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Stratification of Sphingosine Kinase-1 Expression and Activity in Rat Kidney

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Key Words
Sphingosine kinase · Kidney · Sphingosine-1-phosphate

Abstract
Sphingosine-1-phosphate, the product of sphingosine kinase (SK) activity, is a sphingolipid metabolite that regulates cell growth, survival and migration. It is also known to affect diuresis, natriuresis and renovascular contraction in rats, although the mechanisms through which it affects these processes are not known. No previous report has addressed the differences among the kidney zones regarding endogenous SK expression and activity. Therefore, we examined SK1 distribution and activity in the various kidney zones: cortex, medulla and papilla. We found that SK1 expression does not correlate with enzyme activity. Study of the expression showed that the enzyme is highly expressed in cortex, followed by medulla and papilla. However, medulla had the highest enzyme activity. In all kidney zones, SK1 expression was mainly cytosolic. Regarding enzyme activity, whereas we found no difference between cytosol, membrane and nucleus in renal medulla, the membrane-bound enzyme presented the highest activity in cortex and papilla. SK1 distribution observed by immunohistochemical staining showed higher expression in cortical proximal convoluted epithelial cells. In medulla, immunostaining was observed as patches of staining, whereas in papilla, positive immunostaining was exclusively restricted to collecting duct cells. We also evaluated the effects of bradykinin and angiotensin II on SK1 activity.

Introduction

Sphingolipids (SLs) were originally thought to serve only as structural components of mammalian cell membranes. However, in recent years, increasing evidence has demonstrated important roles for SLs and their metabolites as critical signaling molecules [Spiegel et al., 2003; Taha et al., 2006]. Central to the SL metabolic pathway

Abbreviations used in this paper

Ang II	angiotensin II
BK	bradykinin
Cer	ceramide
ECL	enhanced chemiluminescence
PBS	phosphate-buffered saline
S1P	sphingosine-1-phosphate
SK	sphingosine kinase
SLs	sphingolipids
Sph	sphingosine

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are the metabolites ceramide (Cer), sphingosine (Sph) and sphingosine-1-phosphate (S1P). While Cer and Sph have been shown to arrest cell growth and promote apoptosis, S1P has been shown to stimulate cell growth and prevent apoptosis [Pyne et al., 2000; Taha et al., 2004]. Recently, S1P has also emerged as a regulator of physiological and pathophysiological processes. Indeed, S1P is considered to be a novel spasmodic substance, since it contracts canine basilar [Tosaka et al., 2001] and coronary [Sugiyama et al., 2000] arteries, as well as rat mesenteric and intrarenal microvessels in vitro [Bischoff et al., 2001]. Moreover, in vivo experiments demonstrated that intravenous injection of S1P reduced renal and mesenteric blood flow without affecting mean arterial pressure or heart rate [Bischoff et al., 2000]. Recently, S1P has also appeared as a novel regulator of renal tubular function, since infusion of S1P induces natriuresis and calciuresis and increases diuresis, with minor if any change in K^+ excretion [Bischoff et al., 2000]. In addition to its extracellular action, intracellularly formed S1P is involved in calcium mobilization, cell proliferation and inhibition of apoptosis [Pyne et al., 2000; Spiegel et al., 2003]. Thus, S1P is a dual modulator of cellular function, acting extracellularly as a first messenger or intracellularly as a second messenger molecule.

S1P is formed by the enzyme Sph kinase (SK) which catalyzes Sph phosphorylation. SK is a key enzyme in the SL metabolic pathway, as it is an essential checkpoint that regulates the relative levels of S1P, Sph and Cer [Kohama et al., 1998]. SK has been detected in most mammalian tissues with different levels of expression, the kidney being an organ where SK is highly expressed [Melendez et al., 2000]. Two isoforms of SK (SK1 and SK2) have been cloned, but some studies reported the existence of at least 3 SKs with different properties [Banno et al., 1998]. Although classically considered a cytosolic enzyme, recent reports have described activities associated with the plasma membrane and the endoplasmic reticulum as well as the cytosol of rat kidney homogenates [Gijsbers et al., 2001].

