT2 abundance was increased in aged WKY and SHR. The expression of 4F2hc was markedly elevated in aged SHR but not in aged WKY. ASCT2 was upregulated in both aged WKY and SHR. Plasma aldosterone levels and urinary noradrenaline levels were increased in aged WKY and SHR though levels of both entities were more elevated in aged SHR. Activation of the renal dopaminergic system is more pronounced in aged SHR than in aged WKY and is associated with an upregulation of renal cortical ASCT2 in WKY and of LAT2/4F2hc and ASCT2 in SHR. This activation may be the consequence of a counter-regulatory mechanism for stimuli leading to sodium reabsorption.

1. Introduction

Dopamine is a major regulator of mammalian proximal tubule salt and water reabsorption. In the mammalian kidney, dopamine is primarily produced in the proximal tubule (Aperia, 2000; Gomes and Soares-da-Silva, 2008; Soares-da-Silva and Vieira-Coelho, 1998). The dopamine precursor L-dihydroxyphenylalanine (L-DOPA) is filtered at the glomerulus and is taken up by the proximal tubule via luminal transporters and converted to dopamine by aromatic L-amino acid decarboxylase (AADC), which is highly expressed in the proximal tubule (Soares-da-Silva and Fernandes, 1991). The regulation of this non-neuronal dopaminergic system depends mainly on the availability of L-DOPA, on its decarboxylation into dopamine and on cell outward amine transfer mechanisms (Pestana and Soares-da-Silva, 1994; Soares-da-Silva and Fernandes, 1991). In the kidney, dopamine is metabolized predominantly by catechol-O-methyl-transferase (COMT) and monoamine oxidase to 3,4-dihydroxyphenylacetic acid (DOPAC), and to homovallinic acid (HVA) (Pestana and Soares-da-Silva, 1994; Soares-da-Silva and Fernandes, 1991). A considerable amount of evidence favours the view that dopamine of renal origin plays a role in the regulation of central blood volume by reducing the tubular reabsorption of sodium as a paracrine or autocrine substance (Jose et al., 2003). The mechanisms through which renal dopamine is thought to produce natriuresis involve the activation of D_1-like receptors that inhibit the activity of both apical (e.g., Na/H exchange and chloride–bicarbonate exchange and Na–P cotransport) and basolateral (Na–K-ATPase and $NaHCO_3$ cotransport) transporters (Aperia et al., 1987; Felder et al., 1990; Jose et al., 1992; Lokhandwala and Amenta, 1991). The availability of dopamine to activate its specific receptors is determined by factors affecting renal synthesis, mainly the amounts of L-DOPA and sodium delivered to the kidney and the degree of degradation of the amine (Soares-da-Silva et al., 1993).

The spontaneously hypertensive rat (SHR) is a genetic model of hypertension characterized by the resistance to the natriuretic effect of dopamine and D_1-like receptor agonists, as a result of a defective transduction of the D_1 receptor signal in renal proximal

Abbreviations: 4F2hc, 4F2 heavy chain; ANOVA, one-way analysis of variance; COMT, catechol-O-methyl-transferase; Ccr, creatinine clearance; DOPAC, 3,4-dihydroxyphenylacetic acid; FE_{Na+}, fractional excretion of Na^+; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFR, glomerular filtration rate; L-DOPA, L-dihydroxyphenylalanine; LAT1, L-type amino acid transporter 1; LAT2, L-type amino acid transporter 2; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEM, standard error of the mean; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat.

* Corresponding author. Tel.: +351 22 5513642; fax: +351 22 5513643.
E-mail address: pss@med.up.pt (P. Soares-da-Silva).
[1] Present address: Department of Biochemistry, Faculty of Medicine, University of Porto, 4200-319 Porto, Portugal.

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tubules (Jose et al., 2010; Sanada et al., 1999; Zeng and Jose, 2011). It has been suggested that increased oxidative stress in renal proximal tubules of the SHR could be a mechanism for defective dopamine D_1 receptor/G-protein coupling (White and Sidhu, 1998). Moreover, recent studies have shown the overexpression of Na^+-independent and pH-sensitive amino acid transporter LAT2 (Slc7a8) in the SHR kidney, which might contribute to enhanced L-DOPA uptake in the proximal tubule and increased dopamine production (Pinho et al., 2004), as an attempt to overcome the defect in D_1 receptor function.

The aging kidney undergoes structural changes that result in quantitative alterations in some renal functions, such as a decline in renal blood flow and glomerular filtration rate (Zhou et al., 2008). An increasing number of studies have shown that old animals may present particular deficiencies in the renal handling of L-DOPA, its subsequent conversion to dopamine (Armando et al., 1995; Soares-da-Silva and Fernandes, 1991) and at the level of receptor number or coupling to G proteins (Kansra et al., 1997). In the presence of age-related diseases, such as heart failure and hypertension, these changes can be aggravated (Fischer and O'Hare, 2010).

This study was aimed at evaluating age-related changes in the activity of the renal dopaminergic system and the regulation of the amino acid transporters that are potentially involved in the uptake of L-DOPA: Na^+-independent LAT1 and LAT2 and Na^+-dependent ASCT2 in SHR and their normotensive Wistar Kyoto (WKY) counterparts.

2. Materials and methods

2.1. Animal preparation and experimental design

Five-week old male WKY and SHR were obtained from Harlan-Interfauna Ibérica (Barcelona, Spain). The rats were housed under controlled conditions (12 h light/dark cycle and room temperature at 22 ± 2 °C) and had free access to tap water and standard rat chow (PANLAB, Barcelona, Spain). The animals were carefully maintained and monitored until 13 or 91 weeks of age. Blood pressure (systolic and diastolic) and heart rate were measured in conscious animals using a photoelectric tail-cuff detector (LE 5000, Letica, Barcelona, Spain). A minimum of 5 measures were made each time and the mean values were used for further calculations. All rat interventions were performed in accordance with the European Directive number 86/609, and the rules of the "Guide for the Care and Use of Laboratory Animals", 7th edition, 1996, Institute for Laboratory Animal Research (ILAR), Washington, DC.

2.2. Metabolic study

Forty eight hours before experiments, 13- and 91-week old rats were placed in metabolic cages (Tecniplast, Buguggiate, Italy) for a 24 h urine collection. The urine samples were collected in vials containing 1 ml of 6 M HCl to prevent spontaneous decomposition of monoamines and amine metabolites. After completion of this protocol, rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). The animals were then sacrificed by exsanguination using cardiac puncture and the blood collected into tubes containing K_3 EDTA for later determination of plasma biochemical parameters. Before excising the kidneys, a cannula was inserted in the right ventricle of the heart and animals were perfused with ice-cold saline (0.9% NaCl) to remove all blood from the kidneys. The kidneys were then excised, weighed, decapsulated, and the renal cortex and medulla rapidly separated by fine dissection. Tissue pieces were immediately frozen in liquid nitrogen and stored at −80 °C for Western blot analysis.

2.3. Plasma and urine biochemistry

The quantification of sodium and potassium in plasma and urine was performed by ion-selective electrodes. Creatinine was measured by the Jaffé method (Chromy et al., 2008). All assays were performed by Cobas Mira Plus analyzer (ABX Diagnostics, Switzerland). Creatinine clearance (in ml/min) was calculated using the formula $C_{cr} = (U_{creat} \times Vu)/(P_{creat} \times 24\,h \times 60)$ where U_{creat} and Vu are the urinary creatinine concentration and urinary 24 h volume and P_{creat} is the plasma creatinine concentration. Aldosterone in plasma samples was performed by radioimmuno assay (Diagnostic Products Corporation; Los Angeles, CA).

2.4. Assay of catecholamines

The assay of catecholamines in urine (L-DOPA, dopamine, DOPAC and norepinephrine) and plasma samples (L-DOPA, dopamine and DOPAC) was performed by HPLC with electrochemical detection, as previously described (Soares-da-Silva et al., 1994, 1993). The lower limit of detection of L-DOPA, dopamine, norepinephrine, and DOPAC ranged from 350 to 1000 fmol.

2.5. Western blotting

Renal cortices from 13- and 91-week old WKY and SHR, were lysed in RIPA buffer containing 150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 2 μg/ml leupeptin and 2 μg/ml aprotinin. Protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin as standard. Lysates were boiled in sample buffer (35 mM Tris–HCl, pH 6.8, 4% SDS, 9.3% dithiothreitol, 0.01% bromophenol blue, 30% glycerol) at 95 °C for 5 min. Samples containing 50–75 μg of protein, were separated by SDS-PAGE with 10% polyacrylamide gel and then electroblotted onto nitrocellulose membranes (Bio-Rad). Blots were blocked for 1 h with 5% non-fat dry milk in PBS (10 mmol/l phosphate-buffered saline) at room temperature with constant shaking. Blots were then incubated with the antibodies rabbit polyclonal anti-LAT1 (1:500; Serotec); goat polyclonal anti-LAT2 (1:500; Santa Cruz Biotechnology); rabbit polyclonal anti-4F2hc (1:500; Santa Cruz Biotechnology); rabbit polyclonal anti-ASCT2 (1:500; Chemicon International); mouse monoclonal anti-β-actin (1:20,000; Santa Cruz Biotechnology) or mouse monoclonal anti-GAPDH (1:60,000; Santa Cruz Biotechnology) in 5% non-fat dry milk in PBS-T overnight at 4 °C. The immunoblots were subsequently washed and incubated with fluorescently labeled goat anti-rabbit (1:20,000; IRDyeTM 800, Rockland); fluorescently labeled donkey anti-goat (1:10,000; IRDye™ 800, Rockland); or the fluorescently labeled goat anti-mouse secondary antibody (1:20,000; AlexaFluor 680, Molecular Probes) for 60 min at room temperature and protected from light. The membrane was washed and imaged by scanning at both 700 and 800 nm, with an Odyssey Infrared Imaging System (LI-COR Biosciences).

2.6. Drugs

All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise stated.

2.7. Data analysis

Arithmetic means are given with standard error of the mean (SEM). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Newman–Keuls test. A P value less than 0.05 was assumed to denote a significant difference.

Table 1
Cardiovascular and physiological parameters in 13- and 91-week old WKY and SHR.

Parameter	WKY		SHR	
	13 weeks $n=6$	91 weeks $n=6$	13 weeks $n=6$	91 weeks $n=6$
Systolic blood pressure (mm Hg)	122 ± 6	148 ± 3*	191 ± 2#	224 ± 5*,#
Diastolic blood pressure (mm Hg)	97 ± 3	88 ± 3	165 ± 2#	132 ± 3*,#
Pulse pressure (mm Hg)	25 ± 2	60 ± 1*	26 ± 2	92 ± 7*,#
Creatinine clearance (ml/min)	2.64 ± 0.26	3.9 ± 0.23*	1.85 ± 0.15#	2.05 ± 0.18#
Urinary protein excretion (mg/24 h)	14.01 ± 0.80	14.77 ± 1.21	26.02 ± 1.60#	41.06 ± 3.38*,#
FE_{Na+} (%)	0.38 ± 0.04	0.23 ± 0.01*	0.37 ± 0.04	0.23 ± 0.04*
Kidney weight/tibia length (% of control)	100 ± 2	114 ± 1*	100 ± 1	122 ± 2*,#

* Significantly different from corresponding values in 13-week old animals ($P < 0.05$).
\# Significantly different from age-matched WKY ($P < 0.05$).

3. Results

3.1. Blood pressure data and renal function

As expected, the systolic and diastolic blood pressures (SBP and DBP) determined by the tail-cuff method were significantly higher in both 13- and 91-week old SHR than in age-matched WKY (Table 1). Moreover, aging was accompanied by increases in SBP in both WKY and SHR. DBP remained unaltered in aged WKY, but a significant decrease was observed in aged versus young SHR (Table 1). No difference in pulse pressure (defined as SBP minus DBP) was found between young WKY and SHR. Pulse pressure increased with age in both WKY and SHR but at 91 weeks of age SHR had higher pulse pressure than age-matched WKY (Table 1). Creatinine clearance (Ccr) levels were decreased in SHR in comparison to age-matched WKY (Table 1). Urinary protein excretion was significantly higher in SHR than in age-matched WKY at 13 and 91 weeks of age and increased significantly with age (Table 1). Moreover, evaluation of FE_{Na+} in WKY and SHR showed a significant decrease in this parameter at the age of 91 weeks in both rat strains (Table 1). Kidney/tibia length ratios were assessed for WKY and SHR (Table 1). Aging was associated with increases in kidney/tibia length ratio in both WKY and SHR. However, increases in kidney size were more marked in SHR (Table 1).

3.2. Activity of the renal dopaminergic system

In the present study, the urinary excretion of dopamine and its metabolite DOPAC was evaluated in 13- and 91-week old WKY and SHR (Fig. 1). No changes were found in urinary L-DOPA indexed to urinary creatinine in aged WKY. However, L-DOPA excretion was decreased in aged SHR, though the difference did not reach statistical significance (Fig. 1A). Aging was accompanied by slight increases in urinary dopamine in WKY and SHR, though not statistically significant. However, urinary dopamine in 91-week old SHR was higher than that in age-matched WKY (Fig. 1B). Urinary DOPAC was significantly increased in aged SHR but not in aged WKY rats (Fig. 1C). More complete information on the L-DOPA renal turnover is obtained when the sum of urinary dopamine and DOPAC is considered. The sum of urinary dopamine and DOPAC was found to be increased in aged SHR but not in aged WKY (Fig. 1D). Furthermore, urinary dopamine + DOPAC was markedly increased in aged SHR in comparison to age-matched WKY (Fig. 1D).

The enhanced urinary excretion of dopamine and DOPAC in the SHR may reflect their enhanced ability to synthesize dopamine. The urinary dopamine/L-DOPA ratio (a measure of renal L-DOPA utilization and of renal dopamine-synthesis efficiency) in 91-week old SHR was markedly higher than in young SHR (Fig. 2A). The dopamine/L-DOPA ratio was also greater in 91-week old SHR than in age-matched WKY (Fig. 2A). No differences were detected with aging in the WKY (Fig. 2A). On the other hand, the ratio between urinary dopamine and the renal delivery of L-DOPA (another index of renal dopamine production) was greater in aged WKY and SHR than in young animals (Fig. 2B). However, the dopamine/renal delivery of L-DOPA ratio was significantly increased in aged SHR when compared to age-matched WKY (Fig. 2B). The renal delivery of L-DOPA, which considers L-DOPA plasma levels and creatinine clearance (plasma L-DOPA × creatinine clearance), decreased with age in WKY and SHR, though the difference did not reach statistical significance in WKY (Fig. 2C). Moreover, the renal delivery of L-DOPA in 91-week old SHR was significantly lower than in age-matched WKY (Fig. 2C). As depicted in Table 2, aging was accompanied by decreases in plasma levels of L-DOPA, dopamine and DOPAC in both WKY and SHR.