The kidney is a highly stratified organ, where the various kidney zones play different functional roles. Since S1P can be either an autocrine or paracrine molecule, it seems that the kidney zone and the cellular type where SK is expressed could be relevant to further understand its renal physiological implications.

No previous report has addressed the difference among the kidney zones regarding SK expression and activity. In this study, we analyzed SK1 expression, activity and subcellular localization in the cortex, medulla and papilla of rat kidney. The results demonstrate that SK is highly expressed in the cortex, and to a lesser extent in the medulla and the papilla. However, enzyme expression does not correlate with its basal level of activity, since the latter is higher in the medullary zone compared to the cortex. Both bradykinin (BK) and angiotensin II (Ang II) differentially regulate the enzyme activity in the kidney zones.

Methods

Reagents

[γ-^{32}P]ATP (3,000 Ci/mmol) was purchased from Amersham (Little Chalfont, UK). SK1 antibody was generously donated by Dr. Obeid and was raised against the C-terminal residues of human SK1. TLC silica gel plates were obtained from Merck (Darmstadt, Germany). X-ray film was obtained from Eastman Kodak Co. (Rochester, N.Y., USA). All other reagents and chemicals were of analytical grade (Sigma, Merck or Mallinckrodt) and purchased from local commercial suppliers.

Isolation of the Kidney Zones

Male Wistar rats (body weight 250–300 g) were sacrificed by cervical dislocation, and both kidneys were removed and kept in ice-cold Krebs-Ringer buffer, pH 7.4, containing 5.5 mM glucose. The medium was gassed with 95% O_2 and 5% CO_2. Each kidney was cut in half along its longitudinal axis, and the papilla (inner medulla), medulla (outer medulla, including its outer and inner strip) and cortex were isolated by scissors and scalpel dissection. The renal papilla, medulla and cortex were sliced (approx. 0.5 mm thick) using a Stadie-Riggs microtome. The papillary, medullary and cortical tissue was treated with BK (1 μM), Ang II (10 μM) or vehicle for 15 min, as appropriate, and then homogenized.

Preparation of Tissue Homogenates

The papillary, medullary and cortical tissue was homogenized with ice-cold lysis buffer (1% v/v Triton X-100, 120 mM NaCl, 50 mM NaF, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 5 mM β-glycerophosphate and 25 mM HEPES, pH 7.6 at 4°C, freshly supplemented with 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 10 μM leupeptin, 1 mM Na_3VO_4 and 1 mM dithiothreitol). The homogenate was subsequently centrifuged for 5 min at 20,000 *g* and 4°C to remove insoluble material. The supernatant was then both assayed for protein quantification and stored at –80°C for later analysis.

Subcellular Fractionation

Papillary tissue was homogenized in glass tubes with a Teflon pestle at 3,000–3,500 rpm with 10 vol of ice-cold SK buffer [Gijsbers et al., 2001; Murate et al., 2001]. The homogenate was filtered and centrifuged at 860 *g* for 15 min. Postnuclear supernatant was kept. Pure nuclear fraction was obtained by washing the pellet twice, resuspended in 3.8 vol of buffer containing 25 mM Tris-HCl, 2.4 M sucrose, 1 mM $MgCl_2$ and 2 mM EGTA, pH 7.4, and centrifuged at 50,000 *g* for 60 min at 4°C. Then, the sediment was washed twice to obtain the pure nuclear fraction. Postnuclear S1 and the washes were pooled and centrifuged at 9,000 *g* for 10 min

at 4°C to sediment mitochondrial fraction. Postmitochondrial supernatant plus subsequent washes were pooled and centrifuged at 105,000 *g* for 60 min at 4°C. The pellet obtained was the microsomal fraction. Protein concentration was determined by the method of Lowry et al. [1951]. The purity of nuclei was assessed by microscopy and by the measurement of glucose-6-phosphatase and 5′-nucleosidase activities to evaluate endoplasmic reticulum and plasma membrane contamination, respectively. These assays showed that the pellet obtained after 2.4 M sucrose centrifugations was highly enriched in nuclei.

SK Assay

SK activity was measured by the method described by Olivera et al. [2000]. Briefly, 5% (v/v) Triton X-100 was used to prepare a stock solution of 2.5 mM Sph. Four hundred microliters of conditioned medium was supplemented to achieve a final concentration of 20 μM Sph, 500 μM [^{32}P]ATP (10 μCi), 5 mM $MgCl_2$, 15 mM NaF, 0.5 mM 4-deoxypyridoxine, 40 mM β-glycerophosphate and 50 μg protein lysate, and incubated at 37°C for 30 min. Lipids were extracted, and samples were resuspended in 50 μl of chloroform. [^{32}P]S1P was resolved by TLC on Silica Gel G60 using 1-butanol/acetic acid/water (60/20/20 v/v) and quantified with a PhosphorImager. Bands corresponding to S1P were also scraped from the plates and counted in a scintillation counter.

Immunoblotting for SK Expression

Total homogenate (30 μg) was subjected to 12% SDS-PAGE. Proteins were transferred to PDVF membranes, blocked with phosphate-buffered saline (PBS)/0.1% Tween-20 containing 5% nonfat dried milk, washed with PBS/0.1% Tween-20, and incubated 1 h with primary antibody against SK in PBS/0.1% Tween-20 containing 5% nonfat dried milk. Blots were washed in PBS/0.1% Tween-20 and incubated 45 min with the secondary antibody in PBS/0.1% Tween-20 containing 5% nonfat dried milk. Detection was performed using enhanced chemiluminescence (ECL; Amersham Biotech). The amount of SK was measured by densitometric analysis of the ECL signals. To ensure that ECL signals were within the linear range, multiple exposures were taken during the short initial phase of ECL reaction. Only those signals that were in the linear range were used for quantification.

Immunohistochemistry for SK Expression

For immunohistochemistry, kidneys were removed and placed in ethanol at 4°C in order to be processed by Sainte-Marie's technique [Sainte-Marie, 1962]. Tissue was fixed in 95% ethanol precooled at 4°C, dehydrated in 4 changes of precooled absolute ethanol, then cleared by passing through 3 consecutive baths of xylene and embedded in paraffin at 56°C. Next, tissue sections (4–5 μm thick) were placed on glass slides. Before incubation with the primary antibody, all tissue sections were incubated with 3% goat normal serum (Vector Laboratories, Burlingame, Calif., USA) in PBS for 1 h at room temperature to reduce nonspecific binding. Incubation with primary antibodies was performed overnight at 4°C in goat serum-containing buffer, followed by 3 washes in PBS. Immunohistochemical staining was performed with an avidin/biotin/peroxidase system (ABC Elite; Vector Laboratories) and 3,3′-diaminobenzidine (Sigma). Specific immunoreaction was detected using the Vectastain ABC Elite kit (Vector Laboratories) and 3′,3′-diaminobenzidine tetrahydrochloride horseradish peroxidase substrate (Zymed Laboratories, South San Francisco, Calif., USA) according to the manufacturer's protocols. Sections were counterstained with hematoxylin before dehydration, mounting in Permount (Fisher Scientific, Pittsburgh, Pa., USA) and photomicroscopy (Olympus BX60; Olympus, Tokyo, Japan).

Statistics

The results are presented as means ± SE of 3 independent experiments, each performed in triplicate. Analysis of variance was used to compare mean basal values versus those after treatments. $p < 0.05$ was considered statistically significant.

Results

Differential SK1 Expression and Activity in Rat Kidney Zones

The 2 isoforms of SK, SK1 and SK2, differ in their distribution and function [Liu et al., 2000; Fukuda et al., 2003], with SK1 being a pro-proliferative and antiapoptotic enzyme and SK2 a proapoptotic one. Both SK isoforms were isolated, purified and characterized first from kidney [Olivera et al., 1998; Liu et al., 2000].

The polyclonal antibody used in our experiments only detects SK1 and it identified a 42-kDa protein by Western blotting. As seen in figure 1a, SK1 is highly expressed in the renal cortex, followed by the medulla and to a lesser extent the papilla. The densitometric analysis revealed that the cortical SK1 is 20 times the amount in medulla and 100 times the amount in papilla (fig. 1b). We further studied the enzyme activity in the different kidney zones. As shown in figure 1c, the medullary zone had the highest activity (6,070 cpm/mg protein/h), followed by the cortex (2,800 cpm/mg protein/h) and the papilla (1,130 cpm/mg protein/h). In order to obtain some correlation between enzyme expression and activity, we normalized the activity with total protein content. As seen in the inset of figure 1c, not many differences were observed for renal medulla and papilla. However, in the cortex, the value obtained was around 40 times lower than those of medulla and papilla.