3.3. Renal expression of LAT1, LAT2, 4F2hc and ASCT2

Age-related changes in the amino acid transporters that are potentially involved in the uptake of L-DOPA were evaluated in the renal cortex of 13- and 91-week old WKY and SHR. As depicted in Fig. 3A LAT1 expression levels were downregulated in 91-week old WKY and SHR rats when compared to young animals. On the other hand, LAT2 abundance was significantly upregulated in 91-week old WKY and SHR, as compared to young animals (Fig. 3B). Aging had no effect on 4F2hc protein abundance in WKY, whereas 4F2hc

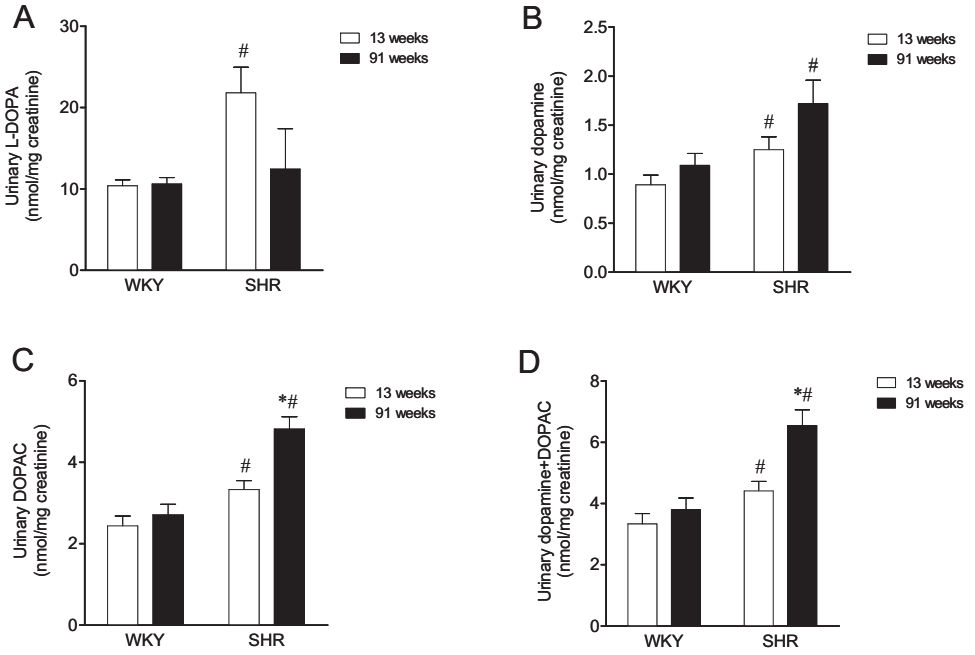

Fig. 1. Urinary excretion of L-DOPA (A), dopamine (B), DOPAC (C), and sum of urinary dopamine and DOPAC (D) indexed to urinary creatinine in 13- and 91-week old WKY and SHR. Each bar represents the mean ± SEM of 6 rats. Significantly different from corresponding values in 13-week old animals ($^{*}P < 0.05$) and significantly different from age-matched WKY ($^{\#}P < 0.05$) using the Newman–Keuls test.

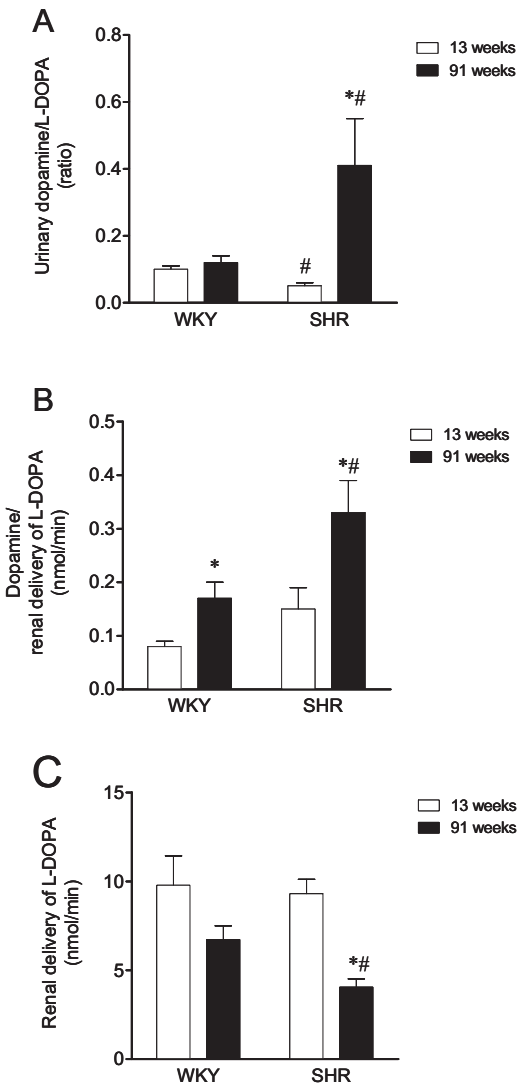

Fig. 2. Urinary dopamine/L-DOPA ratios (A), dopamine/renal delivery of L-DOPA ratios (B) and renal delivery of L-DOPA (C) in 13- and 91-week old WKY and SHR. Each bar represents the mean ± SEM of 6 rats. Significantly different from corresponding values in 13-week old animals ($^{*}P < 0.05$) and significantly different from age-matched WKY ($^{\#}P < 0.05$) using the Newman–Keuls test.

expression levels were significantly increased at 91 weeks of age in SHR when compared to 13-week old SHR (Fig. 3C). ASCT2 was increased at 91 weeks of age in both WKY and SHR when compared with 13-week old animals (Fig. 3D).

3.4. Neurohumoral parameters

Aging was accompanied by increases in plasma aldosterone levels in both WKY and SHR. However, at 91 weeks of age SHR had increased circulating aldosterone levels when compared to age-matched WKY (Fig. 4A). Plasma aldosterone levels were accompanied by decreases in the UNa^{+}/K^{+} ratio in both WKY and SHR though the mineralocorticoid response to endogenous aldosterone was markedly greater in 91-week old SHR than in age-matched WKY rats (Fig. 4B). Similar to plasma aldosterone levels, urinary excretion of noradrenaline was also greater in aged WKY and SHR than in young animals, but levels in 91-week old SHR were higher than in age-matched WKY rats (Fig. 4C).

4. Discussion

The present study evaluated age-related changes in dopaminergic activity and amino acid transporter expression in WKY and SHR. The results indicate that aged SHR have increased renal cortical LAT2/4F2hc and ASCT2 abundance and increased efficiency in the formation of renal dopamine. Moreover, activation of the renal dopaminergic system is accompanied by an increase in the activity of the sympathetic and renin–angiotensin–aldosterone systems.

There are conflicting results concerning the effect of age on renal dopamine production. Although several studies have shown an association between age and deficiencies in the renal handling of L-DOPA (Armando et al., 1995; Kansra et al., 1997; Soares-da-Silva and Fernandes, 1991; Vieira-Coelho et al., 1999), other authors have reported no alterations in renal dopamine production with aging (Komori et al., 1997; Lehmann et al., 1985; Nicolau et al., 1985). In the present study, no significant changes with aging were found in urinary excretion of L-DOPA, dopamine and DOPAC or in the urinary dopamine/L-DOPA ratio in WKY. However, results indicated that young WKY had increased renal delivery of L-DOPA when compared to aged rats though the urinary excretion of L-DOPA was the same. This may have led to the accumulation of plasma levels of L-DOPA in young WKY. Since the urinary dopamine/L-DOPA ratio only takes into account levels of urinary dopamine and L-DOPA no differences were found between young and aged WKY, regarding the ability to form dopamine. However, a significant increase in the dopamine/renal delivery of L-DOPA ratio in aged WKY indicates that the ability to produce dopamine may be increased in aged WKY.

In comparison to aged WKY, aged SHR had increased urinary excretion of dopamine and DOPAC. Additionally, urinary dopamine/L-DOPA and urinary dopamine/renal delivery of L-DOPA ratios were increased in aged SHR, indicating that aged SHR may have an enhanced ability to produce dopamine than aged WKY. Previous reports by other authors have shown that dopamine production and excretion in the SHR were normal or increased when compared with those in WKY, though most studies were conducted in young animals (Herlitz et al., 1982; Kuchel et al., 1987; Racz et al., 1985; Yoshimura et al., 1990). Our group reported that SHR maintained on a normal-salt diet had, at 4 weeks of age but not at 12 weeks, overexpression of LAT2 and increased tubular uptake of L-DOPA (Pinho et al., 2007). Accordingly, the enhanced ability to take up L-DOPA in the pre-hypertensive SHR was suggested to take place as an attempt to overcome the deficient dopamine-mediated natriuresis generally observed in this genetic

Table 2
Plasma levels of L-DOPA, dopamine and DOPAC in 13- and 91-week old WKY and SHR.

Parameter	WKY		SHR	
	13 weeks $n=6$	91 weeks $n=6$	13 weeks $n=6$	91 weeks $n=6$
L-DOPA (pmol/ml)	4.07 ± 0.31	2.00 ± 0.21*	5.33 ± 0.36#	2.89 ± 0.21*
Dopamine (pmol/ml)	10.67 ± 1.02	0.56 ± 0.09*	15.44 ± 2.97	0.42 ± 0.07*
DOPAC (pmol/ml)	4.62 ± 0.58	1.75 ± 0.41*	5.01 ± 0.60	2.90 ± 0.63*

* Significantly different from corresponding values in 13-week old animals ($P < 0.05$).
# Significantly different from age-matched WKY ($P < 0.05$).

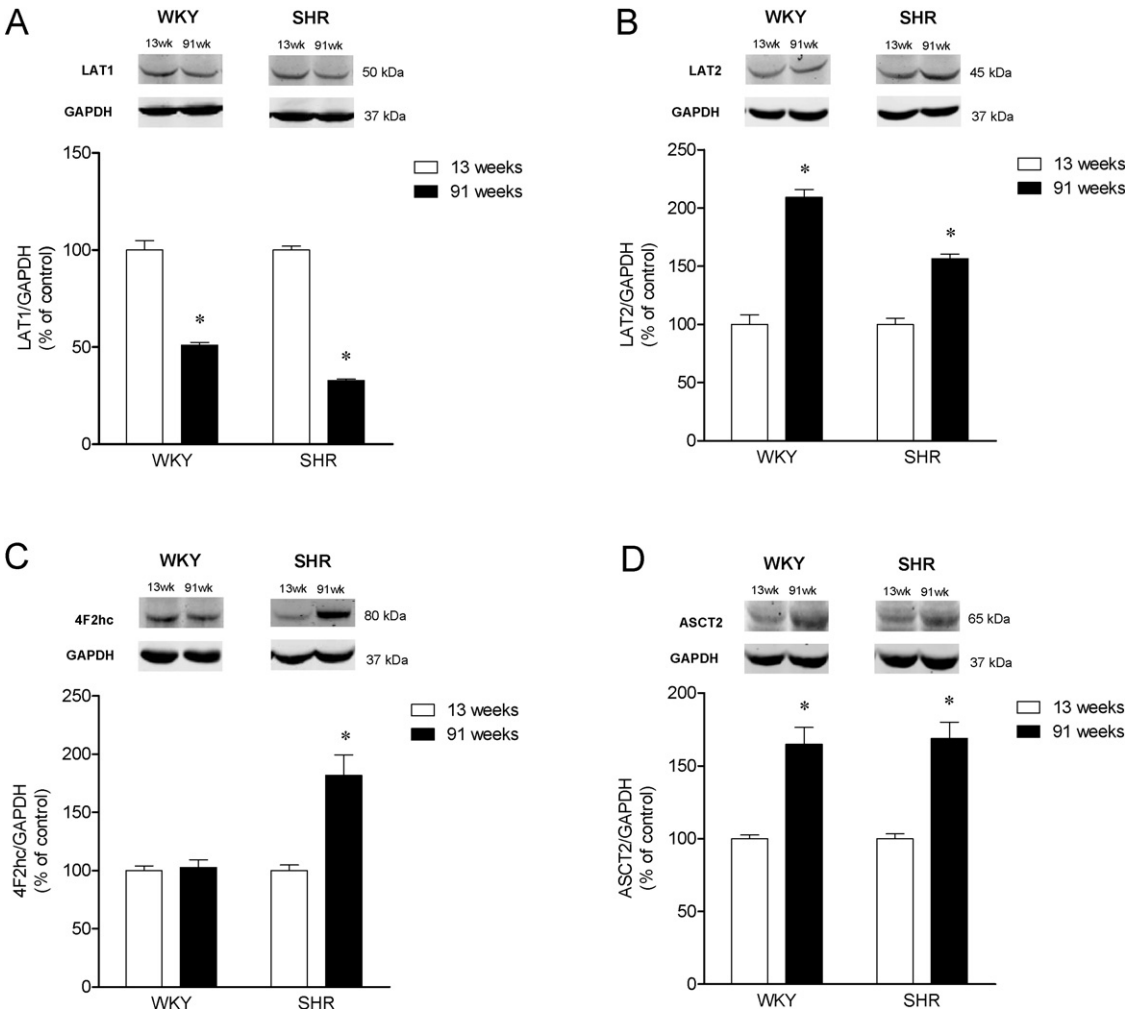

Fig. 3. Expression of LAT1 (A), LAT2 (B), 4F2hc (C) and ASCT2 (D) in the renal cortex of 13- and 91-week old WKY and SHR. Representative immunoblots are depicted on top of the bar graphs. Values are normalized to the level of GAPDH expression in each condition and expressed as % of 13 week-old rats. Each bar represents the mean ± SEM ($n = 4$ per group). Significantly different from values in 13-week old animals (*$P < 0.05$) using the Newman–Keuls test.

model of hypertension (Jose et al., 2002; Pinho et al., 2007). Moreover, at 4 and 12 weeks of age no differences in the urinary excretion of dopamine or DOPAC, or in plasma aldosterone levels were found between age-matched WKY and SHR (Pinho et al., 2007).