Subcellular Study of SK1 in Rat Kidney Zones

Renal SK1 was first purified and characterized from total rat kidney homogenates. This activity was further demonstrated in cytosol and in vesicles derived from endoplasmic reticulum and plasma membrane obtained from total homogenates of rat kidney [Gijsbers et al., 2001]. Since it has been reported that SK has different cellular functions depending on its subcellular location [Gijsbers et al., 2001], it was of interest to study the en-

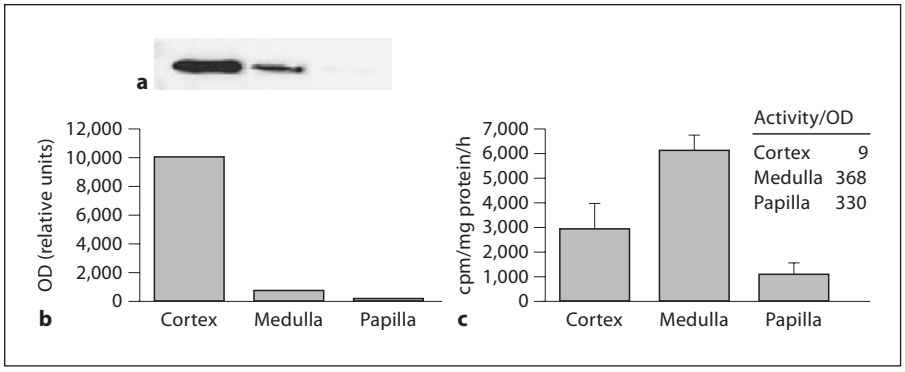

Fig. 1. Differential SK1 expression and activity in the papilla, medulla and cortex of rat kidney. The papillary, medullary and cortical tissues were homogenized, and 30 μg of lysate was subjected to 12% SDS-PAGE analysis, transferred to PDVF and immunoblotted for SK1 protein. **a** Representative Western blot of SK1 (42 kDa) expression in cortical, medullary and papillary homogenates. **b** Densitometric analysis of Western blot from **a**. **c** SK activity was measured as radioactive S1P produced per milligram of protein per hour. The inset shows the ratio between enzyme activity and densitometric values of protein expression. Bars represent the means ± SEM of 3 separate experiments performed in triplicate. OD = Optical density.

zyme compartmental distribution and activity. For this purpose, we isolated cytosolic, nuclear and membrane fractions from homogenates obtained from the 3 zones and determined SK expression and activity. Most SK activity corresponded to SK1, since the assay conditions used were optimal for SK1, while SK2 activity was blocked [Olivera et al., 2000; Maceyka et al., 2005]. As seen in figure 2a, d, g, SK1 expression was higher in cytosol, accounting for 49, 51 and 62% of SK1 protein in the cortex, medulla and papilla, respectively. Microsomal enzyme represented 33, 41 and 34%, respectively, of total SK1 mass, while the nuclear expression was only 18, 8 and 4%, respectively. Regarding enzyme subcellular activity (fig. 2b, e and h), in the renal cortex and papilla the cytosolic contribution was low (20 and 17%, respectively) and most of the activity was membrane-associated (50 and 57%, respectively), while nuclear fraction brought about one third of total activity in both kidney zones. By contrast, in the renal medulla, the enzyme activity was almost equally distributed in the 3 fractions studied.

In terms of specific activity, all cortical compartments showed very low values as seen in figure 2c, being lowest in cytosol, while membrane-bound and nuclear activities were similar. In the medulla, nuclear specific activity was 4 times higher than that of the microsomes and cytosol. In the papilla, great differences were observed among the various fractions. Nuclear specific activity was the highest, being 20 and 10 times higher than those of cytosolic and membrane fractions, respectively.

BK and Ang II Modulate SK1 Activity

In order to evaluate if intrarenal hormones that play important roles in the regulation of renal physiology affect SK1 activity, tissue slices were incubated with BK, Ang II and vehicle, and SK activity was measured as described. As seen in figure 3a, cortical SK activity was downregulated by BK and Ang II by 60 and 40%, respectively, compared to control-treated tissue. By contrast, medullary and papillary activities were stimulated by both hormones (fig. 3b, c). In the medulla, there was an increase in activity of 110% for BK and 98% for Ang II; in the papilla the stimulatory effect was even higher (160% for BK and 190% for Ang II).

Immunohistochemical Staining of Rat Renal Tissue

The distribution of SK1 was examined in ethanol-fixed and paraffin-embedded tissue sections. In order to compare SK1 expression in the various cell types, the intensity of immunostaining was graded, with grade 3 corresponding to the highest intensity. As seen in figure 4, the cortical region showed strong staining (fig. 4a). Grade 3 staining was observed in proximal convoluted epithelial cells (fig. 4b, black arrow). Distal convoluted tubular cells (red arrow) and cortical collecting duct cells (arrow head) were also positive, graded 2+ and 1+, respectively (fig. 4b).

Patches of staining were observed at low magnification in both outer and inner strips of the outer medulla (fig. 4d). At higher magnification (fig. 4e) all tubular structures appeared positively immunostained. As seen