The renal cortical abundance of Na^+-independent LAT1 and LAT2, 4F2hc and Na^+-dependent ASCT2, amino acid transporters potentially involved in renal tubular uptake of L-DOPA, was evaluated in 13- and 91-week old WKY and SHR rats. The system L-type amino acid transporters is a major route for providing living cells with neutral amino acids including several essential amino acids that cells are unable to synthesize such as leucine, isoleucine, valine, phenylalanine, tryptophan, methionine and histidine (Christensen, 1990; Silbernagl, 1979). Although the transport of leucine by LAT1 in pig LLC-PK1 renal cells has been previously described (Soares-da-Silva and Serrao, 2004), LAT1 has a very limited tissue distribution in the kidney (Pinho et al., 2007). Global gene expression monitoring by cDNA microarrays showed a decline in the expression of y^+LAT1 and B^0AT1 with age in the renal cortex (Melk et al., 2005). Similarly, in the present study aging was accompanied by decreases in LAT1 abundance in WKY and SHR. LAT2 is a major Na^+-independent amino acid transporter expressed mainly in transporting epithelia, such as in the kidney and intestine (Broer, 2008), and its functionality is dependent on the abundance of 4F2hc (Pineda et al., 1999). The heterodimerization of LAT2 with 4F2hc is necessary for the transporter to reach the cell surface (Nakamura et al., 1999). Therefore, increases in 4F2hc and LAT2 abundance may translate in increases in LAT2 functionality in aged SHR. On the other hand, the abundance of 4F2hc does not vary with age in WKY, which would limit the translocation of LAT2 to the cell surface. At the apical membrane of renal proximal tubule cells only Na^+-dependent amino acid transporters ASCT2 and B^0AT1 are capable of transporting amino acids with similar characteristics to substrates transported through system L. Analogous to the LAT2 abundance profile, ASCT2 was found to be upregulated in aged WKY and SHR. Overall, these results suggest that activation of the renal dopaminergic system is accompanied by increases in LAT2/4F2hc functionality and ASCT2 overexpression in aged SHR. In contrast, LAT2/4F2hc functionality may not have a role in L-DOPA uptake in the renal cortex of aged WKY.

Plasma aldosterone and renal noradrenaline levels were higher in aged SHR than in aged WKY, indicating a marked neurohumoral activation in aged SHR. The result of these hemodynamic and neurohumoral alterations was an increase in renal sodium transport (as indicated by a decrease in urinary UNa^+/K^+ ratio), proteinuria and reductions of the renal delivery of L-DOPA in aged SHR. Another indication of aldosterone actions is the marked increases in kidney size in aged SHR rats. Aldosterone directly modulates renal cell proliferation and differentiation via stimulation of rapidly activated protein

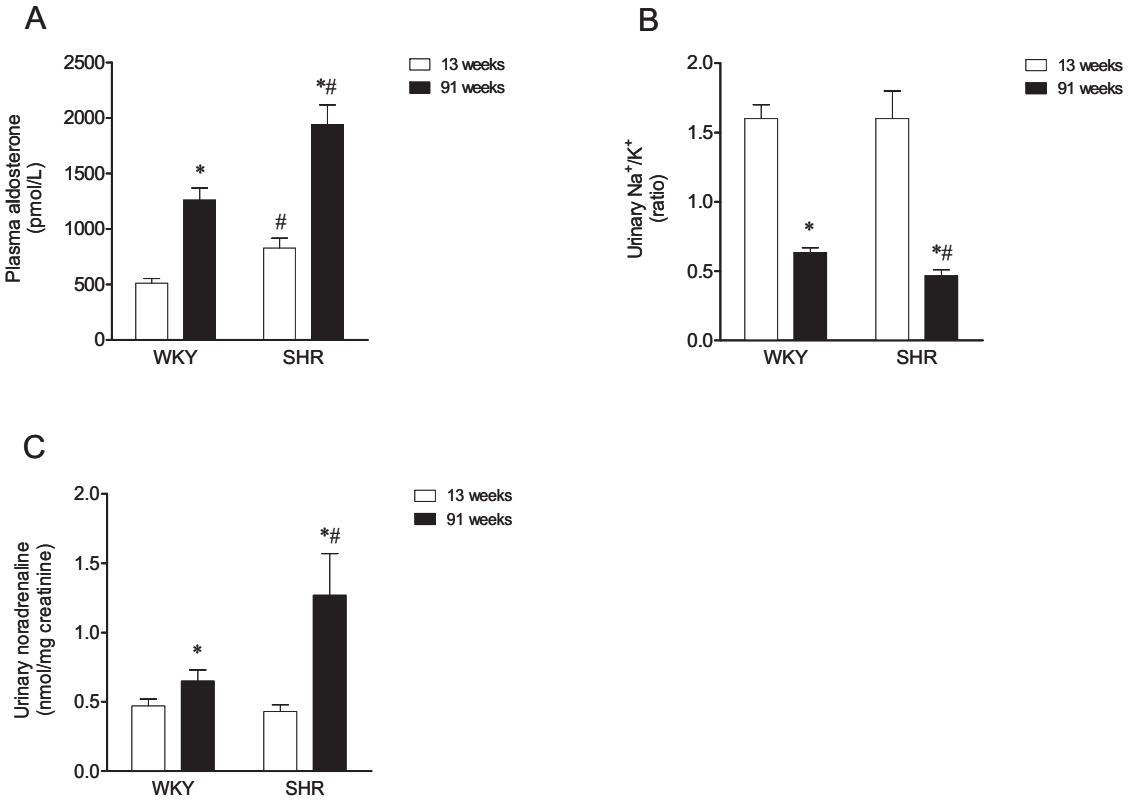

Fig. 4. Plasma levels (pmol/l) of aldosterone (A) changes in urinary Na^+/K^+ ratio (B) and urinary noradrenaline levels (nmol/mg creatinine) in 13- and 91-week old WKY and SHR. Each column represents the mean ± SEM of 6 rats. Significantly different from corresponding values in 13-week old animals ($^*P < 0.05$) and significantly different from age-matched WKY ($^\#P < 0.05$) using the Newman–Keuls test.

kinase cascades as part of normal kidney development (Thomas et al., 2010). The renal dopaminergic and renin–angiotensin–aldosterone systems (RAAS) control renal electrolyte balance through various receptor mediated pathways with counter-regulatory interactions. In order to conserve sodium during low sodium intake, the RAAS is upregulated in order to produce angiotensin II (Ang II). Stimulation of the principal membrane bound cell surface receptor for Ang II, the AT_1R, leads to sodium reabsorption. In order to eliminate sodium during high sodium intake the local renal production of dopamine is increased leading to inhibition of sodium reabsorption (Felder and Jose, 2006). The natriuretic renal dopaminergic system opposes the anti-natriuretic activity of the RAAS by downregulating the AT_1R, upregulating the AT_2R and inhibiting ROS generation. Each of the individual dopamine receptors has been shown to oppose the activity of the AT_1R, with the D_1R, D_3R, and D_5R physically interacting with the AT_1R (Gildea, 2009). Taken together, it is suggested that the renal dopaminergic system might be a compensatory mechanism activated by stimuli that lead to sodium reabsorption in aged WKY and SHR. However, this counter-regulatory mechanism is considerably more enhanced in aged SHR. A similar mechanism has been shown in patients with heart failure. Stimuli leading to activation of anti-natriuretic systems and sodium retention are accompanied by activation of the renal dopaminergic system characterized by an increase in the renal utilization of filtered L-DOPA (Alvelos et al., 2004; Ferreira et al., 2001, 2002).

The specific effects of aldosterone on the expression of 4F2hc and LAT2 have recently been explored by our group (Pinho et al., 2009). Eight-week old Wistar rats were submitted to high salt intake (1% NaCl in their drinking water) and treated chronically with aldosterone and/or spironolactone, a mineralocorticoid receptor (MR) antagonist. Treatment with aldosterone significantly increased LAT2 mRNA expression via the MR (abolished by spironolactone), though protein levels remained unchanged. On the other hand, aldosterone treated rats had decreased 4F2hc protein expression in a spironolactone-independent manner. These effects of aldosterone were accompanied by decreases in urinary dopamine and DOPAC in a spironolactone-sensitive manner (Pinho et al., 2009).

Studies have shown that cardiac function and coronary hemodynamics progressively deteriorate with aging in both SHR and WKY and that very old WKY tend to develop a significant degree of isolated systolic hypertension (Susic et al., 1998, 2001). In the present study SBP was found to be increased in aged WKY and SHR, displaying the same trend as the plasma aldosterone levels. Pulse pressure has been reported to increase significantly with age in SHR but not in WKY (Chamiot-Clerc et al., 2001). However, these studies were conducted in rats between 3 and 78 weeks of age. The findings show that aged SHR has in fact an intense dopaminergic response but SBP and pulse pressure values remain increased. The cause for this outcome is possibly related to the defective transduction of the D_1 receptor signal in renal proximal tubules usually attributed to this strain (Jose et al., 2010). On the other hand, the activation of the renal dopaminergic system is not as effective in aged WKY and SBP and pulse pressure are increased in these animals.

In conclusion, aging in WKY and SHR is accompanied by increases in renal cortical ASCT2 abundance in the former and in increases in LAT2/4F2hc and ASCT2 abundances in the latter. Moreover, the dopaminergic response is more enhanced in aged SHR than in aged WKY and this is probably a result of a compensatory mechanism activated by stimuli leading to sodium reabsorption.

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CHAPTER IV - Activation of the renal aldosterone/MR system in aging and hypertension

***Pinto V**, Pinho MJ, Silva E, Simão S, Serrão MP, Gomes P, Soares-da-Silva P The renal aldosterone/mineralocorticoid receptor system in aged WKY and SHR. Submitted for publication.*

The renal aldosterone/mineralocorticoid receptor system in aged WKY and SHR

Vanda Pinto[1], Maria João Pinho[1], Elisabete Silva[1], Sónia Simão[1], Joana Afonso[1], Maria Paula Serrão[1], Pedro Gomes[1], Celso Gómez-Sánchez [2], Elise Gómez-Sánchez [2], Pedro A. Jose[3] and Patrício Soares-da-Silva[1]

[1]Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Porto, 4200-319 Porto, Portugal; [2]Veterans Administration Medical Center, Jackson, Mississippi and Division of Endocrinology, University of Mississippi Medical Center, Jackson, Mississippi;[3]Center for Molecular Physiology Research, Children's National Medical Center, George Washington University School of Medicine & Public Health, Washington, D.C.

Aging is associated with alterations in renal physiology which may be aggravated by diseases such as hypertension. In the present study we hypothesised that age-associated changes in the renal aldosterone/mineralocorticoid receptor (MR) system may differ between spontaneously hypertensive rats (SHR), and Wistar-Kyoto rats (WKY). WKY became obese; body mass index (BMI) significantly increased with age. Fat mass accumulation may operate as a confounding factor therefore WKY were submitted to 15% food restriction (WKY-FR). Renal oxidative stress was increased in aged WKY and SHR. Long-term FR significantly reduced the BMI and decreased renal oxidative stress when compared to age-matched WKY and SHR. The renal medulla of aged WKY and SHR had increased MR action, assessed by the urinary Na^+/K^+ ratio which correlated positively with increased plasma aldosterone levels, nuclear MR content and expressions of Na^+,K^+-ATPase α_1-subunit and the α-subunit of the epithelial sodium channel (αENaC). In contrast, when compared to aged WKY and SHR, aged WKY-FR had decreased plasma aldosterone levels and decreased activation of the aldosterone/MR system in the renal medulla. Systolic and diastolic blood pressures in SHR increased with age and were significantly higher than that in age-matched WKY. In conclusion, renal oxidative stress and plasma aldosterone in aged WKY increased to levels observed in SHR but were not sufficient to result in sustained increases in blood pressure. Activation of the aldosterone/MR system is intensified by aging in SHR whereas hyperaldosteronism in WKY is associated with increases in body fat mass and not with aging *per se*.

A broad-spectrum of physiological, functional and morphological changes in the kidney is associated with age, resulting in an almost inevitable decline of renal function (Kielstein et al. 2003; Epstein 1996). Previous studies have shown renal function decline to be associated with both structural (glomerulosclerosis, tubular atrophy and interstitial fibrosis) and functional (decreases in glomerular filtration rate (GFR), proteinuria, reduced ability to concentrate or dilute urine, impairment of electrolyte and ion transport, alteration in hormonal functions, reduced drug excretion) changes in the

kidney (reviewed in refs (Martin and Sheaff 2007) and (Zhou et al. 2008)).

In the presence of age-related diseases, such as heart failure and hypertension, these changes can be accelerated (Fischer and O'Hare). The prevalence of hypertension increases with age (Mosterd et al. 1999). Hypertension also correlates with altered kidney function and structure, which has been suggested to play a role in the development of this disorder (Mullins et al. 2006). This became particularly evident with renal cross-transplantation between normotensive and hypertensive strains. Normotensive rats receiving a kidney from hypertensive rats were found to develop hypertension (Bianchi et al. 1974). The spontaneously hypertensive rat (SHR) is a genetic model of hypertension characterized by the resistance to the natriuretic effect of dopamine and D_1-like receptor agonists, as a result of a defective transduction of the D_1 receptor signal in renal proximal tubules (Jose et al. 2010). It has been suggested that increased oxidative stress in renal proximal tubules of the SHR could be a mechanism for defective dopamine D_1 receptor/G-protein coupling (White and Sidhu 1998). Moreover, recent studies have shown the overexpression of Na^+-independent and pH-sensitive amino acid transporter LAT2 (Slc7a8) in the SHR kidney, which might contribute to enhanced L-DOPA uptake in the proximal tubule and increased dopamine production (Pinho et al. 2004), as an attempt to overcome the defect in D1 receptor function.