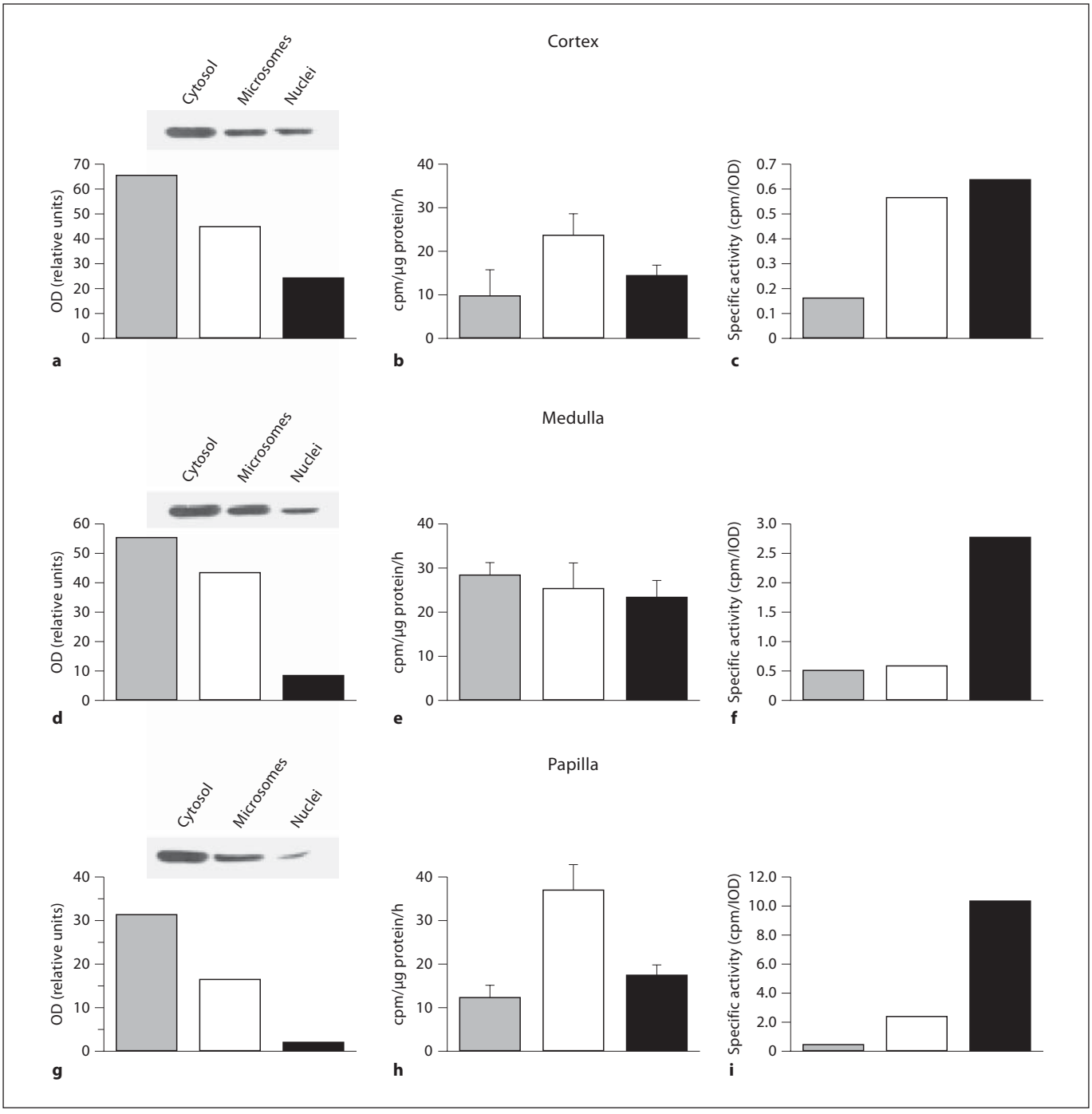

Fig. 2. Subcellular study of SK1 in rat kidney zones. The papillary, medullary and cortical tissues were homogenized and subcellular compartments were isolated as described in Methods. SK1 expression, activity and specific activity were determined in the cortex (**a**, **b**, **c**), medulla (**d**, **e**, **f**) and papilla (**g**, **h**, **i**). **a**, **d**, **g** Densitometric analysis of SK-1 expression. **b**, **e**, **h** SK-1 activity. **c**, **f**, **i** Specific activity. Bars represent the means ± SEM of 3 separate experiments performed in triplicate. OD = Optical density; IOD = immunoblot optical density.

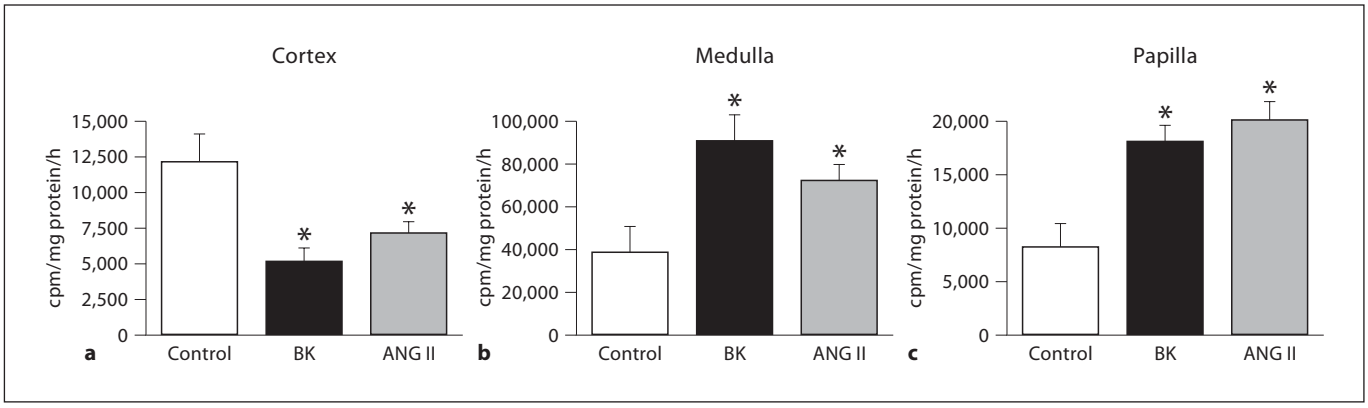

Fig. 3. Hormonal modulation of SK1 activity in the rat renal zones. After being treated with either BK 1 μM or Ang II 0.01 μM for 15 min, total homogenates were obtained from the cortex (**a**), medulla (**b**) and papilla (**c**) and SK1 activity was measured as described in Methods. Bars represent the means ± SEM of 3 separate experiments performed in triplicate. * $p < 0.05$.

in figure 4e, brown immunostaining was seen in collecting ducts as well as thick and thin loops of Henle. The immunostaining appeared homogenously distributed in cytoplasm with no differences between apical and basolateral domains.