Aldosterone is a major regulator of extracellular fluid (ECF) volume and is the principal determinant of K^+ homeostasis (Bhargava et al. 2004). Acting on the mineralocorticoid receptors (MR) it stimulates Na^+ reabsorption, K^+ and H^+ secretion by the distal nephron, particularly in the collecting duct (O'Neil 1990). The MR is primarily localized in the cytosol of the cell in the absence of ligand (Nishi et al. 2001). Binding of aldosterone to the MR triggers its nuclear translocation and activity as a transcription factor (Fuller and Young 2005; Loffing et al. 2001). Inappropriate aldosterone secretion in relation to sodium balance leads to hypertension in the case of hyperaldosteronism, or to hypotension in the case of hypoaldosteronism (Epstein 2001). Although we recently showed that plasma aldosterone levels are increased in aged WKY (Pinto et al. 2011) the available data on the relationship between age and the function of the renin-angiotensin-aldosterone system in normotensive healthy adults are conflicting. Some authors noted diminished urinary aldosterone or plasma renin and aldosterone values (Weidmann et al. 1975), but others reported that plasma aldosterone remains unchanged with aging (Abd-Allah et al. 2004). Our group has recently reported the effects of aging (from 13- to 91-weeks of

age) on Na^+,K^+-ATPase expression and activity in the kidney of WKY rats (Silva et al. 2010). Furthermore, Simão et al., reported that aged WKY and SHR display increased levels of oxidative stress in renal tissue that were suggested to be related to increases in body weight (Simao et al. 2011). In the present study we investigated age-related changes in the renal regulation of the aldosterone/MR system in normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR). In comparison with SHR, aging in WKY was accompanied by marked increases in body weight, renal oxidative stress and a relative greater increase in plasma aldosterone levels. In WKY these changes were attenuated by long-term food restriction.

Materials and Methods

Animal preparation and experimental design

All rat interventions were performed in accordance with the European Directive number 86/609, and the rules of the "Guide for the Care and Use of Laboratory Animals", 7th edition, 1996, Institute for Laboratory Animal Research (ILAR), Washington, DC. Five-week old male WKY and SHR rats were obtained from Harlan-Interfauna Ibérica (Barcelona, Spain) and carefully maintained and monitored until 13 and/or 52 weeks of age. One group of WKY rats were either fed *ad-libitum* or subjected to 15% food restriction (FR) until 13 weeks of age while another group was fed *ad-libitum* or subjected to 15% FR until 52 weeks of age. The rats were housed under controlled conditions (12 h light/dark cycle and room temperature at 22±2 ºC) and had free access to tap water and fed standard rat chow (PANLAB, Barcelona, Spain). Blood pressure (systolic and diastolic) was measured using a photoelectric tail-cuff detector (LE 5000, Letica, Barcelona, Spain). Body mass index (BMI) was determined in all animals as reported previously by other authors (Novelli et al. 2007), using the formula: BMI = body weight (g) / length^2 (cm^2), where "length" corresponds to the "nose-to-anus" length.

Metabolic study

Forty-eight hours before the experiments, 13- or 52-week old rats were placed in metabolic cages (Tecniplast, Buguggiate, Italy) for a 24 h urine collection. The urine samples were collected in vials that were subsequently stored at -80 ºC until assayed. After completion of this protocol, rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). The animals were then sacrificed by exsanguination using cardiac puncture and the blood collected into tubes containing K_3 EDTA for later determination of plasma biochemical parameters. Before excising their kidneys, a cannula was inserted in the right ventricle of the heart and animals were perfused with ice-

cold saline (0.9 % NaCl) to remove all blood from the kidneys. The kidneys were then excised, weighed, decapsulated, and the renal cortex and medulla rapidly separated by fine dissection.

Plasma and urine biochemistry

All biochemical assays were performed by Cobas Mira Plus analyzer (ABX Diagnostics for Cobas Mira, Switzerland). Plasma aldosterone was assayed by radioimmunoassay (Diagnostic Products Corporation; Los Angeles, CA).

H_2O_2 production by renal medulla and cortex

H_2O_2 was measured fluorometrically using the Amplex Red Hydrogen Peroxide Assay kit (Molecular Probes Inc., Eugene, OR, USA). Amplex Red is a fluorogenic substrate with very low background fluorescence that reacts with H_2O_2 with a 1:1 stoichiometry to produce a highly fluorescent reagent. Renal cortex and medulla were cut into square pieces and incubated at 37 ºC in Krebs-HEPES buffer (in mM: NaCl 118, KCl 4.5, $CaCl_2$ 2.5, $MgCl_2$ 1.20, K_2HPO_4 1.2, $NaHCO_3$ 25.0, Na-HEPES 25.0, and glucose 5; pH 7.4) for 90 min. H_2O_2 released from the tissue was detected using the Amplex Red Hydrogen Peroxide Assay kit. Fluorescence intensity was measured in a multiplate reader (Spectromax Gemini Molecular Devices) at an excitation wavelength of 530 nm and emission wavelength of 590 nm at room temperature. After subtracting background fluorescence, the concentrations of renal cortical and medullary H_2O_2 (in pmol/mg) were calculated using a resorufin–H_2O_2 standard calibration curve generated from experiments using H_2O_2 and Amplex Red.

Malondialdehyde (MDA) determination

Briefly, urine samples were combined with 8.1% SDS for 10 min. Equal volumes of 28% trichloroacetic acid (TCA) and 0.6% thiobarbituric acid (TBA) were added and heated at 95 ºC during 1 hour. After cooling at room temperature, a mixture of chloroform/methanol (2:1) were added and centrifuged at 5000 rpm for 10 min. Supernant absorbance was measured at 532 nm. The content of urinary malondialdehyde MDA was calculated using a MDA standard calibration curve and results were expressed as nanomoles of MDA per 24 h urine volume.

Western Blotting

Isolated renal cortex and medulla of 13- and 52-week old WKY, WKY-FR and SHR were sliced very thinly and lysed by the addition of RIPA buffer containing 150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 100 µg/ml phenylmethylsulfonyl fluoride (PMSF), 2 µg/ml leupeptin and 2 µg/ml aprotinin, as described previously (Amaral et al. 2009).

Nuclear protein was prepared as described by other authors (Kanematsu et al.). Briefly, renal medullary and cortical tissues were homogenized in ice-cold buffer (A) containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM PMSF, 2 µg/ml leupeptin, 2 µg/ml aprotinin and 10% Nonidet P-40. After centrifugation of the homogenate at 1,000 x g for 5 min at 4°C, the supernatants and pellets were collected separately. The supernatants were centrifuged again at 6,000 x g for 10 min. For nuclear fraction isolation, the pellets from the first centrifugation, which contain cell nuclei, were washed with buffer A and then incubated with ice-cold buffer (B) containing 5 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 300 mM NaCl, 400 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2 µg/ml leupeptin, 2 µg/ml aprotinin, and 26% glycerol for 30 min to release nuclear proteins. Next, the reaction mixtures were centrifuged at 24,000 x g for 30 min, and the supernatant (containing nuclear-enriched protein fraction) was collected and frozen in liquid nitrogen until use as nuclear extracts for western blot analysis. Protein concentrations in nuclear protein enriched fractions and homogenates of total protein were determined by the Bradford assay. Proteins were subjected to SDS-10%PAGE and then electroblotted onto nitrocellulose membranes (Bio-Rad). Blots were blocked for 1 h with 5% non-fat dry milk in TBS (10 mmol/l tris-buffered saline) at room temperature with constant shaking and subsequently incubated with antibodies mouse polyclonal anti-MR (1:500,(Gomez-Sanchez et al. 2006); kindly supplied by Dr Elise Gómez-Sánchez, (University of Mississippi Medical Center, Jackson, Mississippi); rabbit polyclonal anti-αENaC (1:500, Chemicon); rabbit polyclonal anti-NHE3 (1:200) (Xu et al. 2000); kindly supplied by Dr Pedro A. Jose, (George Washington University School of Medicine & Public Health, Washington, D.C.); mouse monoclonal anti-α1-subunit of Na^+,K^+-ATPase (1:1000, Santa Cruz Biotechnology) and mouse monoclonal anti-GAPDH (1:20,000, Santa Cruz Biotechnology) in 5% non-fat dry milk in TBS-T overnight at 4 ºC. Mouse monoclonal anti-β-actin (1:20,000, Santa Cruz Biotechnology) was used as an internal loading control for homogenates of nuclear proteins as previously described by other authors (Kariagina et al. 2005; Olave et al. 2002). Since β-actin expression did not vary between the different groups it was regarded as a safe loading control. The immunoblots were subsequently washed and incubated with fluorescently labeled goat anti-rabbit (1:20,000; IRDyeTM 800, Rockland) or the fluorescently labeled goat anti-mouse secondary antibody (1:20,000; AlexaFluor 680, Molecular Probes) for 60 min at room temperature and protected from light. The membrane was washed and imaged

by scanning at both 700 and 800 nm, with an Odyssey Infrared Imaging System (LI-COR Biosciences).

Drugs

All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise stated.

Data analysis

Arithmetic means are given with standard error of the mean (SEM). One-way ANOVA followed by the Newman–Keuls test for multiple comparisons test was used to determine differences between single groups when more than 2 groups were represented. A *P* value less than 0.05 was assumed to denote a significant difference.

Results

Physiological parameters in aged WKY, WKY-FR and SHR

As expected, systolic (SBP) and diastolic (DBP) blood pressures in the SHR were significantly higher than that in age-matched WKY (**Figure 1A and 1B**). Blood pressure in the SHR was found to increase with age, whereas SBP and DBP in WKY were not significantly affected by aging (**Figure 1A and 1B**). Body weight of WKY and SHR increased steadily until 52 weeks of age (**Figure 1C**). By 52 weeks of age WKY had significantly greater body weight than SHR (**Table 1**). Moreover, at 52 weeks of age WKY had a BMI (g/cm^2) of 0.80±0.01, significantly above the considered "normal" range (0.45±0.02 to 0.68±0.05) determined by other authors for adult Wistar rats (Novelli et al. 2007). For this reason WKY were submitted to 15% FR (WKY-FR) from 5 to 13 or 52 weeks of age. WKY-FR had at 13 weeks of age, similar body weight to those fed *ad libitum* (**Table 1**).

FR resulted in a 14% body weight reduction at the age of 52 weeks in WKY-FR compared to WKY fed *ad libitum* and in a significant decrease in the BMI value (**Table 1**). As electrolyte excretion may be conditioned by food ingestion, all groups were placed in metabolic cages and fed *ad libitum* 48 hours before experiments. No differences in the amount of food ingested during this time period were observed between groups (data not shown).

Urinary Na^+ was lower in 13-week-old SHR than in age-matched WKY. At 13 weeks of age WKY-FR had increased urinary Na^+ when compared to age-matched WKY. On the other hand, aging had no effect on urinary Na^+ levels in WKY, WKY-FR and SHR (**Table 1**). No differences were found in urinary K^+ levels between young WKY, WKY-FR or SHR. Aging was accompanied by increases in urinary K^+ in all groups.

Table 1. Physiological parameters in 13- and 52-week old WKY, WKY-FR and SHR.

	WKY		WKY-FR		SHR	
	13 weeks	**52 weeks**	**13 weeks**	**52 weeks**	**13 weeks**	**52 weeks**
Body weight (g)	321±12	578±9*	324±8	498±10$^{*\#\lambda}$	272±5$^{\#}$	392±6$^{\#}$
BMI (g/cm^2)	0.60±0.01	0.80±0.01*	0.60±0.01	0.73±0.01$^{*\#\lambda}$	0.59±0.01	0.68±0.01$^{*\#}$
UNa$^+$ (mmol/24h)	1.77±0.11	1.36±0.23	2.33±0.05$^{\#}$	1.51±0.08	1.32±0.06$^{\#}$	1.07±0.13
UK$^+$ (mmol/24h)	1.10±0.06	1.56±0.10*	1.11±0.06	1.35±0.05$^{*\#}$	0.86±0.08	1.30±0.07$^{*\#}$
Plasma cholesterol (mg/dl)	78±4	82±7	65±2$^{\#}$	65±2$^{\#\lambda}$	50±2$^{\#}$	51±2$^{\#}$
Plasma creatinine (mg/dl)	0.39±0.04	0.50±0.01*	0.43±0.03	0.57±0.03*	0.41±0.03	0.51±0.02*

Significantly different from corresponding values in 13-week old animals (*P<0.05). Significantly different from age-matched WKY (#P< 0.05). Significantly different from 52-week old SHR (λP< 0.05).

At 52 weeks of age urinary K^+ was significantly lower in WKY-FR and SHR than in age-matched WKY (**Table 1**). SHR had increased levels of urinary protein (UPRT) when compared to age-matched WKY. Furthermore, while no changes in UPRT were observed in aged WKY or WKY-FR, urinary protein excretion in SHR increased with age (**Table 1**).

Cholesterol levels were unaffected by aging in all WKY groups although levels were decreased in young and aged WKY-FR animals when compared to age-matched WKY (**Table 1**). Plasma creatinine levels increased with age in WKY, WKY-FR and SHR, although no differences were observed between age-matched animals (**Table 1**).

Renal oxidative markers in aged WKY, WKY-FR and SHR

In order to examine the renal oxidative status with aging, H_2O_2 production and lipid peroxidation levels were measured in the medulla and cortex of 13- and 52-week old WKY, WKY-FR and SHR (**Figure 2**). Aging was associated with increases in H_2O_2 production in the renal medulla of all rats, however, at 52 weeks of age, WKY-FR had lower H_2O_2 production than age-matched WKY and SHR (**Figure 2A**). The production of H_2O_2 in the renal cortex of WKY, WKY-FR and SHR was similar to that observed in the renal medulla (**Figure 2B**).

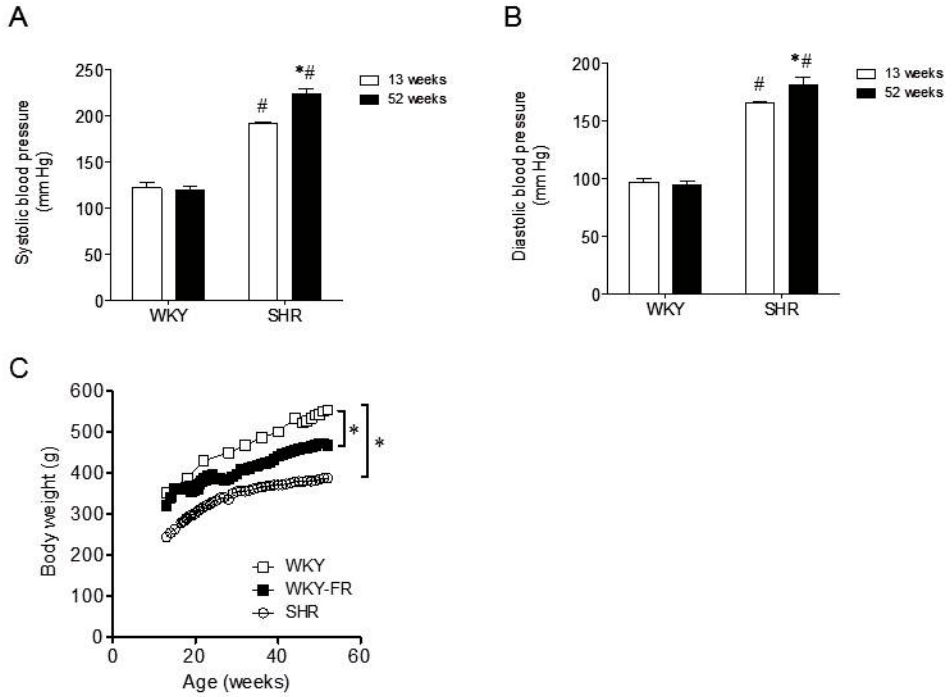

Figure 1. Changes in systolic (A) and diastolic (B) blood pressure of 13- and 52-week old WKY and SHR. Significantly different from values in 13-week old rats (*P<0.05) and significantly different from age-matched WKY (#P< 0.05) using the Newman–Keuls test. (C) Changes in body weight of WKY, WKY-FR and SHR rats as a function of time. Significantly different from WKY at 52 weeks of age (*P<0.05) using the Newman–Keuls test. Each bar represents the mean ± SEM of 6-12 rats. Each point represents the mean ± SEM of 6–12 rats.

Young WKY-FR had decreased H_2O_2 production in comparison to age-matched WKY and SHR. Aging was accompanied by increases in H_2O_2 production in all groups. By 52 weeks of age no differences were found between age-matched WKY and SHR, but WKY-FR had lower H_2O_2 production levels than WKY and SHR (**Figure 2B**).

We next measured the concentration of urinary malondialdehyde (MDA), a marker of lipid peroxidation (**Figure 2C**). Lipid peroxidation levels were similar in 13-week old WKY, WKY-FR and SHR. MDA levels increased with age in both WKY and SHR, but not in WKY-FR. No differences in MDA levels were observed between aged WKY and SHR. In contrast, lipid peroxidation levels were lower in 52-week old WKY-FR than in age-matched WKY and SHR

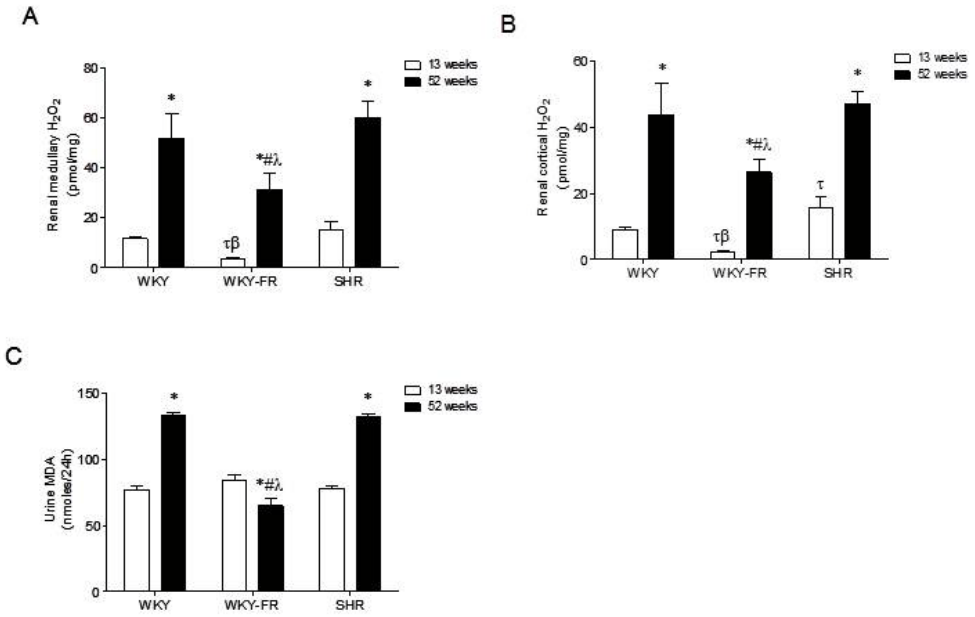

Figure 2. Production of H_2O_2 by renal medulla (A) and cortex (B) of 13- and 52-week old WKY, WKY-FR and SHR. Urinary levels of lipid peroxidation (MDA levels) in 13- and 52-week old WKY, WKY-FR and SHR (C). Each bar represents the mean ± SEM of 3-7 rats. Significantly different from 13-week old WKY ($\tau P < 0.05$), significantly different from 13-week old SHR ($\beta P < 0.05$), significantly different from corresponding values in 13-week old animals (*P<0.05), significantly different from 52-week old WKY (#P< 0.05) and significantly different from 52-week old SHR ($\lambda P < 0.05$) using the Newman–Keuls test.

Plasma levels of aldosterone and the urinary Na^+/K^+ ratio (UNa^+/K^+)

Plasma aldosterone levels were greater in 13-week old SHR when compared to age-matched WKY (**Figure 3A**). At this age, no differences were found between WKY and WKY-FR. Aging was accompanied by marked increases in plasma aldosterone levels in all groups. By 52 weeks of age no differences were observed between WKY and SHR. However, WKY-FR had significantly lower plasma aldosterone levels when compared to age-matched WKY and SHR (**Figure 3A**). The mineralocorticoid effect was assessed by the urinary Na^+/K^+ (UNa^+/K^+) ratio (**Figure 3B**). The UNa^+/K^+ ratio was significantly increased in 13-week old WKY-FR in comparison to age-matched WKY and SHR. Aging decreased the UNa^+/K^+ ratio in all animal groups, however, at 52 weeks of age WKY-FR had higher UNa^+/K^+ ratio when compared to age-matched WKY and SHR. No differences in the UNa^+/K^+ were observed between 52-week old WKY and SHR (**Figure 3B**).

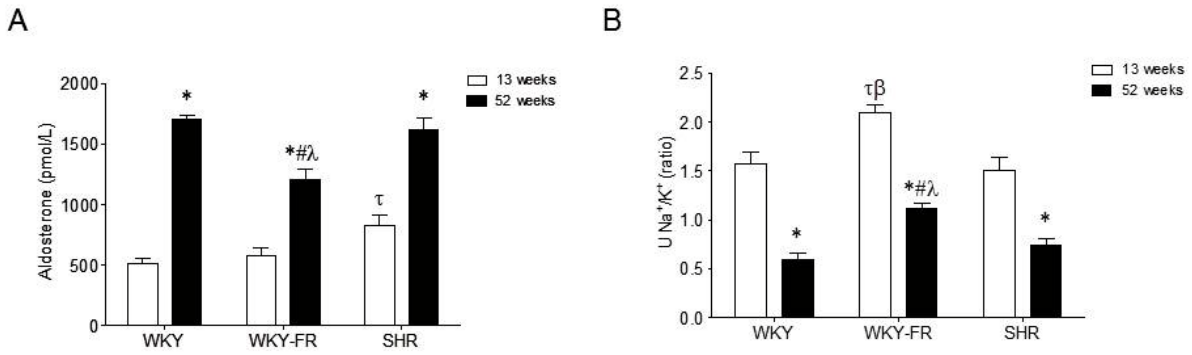

Figure 3. Plasma levels of aldosterone (pmol/L) (A) and changes in urinary Na^+/K^+ (UNa^+/K^+) ratio (B) in 13- and 52-week old WKY and SHR. Each column represents the mean ± SEM of 6-12 rats. Significantly different from 13-week old WKY (τP< 0.05), significantly different from 13-week old SHR (βP< 0.05), significantly different from corresponding values in 13-week old animals (*P<0.05), significantly different from 52-week old WKY (#P< 0.05) and significantly different from 52-week old SHR (λP< 0.05) using the Newman–Keuls test.

Renal abundance of total and nuclear MR in aged WKY, WKY-FR and SHR

Aldosterone regulates sodium reabsorption in epithelial tissues such as the kidney via the activation of intracellular MR. Therefore, total and nuclear abundance of MR was measured in the renal medulla and cortex of 13- and 52-week old WKY, WKY-FR and SHR (**Figure 4 and 5**). In the renal medulla, no differences in total MR abundance were found between 13-week old WKY, WKY-FR and SHR (**Figure 4A**). Total MR abundance was similar in WKY and SHR at 52 weeks of age. Aged WKY-FR had higher total MR abundance than age-matched WKY and SHR (**Figure 4A**). The nuclear content of MR in the renal medulla was increased in 13-week old SHR in comparison to age-matched WKY (**Figure 4B**). Aged WKY, WKY-FR and SHR had significantly increased MR nuclear abundance when compared to 13-week old animals. However, MR nuclear abundance was significantly greater in 52-week old SHR than in age-matched WKY. Aged WKY-FR had lower MR nuclear abundance in comparison to age matched WKY and SHR (**Figure 4B**).

In the renal cortex, no differences were observed in the abundance of total or nuclear MR between 13-week old WKY, WKY-FR and SHR (**Figure 5A and 5B**). Total MR abundance decreased in the renal cortex of aged WKY, but increased in aged SHR (**Figure 5A**). In WKY-FR, no age-related changes in total MR abundance were observed. Total MR was greater in 52-week old WKY-FR than in WKY. Conversely, total MR levels were lower in aged WKY-FR than in age-matched SHR (**Figure 5A**). Nuclear

MR content was decreased in the renal cortex of aged WKY, WKY-FR and SHR (**Figure 5B**). No differences were observed between 52-week old WKY and SHR. In contrast, WKY-FR had significantly higher nuclear MR content than age-matched WKY and SHR (**Figure 5B**).

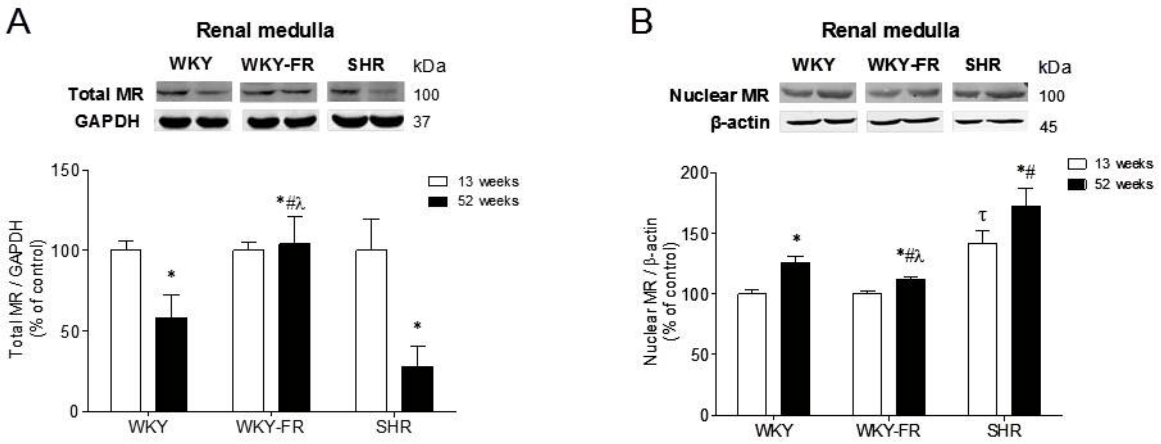

Figure 4. Total mineralocorticoid receptor (MR) expression (A) and nuclear MR content (B) in the renal medulla of 13- and 52-week old WKY, WKY-FR and SHR. Values are normalized to the level of GAPDH or β-actin expression for each protein of interest and expressed as percentage of control (13-week old WKY). Each column represents the mean ± SEM (n=4 per group). Significantly different from 13-week old WKY ($\tau P < 0.05$), significantly different from corresponding values in 13-week old animals ($^*P<0.05$), significantly different from 52-week old WKY ($\#P < 0.05$) and significantly different from 52-week old SHR ($\lambda P < 0.05$) using the Newman–Keuls test.

The effect of aging on the renal expression of aldosterone effectors in WKY, WKY-FR and SHR

We evaluated the expression of several sodium transport proteins that have been reported to be regulated by aldosterone, such as NHE3, Na^+,K^+-ATPase α_1-subunit, and αENaC in the renal medulla and renal cortex of 13- and 52-week old WKY, WKY-FR and SHR. No differences in the renal medullary abundance of NHE3, Na^+,K^+-ATPase α_1-subunit and αENaC were found between WKY, WKY-FR, and SHR at 13 weeks of age (**Figure 6**). Moreover, no significant differences in NHE3 expression were found between age-matched WKY, WKY-FR and SHR in the renal medulla nor did NHE3 abundance change with aging (**Figure 6B**). Expression of Na^+,K^+-ATPase α_1-subunit in the renal medulla was significantly increased in aged WKY and SHR, but not in WKY-FR (**Figure 6C**). No differences were found between age-matched WKY and SHR (**Figure 6C**).