In the papilla, positive immunostaining was exclusively observed in the tubular structures (graded 3+), while interstitial tissue presented no brown staining (fig. 4g, h).

The observation of individual proximal tubular cells at higher magnification (fig. 4c) showed that cytoplasmic staining was not homogeneous but followed a punctuated pattern mostly located in the basolateral side of the cell. Also at higher amplification, the image of the individual distal convoluted tubular cells showed very low positive staining (fig. 4f). In cortical blood vessels, immunostaining was observed in endothelial cells (fig. 4i).

Discussion

SK1 is considered the major SK in all mammalian tissues, but its contribution varies among tissues [Murate et al., 2001]. The kidney is one of the organs where SK1 messenger RNA is highly expressed [Melendez et al., 2000] and where the enzymatic activity is also high when compared with other tissues [Gijsbers et al., 2001; Fukuda et al., 2003]. Since these studies have been performed in total kidney homogenates, the main contribution to the overall expression and activity mostly corresponds to the renal cortex. No previous report has addressed the differences among the kidney zones regarding SK expression and activity. In the present report, we demonstrate for the first time that SK1 expression and activity are highly stratified in the kidney, showing a decreasing gradient of expression from the cortex to the papilla. Additionally and consistently with previous observations reported by Gijsbers et al. [2001], we found that, although the most abundant, cytosolic enzyme accounts only for less than 30% of total SK1 activity in the 3 kidney zones. Moreover, SK besides serving as mediator of S1P synthesis also clears Cer and Sph, which are known regulators of cellular fate and function. Translocation of SK from the cytosol to membranes or nuclear fraction has been considered a step for enzyme activation by exogenous stimuli [Johnson et al., 2002]. Consistent with this notion, nuclear and microsomal-associated SK1 appeared more active than the cytosolic enzyme (fig. 2c, f, i). We have previously reported [Facchinetti et al., 2003] that SK1 immunolabeling of adult rat renal papilla shows membrane perinuclear staining, whereas neonate rat renal papilla shows more plasma membrane-associated SK1. In neonate rat, most papillary cells, which are highly undifferentiated, are found in different phases of the cell cycle, and a great deal of cells have an embryonic phenotype with migrating cell characteristics [Marquez et al., 2002]. The difference observed between adult and neonate rat renal papilla regarding SK1 localization points to the importance that translocation of the enzyme to different compartments has on the behavior of kidney cells. Further experiments will address the effect that SK1 nuclear localization has on papillary, cortical and medullary kidney cells.

Cortical SK1 appears less active than medullary and papillary enzymes as judged by their specific activity