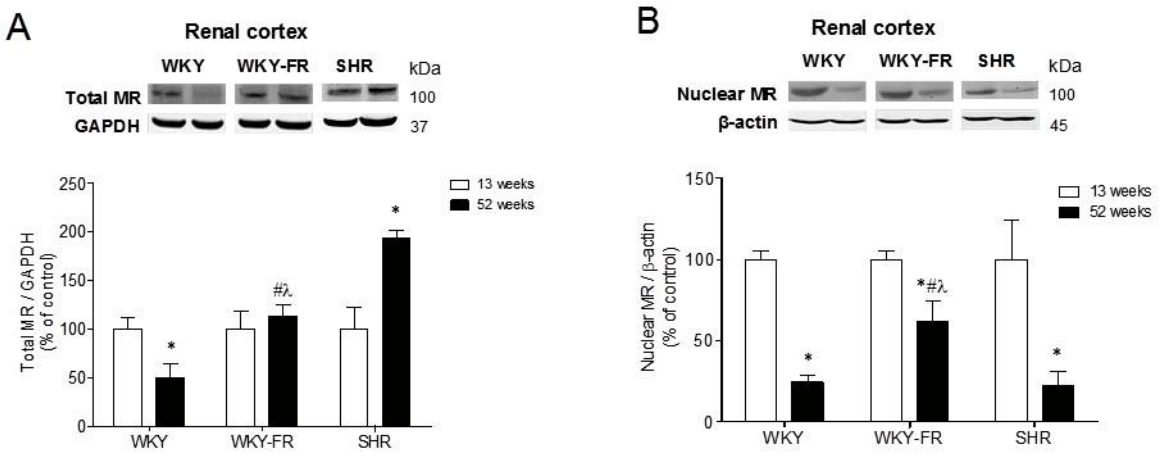

Figure 5. Total mineralocorticoid receptor (MR) expression (A) and nuclear MR content (B) in the renal cortex of 13- and 52-week old WKY, WKY-FR and SHR. Values are normalized to the level of GAPDH or β-actin expression for each protein of interest and expressed as percentage of control (13-week old WKY). Each column represents the mean ± SEM (n=4 per group). Significantly different from 13-week old WKY ($\tau P < 0.05$), significantly different from corresponding values in 13-week old animals ($*P<0.05$), significantly different from 52-week old WKY ($\#P < 0.05$) and significantly different from 52-week old SHR ($\lambda P < 0.05$) using the Newman–Keuls test.

Similar to what other authors have observed (Tiwari et al. 2009), we identified the presence of 3 different MW bands for αENaC expression in the renal medulla of WKY and SHR (**Figure 6**). The 65 kDa band density for αENaC was greater in aged WKY, WKY-FR and SHR than in young rats. Furthermore, at 52 weeks of age, WKY-FR had significantly decreased 65 kDa band density in comparison to age-matched WKY and SHR (**Figure 6D**). Aging was associated with decreases in the 75 kDa band density in both WKY and SHR, but not in WKY-FR, and no differences were found between age-matched WKY and SHR (**Figure 6E**). At 52 weeks of age WKY-FR had significantly increased 75 kDa band density in comparison to age-matched WKY and SHR (**Figure 6E**). No age-related or strain-related changes in 90 kDa band density for αENaC in the renal medulla were observed (**Figure 6F**).

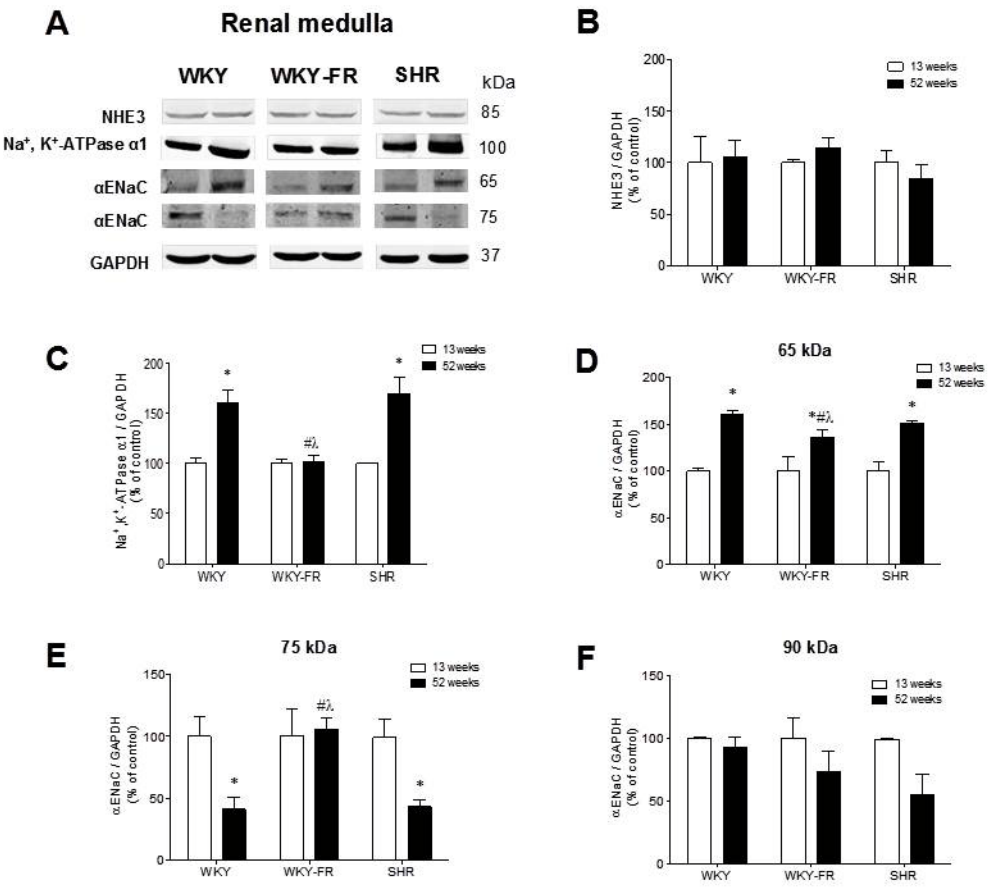

Figure 6. Representative immunoblots of renal medullary homogenates from 13- and 52-week old WKY, WKY-FR and SHRs probed with NHE3, α_1-subunit Na^+,K^+-ATPase, αENaC and GAPDH antibodies (A). Protein expression of NHE3 (B) and α_1-subunit Na^+,K^+-ATPase (C). D-F, Quantification of several bands of αENaC: 65 kDa (D), 75 kDa (E) 90 kDa (F). Values are normalized to the level of GAPDH expression for each protein of interest and expressed as percentage of control (13-week old WKY). Each column represents the mean ± SEM (n=4 per group). Significantly different from corresponding values in 13-week old animals (*P<0.05), significantly different from 52-week old WKY (#P<0.05) and significantly different from 52-week old SHR (λP< 0.05) using the Newman–Keuls test.

The renal cortical expression profiles of NHE3, Na^+,K^+-ATPase α_1-subunit and αENaC of WKY, WKY-FR and SHR were also evaluated (**Figure 7**). Aging was associated with decreases in NHE3 abundance in WKY and SHR but not in WKY-FR (**Figure 7B**). At 52 weeks of age WKY-FR had significantly increased NHE3 abundance in comparison to age-matched WKY and SHR (**Figure 7B**). No significant

differences in Na^+,K^+-ATPase α_1-subunit abundance in the renal cortex were found between WKY and SHR at any age nor did FR have an effect on Na^+,K^+-ATPase α_1-subunit expression (**Figure 7C**). Age-related decreases in cortical abundance of NHE3 in WKY and SHR (**Figure 7B**) correlated positively with the decreases of the 75 and 90kDa band densities of αENaC (**Figure 7D and 7E, respectively**).

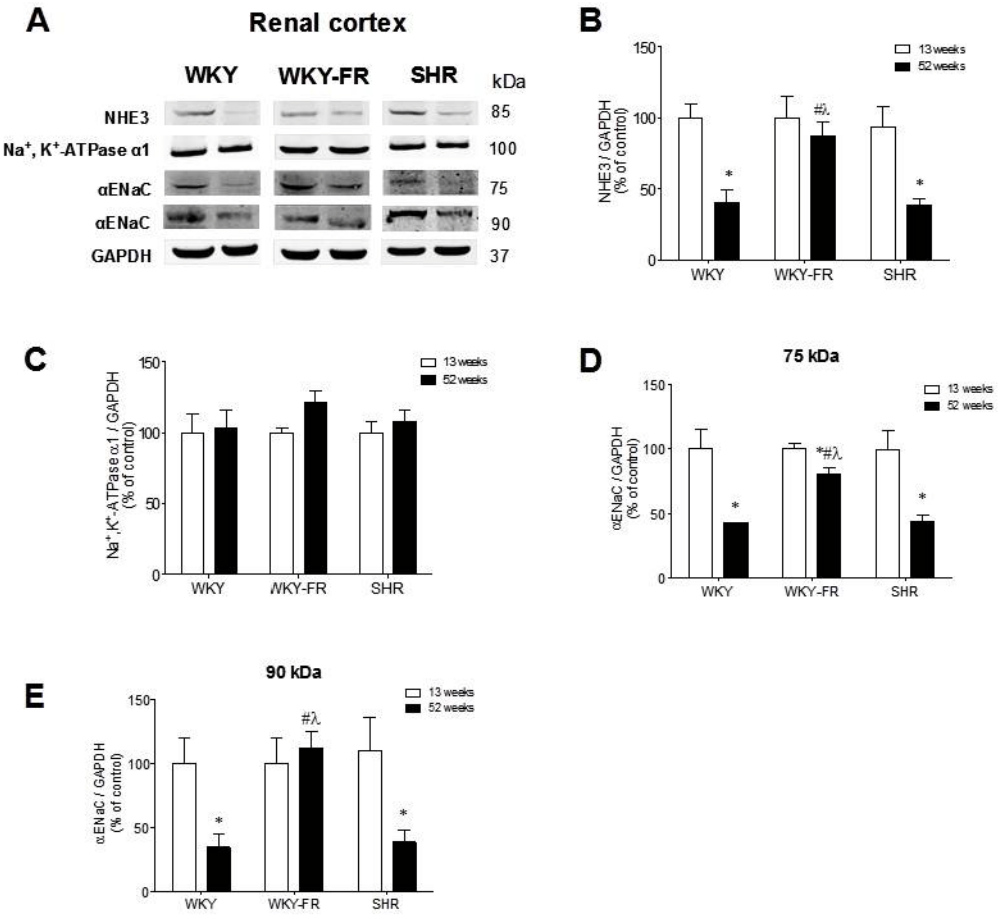

Figure 7. Representative immunoblots of renal cortical homogenates from 13- and 52-week old WKY, WKY-FR and SHRs probed with NHE3, α_1-subunit Na^+,K^+-ATPase, αENaC and GAPDH antibodies (A). Protein expression of NHE3 (B) and α_1-subunit Na^+,K^+-ATPase (C). Quantification of αENaC: 75 kDa (D) and 90 kDa (E). Values are normalized to the level of GAPDH expression for each protein of interest and expressed as percentage of control (13-week old WKY). Each column represents the mean ± SEM (n=4 per group). Significantly different from corresponding values in 13-week old animals (*P<0.05), significantly different from 52-week old WKY (#P<0.05) and significantly different from 52-week old SHR (λP< 0.05) using the Newman–Keuls test.

No differences in the 75 and 90kDa band densities of αENaC were found between age-matched WKY and SHR (**Figure 7D and 7E, respectively**). Although the 90kDa band density of αENaC did not vary with age in WKY-FR (**Figure 7E**), the 75kDa band density was slightly, but significantly, decreased in 52-week old versus 13-week old WKY-FR (**Figure 7D**). In this study we found that the density of the 65 kDa band for αENaC in renal cortex was too low to be quantified accurately.

The magnitude of the effect of aging in WKY, WKY-FR and SHR

The modifying effect of age on the expression levels of NHE3, α_1-subunit Na^+,K^+-ATPase and αENaC in the renal medulla and renal cortex of WKY and SHR was summarized in Fig 8A and 8B. The data was analyzed and plotted as a percent of control (respective young animals). The effect of age on the renal medullary and cortical expression profiles of αENaC, NHE3 and the α_1-subunit Na^+,K^+-ATPase were very similar between WKY and SHR (**Figure 8A and 8B**). On the other hand, in the renal medulla, aging was accompanied by greater increases in αENaC (65 kDa band) and the α_1-subunit Na^+,K^+-ATPase abundances in SHR than in WKY-FR (**Figure 8A**). In the renal cortex, age-related decreases in αENaC and NHE3 abundances were more accentuated in SHR than in WKY-FR (**Figure 8B**).

Discussion

The findings reported here demonstrate that aging in WKY and SHR is accompanied by exacerbated oxidative stress at the kidney level and increased aldosterone/MR system activation. Despite similar oxidative stress status and plasma aldosterone levels in aged WKY and SHR, aged WKY did not develop hypertension. Moreover, long-term FR reduced age-related increases in renal oxidative stress and circulating aldosterone.

Similarly to results obtained by other authors (Natalucci et al. 2003; Ruetten et al. 1999), this study showed that WKY rats become obese with age, which mainly results from the accumulation of fat mass. Studies have shown that body weight of aging WKY rat increases significantly when compared with age-matched SHR (Natalucci et al. 2003; Ruetten et al. 1999). A 63% weight gain from the ages of 12 weeks to 40 weeks in WKY rats has been reported while body weight remained unaltered in aging SHR (Natalucci et al. 2003). SHR are considered hyperactive compared to WKY (Hendley et al. 1992).

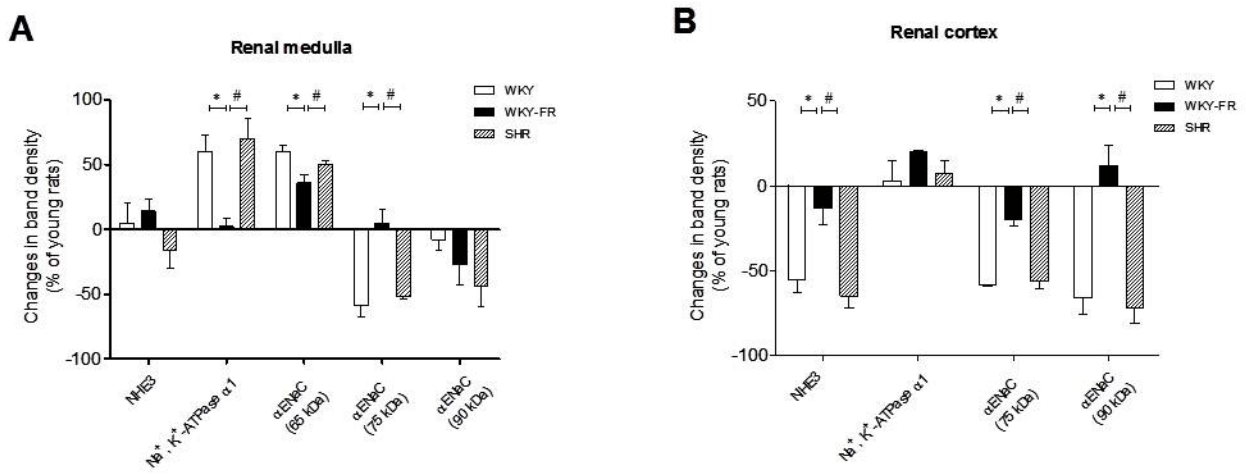

Figure 8. Differences in the magnitude of the effect of aging on the expression of several aldosterone effectors in WKY, WKY-FR and SHR in the renal medulla (A) and the renal cortex (B). Each column represents the mean ± SEM (n=4 per group). Significantly different from WKY (*P<0.05), significantly different from WKY-FR (#P< 0.05) using the Student's *t*-test.

In contrast, WKY is particularly inactive compared to other often-used rat strains and has even been proposed as an animal model of endogenous depression (Wieland et al. 1986; Will et al. 2003). Therefore, the greater increase in body weight observed in WKY may be due to reduced physical activity energy expenditure, slow metabolism and decreased resting energy expenditure. In contrast, SHR may burn more calories than WKY through increased physical activity energy expenditure and an increased resting metabolic rate. There is an increasing amount of evidence that body fat mass is associated with increased aldosterone levels (Bomback and Klemmer 2009). Two mechanisms have been suggested to explain elevated aldosterone levels in obesity. First, that human adipose tissue produces several components of the renin-angiotensin-aldosterone system, mainly adipose tissue-derived angiotensinogen. In line with this view, it has been shown that weight loss reduces systemic RAS activity (Goossens et al. 2007). Second, that increased fatty acid production in obese humans, especially nonesterified fatty acids, might stimulate aldosterone production, in a renin-independent manner (Goodfriend et al. 1999). Adipocyte-derived aldosterone-releasing factors, although not yet identified, have been found to stimulate aldosterone secretion from

adrenocortical cells (Ehrhart-Bornstein et al. 2003).

Increased production of H_2O_2 was observed in the medulla and cortex of 52-week old WKY and SHR, which is in agreement with growing evidence implicating an age-related decline in activity of antioxidant systems (Harman 1998). However, no differences in H_2O_2 production in the renal medulla and cortex were observed between 52-week old WKY and SHR. Moreover, aged WKY and SHR also exhibited significantly increased urinary MDA levels, a frequently used index of cell lipid peroxidation. Several reports have shown an age-associated increase in the concentrations of lipid peroxidation products (Pratico 2002). We propose that oxidative stress in WKY may be associated with their age-related weight gain. Production of ROS is increased in adipose tissue of obese mice, accompanied by augmented expression of NADPH oxidase and decreased expression of antioxidative enzymes (Furukawa et al. 2004). Accordingly, our results demonstrated that renal H_2O_2 levels were decreased WKY-FR when compared to age-matched WKY and SHR fed *ad libitum*. As previously reported, SBP and DBP were significantly increased in SHR in comparison to age-matched WKY (Gerova and Kristek 2008). Studies have suggested that hypertension might result from increased levels of oxidative stress in the renal medulla (Makino et al. 2002). Reduction of SOD activity in the renal medulla by the chronic infusion of the SOD inhibitor DETC into the renal medullary interstitium resulted in an increase of medullary superoxide anion concentrations, a reduction of blood flow to the renal medulla, and a sustained increase of blood pressure (Makino et al. 2002). Our findings demonstrate that despite similar increases in renal oxidative stress and plasma aldosterone levels, SBP and DBP in WKY were significantly lower than age-matched SHR and that renal oxidative stress and increases in sodium reabsorption may not be sufficient to cause hypertension.

The mineralocorticoid response was assessed via the UNa^+/K^+ ratio in WKY, WKY-FR and SHR. The UNa^+/K^+ ratio has been used previously by other authors to examine long-term changes in the aldosterone response (Hanukoglu et al. 2008). All groups were placed in metabolic cages and fed *ad libitum* 48 hours before sacrifice and no differences in the amount of food ingested during this time period were observed between groups. Given that food consumption was similar between groups we assumed that any potential changes in urinary Na^+ and K^+ excretion would be a result of dysregulated renal ion transporters. In our study we found that aging from 13 to 52 weeks was accompanied by a decrease in the UNa^+/K^+ ratio in WKY and SHR and an increase in plasma aldosterone levels. This

decrease was mainly due to an increase in urinary K^+ excretion (Na^+ remained constant with aging). When aldosterone levels are elevated the kidney can overcome aldosterone's Na^+-retaining action by activation of the so-called "aldosterone escape" mechanism, in which renal tubule Na^+ reabsorption decreases despite the continued presence of aldosterone (Schrier 2010). Aldosterone initially decreases urinary Na^+ by increasing sodium retention. However, urinary Na^+ quickly returns to baseline levels through pressure natriuresis and decreased proximal Na^+ reabsorption (Schrier 2010).

The role of aldosterone in the pathogenesis of hypertension is gaining wider recognition with the finding that nearly 1 in 10 hypertensives has inappropriate aldosterone activity (Lim 2002). In this study we report that SHR, a well known experimental model for human essential hypertension, develops hyperaldosteronism with age which is accompanied by increases in MR nuclear trafficking at the renal medulla level and decreases in the UNa^+/K^+ ratio. Studies have shown that aldosterone induces a rapid nuclear accumulation of the MR (Fejes-Toth et al. 1998). Aldosterone-bound MR acts as a transcription factor, therefore, the measurement of nuclear MR content may indicate the level of MR signalling. Moreover, this method has been used by other authors to evaluate MR- and GR (glucocorticoid receptor)-mediated signalling (Nagase et al. 2007; Kariagina et al. 2005). Our results suggest that aging is accompanied by increases in nuclear MR trafficking at the renal medullary level, as a consequence of increased plasma levels of aldosterone. These findings correlate with increased expression of Na^+,K^+-ATPase α_1-subunit and 65 kDa αENaC in the renal medulla of aged rats. In agreement with other studies, we observed the presence of several MW bands for renal αENaC (Tiwari et al. 2009). It has been suggested that higher MW bands for αENaC may represent immature, uncleaved forms of the protein and that the lower bands may be “active” forms of the protein (Ergonul et al. 2006; Guipponi et al. 2002) and that unique MW bands for αENaC may be due to differential activation by steroids (Tiwari et al. 2009). No differences in NHE3 expression were found between age-matched WKY and SHR, although we had previously reported an increase in NHE3 expression in SHR in renal proximal tubule epithelial cells at 4 and 12 weeks of age (Pinho et al. 2007; Pedrosa et al. 2004) that could be related to differential expression in nephron segments other than the proximal tubule. We found that aging is associated with decreases in MR nuclear content, and NHE3 and αENaC expression in the renal cortex. These findings may correspond to a counter-regulatory mechanism activated by increased plasma

aldosterone levels. Potentially counteracting protective mechanisms may be activated via the reduction of cortical nuclear MR and ultimately through the reduction of cortical αENaC and NHE3 expression in an effort to maintain sodium balance in aged WKY and SHR. The dramatic decrease in NHE3 protein expression levels concurs with previous studies that show a decrease in activity and abundance of NHE3 in the kidney of aged rats (Mac Laughlin et al. 2001). We have reported previously that immortalized proximal tubular epithelial (PTE) cells from 4-12 week old SHR have higher Na^+-K^+ ATPase activity and expression than PTE cells from 4-12 week old WKY (Pedrosa et al. 2007). However, the abundance of the renal α_1-subunit of Na^+,K^+-ATPase in freshly isolated renal proximal tubules from 12-week old SHR was found to be similar to that in WKY (Pinho et al. 2007). Likewise, in the present study, no differences in α_1-subunit Na^+,K^+-ATPase expression between 13- and 52-week old age-matched WKY and SHR in the renal cortex were found. However, confirming our previous studies (Silva et al. 2010), α_1-subunit of Na^+,K^+-ATPase expression was increased in the renal medulla with aging.

Plasma aldosterone levels and MR signalling and functionality in the renal medulla were decreased in aged WKY-FR in comparison to age-matched WKY and SHR. Therefore, it is suggested that in WKY, the activation of the aldosterone/MR system is related to increases in body fat mass, and not to the aging process. On the other hand, despite normal body fat mass, SHR already had increased plasma aldosterone levels at 13 weeks of age when compared to age-matched WKY, and the aldosterone/MR system was further intensified by aging in these animals.

In summary, our findings show that age-related increases in body fat mass may be responsible for exacerbated renal oxidative stress, hyperaldosteronism, and increased renal medullary MR trafficking in WKY. Interestingly, despite the existence of multiple independent studies suggesting that aldosterone and ROS broadly contribute to the development of hypertension, blood pressure in WKY remained unaltered throughout this study. Nevertheless, long-term FR in WKY had the beneficial effect of mitigating ROS production, reducing plasma aldosterone levels, and decreasing aldosterone/MR system activation in the renal medulla. These findings shed light on the importance of FR in controlling oxidative stress and the renal aldosterone/MR system. Additionally, we report the aldosterone/MR system is intensified by aging in SHR. The importance of aging in the development of hyperaldosteronism in SHR is undermined. Aging is accompanied by increases in oxidative stress levels and renal aldosterone/MR system activation, which

may play a role in the progression and worsening of hypertension in these animals.

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DISCUSSION AND CONCLUSIONS

Despite the progress made in the molecular identification of cell membrane transport systems, there is still a lack of information on the regulation of renal amino acid transporters. In this study we hypothesised that oxidative stress could modulate the amino acid transporters that are potentially involved in the uptake of L-DOPA, the precursor of dopamine. ROS, originally considered to cause cell damage, are now recognized to be key signaling molecules that mediate diverse biological responses such as induction of host defense genes, activation of transcription factors, phosphorylation of kinases and mobilization of ion transport systems (Droge 2002). On the other hand, it has been suggested that ROS are associated with hypertension, at least in animal models and that a greater understanding of the (patho)biology of ROS may lead to new mechanistic insights and novel diagnostics and treatments for hypertension. The data presented in this work suggests that L-DOPA transporters can be regulated by oxidative stress, mainly in the SHR, possibly as an adaptive mechanism to maintain sodium homeostasis and decrease ROS levels.

In **Chapter I** we addressed the question of whether oxidative stress has a role in the regulation of ASCT2 in immortalized PTE cells from WKY and SHR. Several established cell lines of renal origin have been used previously in our laboratory for analyzing the function of renal amino acid transporters that can potentially transport L-DOPA, such as opossum kidney (OK) cells (Gomes et al. 1997), LLC-PK_1 cells (Soares-da-Silva et al. 2004) and immortalized renal PTE cells from SHR and WKY (Pinho et al. 2004). LLC-PK_1 cells, a

cell line derived from porcine renal tubule epithelial cells that retain several properties of proximal tubular epithelial cells in culture (Hull et al. 1976), express both LAT1 and LAT2 transcripts (Soares-da-Silva et al. 2004). In this cell line, transport of [^{14}C]-L-leucine occurs through the sodium-independent pH-insensitive and high-affinity LAT1 transporter, whereas [^{14}C]-L-DOPA is mainly transported through the sodium-independent pH-insensitive and low-affinity LAT2 transporter and a minor component through a sodium-dependent transporter (Soares-da-Silva et al. 2004). Furthermore, in LLC-PK$_1$ cells, LAT2 gene silencing was shown to reduce markedly the inward and outward transfer of [^{14}C]-L-DOPA (Soares-da-Silva et al. 2004). Immortalized renal proximal tubular epithelial (PTE) cells from SHR and WKY have been used previously in an attempt to better characterize the differences in renal handling of L-DOPA and to evaluate the diversity and regulation of amino acid transport systems in hypertension (Woost et al. 1996; Pedrosa et al. 2004; Pedrosa et al. 2004). Immortalized renal PTE cells from SHR were found to overexpress LAT2 (Pinho et al. 2003). In immortalized WKY PTE cells L-DOPA uptake was almost exclusively through LAT2 whereas in immortalized SHR PTE cells 50% of L-DOPA uptake occurred through LAT1, 25% through LAT2, and 25% through sodium-dependent mechanisms, (Pinho et al. 2004). These sodium-dependent mechanisms may involve ASCT2 and B^{0}AT1, which are the only transport systems that are capable of transporting amino acids with similar characteristics to substrates transported through system L. The rat SLC1A5 gene that codes for ASCT2, is located on chromosome 1 and was associated to hypertension by several linkage analysis studies (Clemitson et al. 2002; Lo et al. 2002).

Quantitative trait loci (QTLs) were identified in chromosome 1 accounting for salt loading-induced variance of blood pressure (Yagil et al. 2003). Interestingly, previous studies showed that high salt intake-induced increases in urinary dopamine were not accompanied by increases in LAT1, LAT2 or 4F2hc expression in 4- and 12-week old WKY and SHR (Pinho et al. 2007). These results indicated that sodium-dependent transporters, such as ASCT2, may play an important role in the regulation of renal dopamine formation during high salt intake.

As shown in **Chapter I**, the renal activity and expression of ASCT2 transporter were lower in SHR than in WKY PTE cells. In view of the fact that ASCT2 is regulated by NO in intestinal Caco-2 cells (Uchiyama et al. 2005), and that NO availability in the kidney is decreased in SHR (Adler et al. 2002), it was suggested that oxidative stress might be downregulating ASCT2 by decreasing intrarenal NO availability. In order to investigate this hypothesis we attempted to explore the short and long term effects of L-NAME (inhibitor of NO production), SNAP (NO donor) and carboxy PTIO (NO scavenger) on the inward transport of [^{14}C]-L-alanine, an ASCT2 preferential substrate, in monolayers of immortalized renal PTE cells from WKY and SHR. No effects were observed with the tested concentrations and the treatment times (unpublished data) which may indicate that NO may not have a role in the regulation of ASCT2 in renal PTE cells.

Previous studies in our laboratory have shown that apocynin, a NADPH oxidase inhibitor, decreased significantly Na^+,K^+-ATPase activity and Na^+,K^+-ATPase α_1-subunit expression in OK cells (Silva et al. 2007). Furthermore, intracellular H_2O_2 can amplify the

response downstream of α_1-adrenoceptor activation (Simao et al. 2008), α_2-adrenoceptor activation (Simao et al. 2008) and Cl^-/HCO_3^- exchanger activity (Pedrosa et al. 2008; Simao et al. 2010) in immortalized SHR PTE cells. Therefore, we hypothesized that H_2O_2 or O_2 could modulate ASCT2 activity. Lineweaver–Burk plots from data obtained in the present study revealed the presence of high- and low-affinity states for the sodium-dependent [^{14}C]-L-alanine uptake processes in both cell lines. At low extracellular sodium concentrations, the sodium-dependent [^{14}C]-L-alanine uptake in both WKY and SHR PTE cells is a high-affinity low-capacity process and increases in extracellular sodium reduced the affinity for the substrate, but increased the capacity to take up [^{14}C]-L-alanine. In **Chapter I** we show that inhibition of H_2O_2 production by apocynin during cell growth significantly reduced Na^+-K_m and V_{max} values of the low-affinity high-capacity component of sodium-dependent [^{14}C]-L-alanine uptake in immortalized SHR PTE cells. Therefore, when H_2O_2 levels are reduced the sodium-dependent [^{14}C]-L-alanine uptake by ASCT2 in SHR PTE cells functions predominantly as a high-affinity low-capacity transporter. It was suggested that oxidative stress may have an effect on the conformations of ASCT2 in SHR PTE cells as they proceed through the transport cycle, which may result in differential sodium binding and unbinding. In fact, in immortalized SHR PTE cells, H_2O_2 has been shown to stimulate Cl^-/HCO_3^- exchanger activity via modification of thiol groups of intracellular and/or transmembrane proteins. In addition, the oxidized conformation of the exchanger enhanced the affinity for HCO_3^- in immortalized SHR PTE cells but not in WKY PTE cells (Simao et al. 2011). Cysteine residues of proteins are especially susceptible

to oxidative stress and, given the important role that disulfides play in protein structure and stability, alterations of reactive cysteine thiol groups may change protein function and activity. Therefore, we concluded that ASCT2 is expressed in immortalized WKY and SHR PTE cells and suggested that H_2O_2 may regulate the sodium binding process of this transporter in immortalized SHR PTE cells only. Further studies are required to determine the effect of apocynin on LAT1 and LAT2 in immortalized WKY and SHR PTE cells.

As indicated in the Introduction section, the dopaminergic system has the ability to stimulate antioxidant activity. D_1-like receptor agonists were found to suppress platelet-derived growth factor (PDGF)-BB–mediated increases in oxidative stress in vascular smooth muscle cells (VSMCs). Furthermore, in HEK-293 cells heterologously expressing human D_1 receptor (HEK-hD_1), fenoldopam, a D_1 receptor agonist was found to inhibit NADPH oxidase activity in a time- and concentration-dependent manner (Yu et al. 2011). On the other hand, several studies suggest that one of the mechanisms linking oxidative stress and hypertension is represented by activation of the RAAS (Fanelli et al. 2011). In **Chapter I**, we demonstrated that in SHR PTE cells, but not in WKY PTE cells, the aldosterone-induced increase in NHE1 activity was prevented by apocynin. Therefore, whereas H_2O_2 may have a "detrimental" role by acting with aldosterone to increase sodium reabsorption in SHR PTE cells, it concurrently contributes to the presence of a more "sophisticated" uptake process for the sodium-dependent [^{14}C]-L-alanine uptake in SHR PTE cells. Although speculative, it is suggested that the presence of two components for the sodium-dependent [^{14}C]-L-alanine uptake, with different affinities and capacities

that vary according to extracellular sodium concentrations, may be more favourable than one. Moreover, this potential adaptive mechanism in SHR PTE cells could be a means for the cell to take up L-DOPA more efficiently.

Several factors may contribute to increases in oxidative stress such as the aging process and increased NADPH oxidase activity intrinsic to essential hypertension. In this setting, the next question we addressed was whether the regulation of the amino acid transporters that are potentially involved in the uptake of L-DOPA (LAT1, LAT2 and ASCT2) is altered in the kidney of aged WKY and SHR. Our group has reported previously that the enhanced ability to take up L-DOPA in the pre-hypertensive SHR was suggested to take place as an attempt to overcome the deficient dopamine-mediated natriuresis generally observed in this genetic model of hypertension (Jose et al. 2002; Pinho et al. 2003). The results presented in **Chapter II** show that aging, from 3 to 12 months of age, was associated with increases in H_2O_2 levels in renal tissue in both WKY and SHR. Furthermore, the abundance of $p22^{phox}$ and Nox4 proteins were increased in the renal cortex of aged WKY and SHR, as well as the expression of the antioxidant enzymes SOD2, SOD3 and catalase. In contrast to our studies, 22/24-week old SHR was reported to have increased plasma (Zhan et al. 2004) and urinary (Suzuki et al. 2008) H_2O_2 levels when compared to age-matched WKY. In 8-month old SHR, renal mitochondrial H_2O_2 production was also found to be increased in comparison to age-matched WKY (de Cavanagh et al. 2006). It was suggested that the apparent conflicting results may be related to the marked increase in body fat mass of aged WKY. The link between oxidative stress and obesity was further

investigated in **Chapter IV**. Nevertheless, in this pro-oxidative environmental milieu, the renal dopaminergic system was found to be activated in aged WKY and SHR as demonstrated in **Chapter III**, although the dopaminergic response is more enhanced in 91-week old SHR than in aged-matched WKY. Moreover, aging was accompanied by the upregulation of renal cortical ASCT2 in WKY and of LAT2/4F2hc and ASCT2 in SHR.

Given the pro-oxidant nature of the RAAS and the fact that the dopaminergic system can counter-regulate the RAAS by decreasing the production of ROS, another goal of this thesis was to evaluate age-related changes in the renal aldosterone/MR system in WKY and SHR. Plasma aldosterone increased with age in WKY and SHR (**Chapter III and Chapter IV**). Therefore it was suggested that the upregulation of ASCT2 in aged WKY and SHR and LAT2/4F2hc and ASCT2 in aged SHR could be an attempt to increase L-DOPA uptake, and consequently dopamine synthesis, in order to normalize sodium levels. There is accumulating evidence that supports the view that the dopaminergic system can directly counteract the RAAS. The natriuretic renal dopaminergic system opposes the anti-natriuretic activity of the RAAS by downregulating the AT_1 receptor and upregulating the AT_2 receptor. Each of the individual dopamine receptors has been shown to oppose the activity of the AT_1 receptor. D_1, D_3, and D_5 receptors even interact physically with the AT_1 receptor (Gildea 2009). In **Chapter IV** we confirmed that aging in WKY and SHR is accompanied by exacerbated oxidative stress at the kidney level and increased aldosterone/MR system activation. Similarly to results obtained by other authors (Ruetten et al. 1999; Natalucci et al. 2003), this study showed that WKY become obese with age, as

a result of fat mass accumulation. In our study 15% food restriction resulted in significant weight loss at the age of 52 weeks, which was accompanied by a decrease in renal oxidative stress, plasma aldosterone levels and MR functionality. These results were found to be in agreement with studies by other authors that have demonstrated that adipose tissue can produce several components of the RAAS, mainly adipose tissue-derived angiotensinogen. Furthermore, adipocyte-derived aldosterone-releasing factors, although not yet identified, have been found to stimulate aldosterone secretion from adrenocortical cells (Ehrhart-Bornstein et al. 2003). Therefore, based on the results presented in **Chapter III** and **Chapter IV** it is proposed that the upregulation of amino acid transporters that may be involved in L-DOPA uptake, such as ASCT2 and LAT2/4F2hc, could be a counteractive mechanism to overcome increases in renal ROS levels and activation of the RAAS during the aging process. However, in aged WKY the exacerbated oxidative stress levels and aldosterone/MR activation may be related to increases in body fat mass and not with aging *per se*. Furthermore, our findings demonstrate that although 52-week old WKY had renal oxidative stress and plasma aldosterone levels similar to those observed in age-matched SHR, blood pressure values were significantly lower. Therefore, it was suggested that increases in renal oxidative stress and sodium reabsorption may not be sufficient to cause hypertension.

The relationship between aldosterone and the expression of amino acid transporters has been previously evaluated by our group in young WKY and SHR (Pinho et al. 2007). At 12 weeks of age, high salt intake for 24 hours increased urinary dopamine in

SHR, but not in WKY. Changes in urinary dopamine paralleled changes in the uptake of L-DOPA in isolated renal tubules from 4- and 12-week old WKY and SHR on normal salt and high salt intake. At 12 weeks of age, high salt intake was accompanied by decreases in LAT1 and LAT2 transcript abundance in WKY and SHR. ASCT2 and B^0AT1 expression was significantly decreased in both 4- and 12-week old WKY and in 4-week old SHR on high salt intake. By contrast, high salt intake increased ASCT2 and B^0AT1 expression in 12-week old SHR (Pinho et al. 2007). We have demonstrated that 8-week old normotensive Wistar rats chronically treated during 8 days with aldosterone had increased renal cortical LAT2 mRNA levels with no changes in LAT1, 4F2hc and ASCT2 transcript levels (Pinho et al. 2009). The effect of aldosterone upon LAT2 mRNA levels was completely prevented by spironolactone, a mineralocorticoid receptor antagonist. At the protein level, aldosterone treatment did not significantly affect LAT1 and LAT2 expression, but markedly reduced the abundance of 4F2hc, although levels were not reversed by spironolactone. The decrease in LAT2 functionality (related to the decrease of 4F2hc abundance) correlated well with the reduction in urinary dopamine (Pinho et al. 2009). Taken together, these results suggested that the transcript abundance of amino acid transporters is age dependent and that can be modulated by aldosterone levels.

There is accumulating data supporting a role for oxidative stress in experimental hypertension; however, it is suggested that increases in ROS at the renal level may not be sufficient to cause hypertension. Moreover, the fact that oxidative stress may have a role in the modulation of the amino acid transporters that are potentially involved in the renal

tubular uptake of L-DOPA could be a defence mechanism activated in response to increases in aldosterone and oxidative stress levels. All together, the work presented in this thesis suggests that amino acid transporters can be regulated directly or indirectly by the oxidative damage that is associated with hypertension and aging.

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RESUMO

O papel do stress oxidativo na regulação dos transportadores de aminoácidos renais na hipertensão e no envelhecimento

O sistema dopaminérgico renal opõe-se à actividade anti-natriurética do sistema renina-angiotensina-aldosterona (SRAA) e inibe a formação de espécies reactivas de oxigénio (ERO). Neste estudo, colocou-se a hipótese de que o stress oxidativo possa desempenhar um papel na regulação dos transportadores de aminoácidos envolvidos na captação de L-DOPA na hipertensão e no envelhecimento. Verificou-se que a expressão e a actividade do ASCT2 são menores nas células epiteliais dos túbulos proximais renais (TPR) imortalizadas provenientes de ratos espontaneamente hipertensos (SHR) do que nas dos TPR de ratos Wistar-Kyoto (WKY). Nas duas linhas celulares, foram identificados componentes, um de alta e outro de baixa afinidade do processo de captação de [^{14}C]-L-alanina (um substrato preferencial do ASCT2), dependente de sódio. O tratamento com apocinina, um inibidor da NADPH oxidase, inibiu o componente de baixa afinidade do processo de captação de [^{14}C]-L-alanina dependente de sódio. Este efeito foi reversível e ocorreu exclusivamente nas células dos TPR dos SHR. Estudos *in vivo* com ratos WKY e SHR indicaram que o envelhecimento estava associado a um aumento dos níveis de H_2O_2 no rim de WKY e SHR. As quantidades de $p22^{phox}$ e de Nox4 estavam aumentadas no cortex renal de WKY e SHR envelhecidos, bem como a expressão das enzimas antioxidantes SOD2, SOD3 e catalase. O processo de envelhecimento também foi acompanhado pelo aumento da expressão de ASCT2 no cortex renal dos WKY e de LAT2/4F2hc e ASCT2 nos SHR. Subsequentemente, foi demonstrado que o sistema aldosterona/receptor mineralocorticoide estava activado de forma semelhante no WKY e SHR envelhecidos mas que a pressão arterial dos WKY continuava a ser inferior às dos SHR. Nos WKY envelhecidos, a restrição alimentar prolongada resultou em perdas significativas de peso, diminuição do stress oxidativo renal

e redução no nível de aldosterona plasmática. No WKY e SHR a modulação dos transportadores de L-DOPA pode ser uma reacção de compensação de activação do SRAA e do aumento nos níveis renais de ERO durante o processo de envelhecimento. Os resultados apresentados podem contribuir para uma melhor compreensão da regulação dos transportadores de aminoácidos renais e do papel das ERO na hipertensão.

ABSTRACT

Oxidative stress on the regulation of renal amino acid transporters in hypertension and aging

The renal dopaminergic system opposes the anti-natriuretic activity of the renin-angiotensin-aldosterone system (RAAS) and inhibits reactive oxygen species (ROS) generation. In this study we hypothesized that oxidative stress could play a role in the regulation of the candidate amino acid transporters for L-DOPA uptake, in hypertension and aging. The activity and expression of ASCT2 transporter were found to be lower in immortalized renal proximal tubular epithelial (PTE) cells from spontaneous hypertensive rat (SHR) than in Wistar-Kyoto (WKY) PTE cells. In both cell lines, high- and low-affinity components were identified for the sodium-dependent [^{14}C]-L-alanine (an ASCT2 preferential substrate) uptake process. In SHR PTE cells, treatment with apocynin, a NADPH oxidase inhibitor, inhibited the low affinity component for the sodium-dependent [^{14}C]-L-alanine uptake. This effect was found to be reversible and exclusive to SHR PTE cells. In vivo studies showed that aging was associated with increases in renal H_2O_2 levels in WKY and SHR. The abundance of $p22^{phox}$ and Nox4 proteins were increased in the renal cortex of aged WKY and SHR, as well as the expression of the antioxidant enzymes SOD2, SOD3 and catalase. Aging was also accompanied by the upregulation of renal cortical ASCT2 in WKY and of LAT2/4F2hc and ASCT2 in SHR. Subsequently, it was shown that the renal aldosterone/mineralocorticoid receptor system was activated in aged WKY and SHR. However, although renal oxidative stress and plasma aldosterone levels were similar to that observed in age-matched SHR, blood pressure values were significantly lower in aged WKY. Moreover, in aged WKY long-term food restriction resulted in significant weight loss which paralleled decreases in renal oxidative stress and reductions in plasma aldosterone levels. In WKY and SHR the modulation of amino acid transporters may be an attempt to overcome activation of the RAAS and increases in renal ROS levels during the aging

process. The data presented in this thesis may contribute to a better understanding of the regulation of renal amino acid transporters and the role of ROS in hypertension.