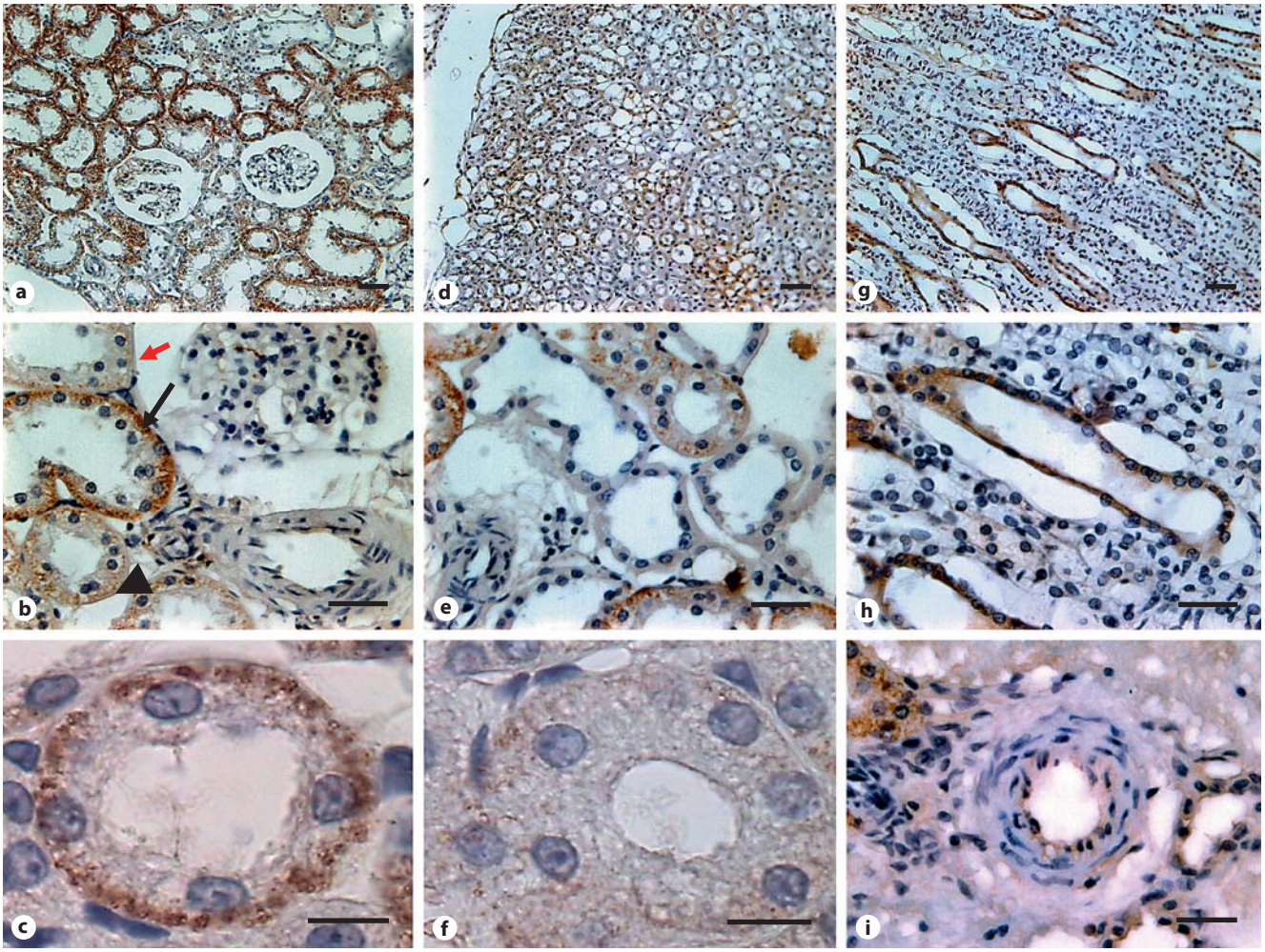

Fig. 4. Immunohistochemistry showing the SK1 expression pattern in the different zones of the kidney. Light micrographs showing the pattern of SK1 expression in rat renal cortex (**a–c**), medulla (**d–f**) and papilla (**g–i**). In the cortex, stronger immunostaining was observed in proximal tubules (**b**, black arrow), whereas a lower expression was found in distal tubules (**b**, red arrow) and glomerulus. In the medulla, all tubular structures appeared positively immunostained, whereas in the papilla positive immunostaining was exclusively observed in the tubular structures (graded 3+), while interstitial tissue was devoid of brown staining (**h**). In cortical blood vessels, positivity was observed in endothelial cells and some positivity was also seen in muscular cells (**i**). **a**, **d**, **g**, **i** Scale bars = 200 μm. **b**, **e**, **h** Scale bars = 100 μm. **c**, **f** Scale bars = 25 μm.

(fig. 1c). The existence of 2 isoforms of SK1, termed SK1a and SK1b, has been reported. They differ in activity and distribution, SK1b being 30–200 times less active than SK1a [Kohama et al., 1998]. Therefore, it could be possible that cortical SK1 corresponded preferably to the SK1b isoform. Moreover, while the medullary and papillary enzymes are upregulated by Ang II and BK, such hormones exert a negative regulation on cortical SK1 (fig. 3), which could also reflect the presence of different isoforms. The divergence in the hormone regulation exerted on SK could be of physiological relevance. Recently, S1P has been identified as a novel class of endogenous regulator of renal function [Bischoff et al., 2001] and as a potent vasoconstrictor agent, exerting its effect predominantly at the level of the blood efferent arteriole [Bischoff et al., 2000]. On the other hand and in contrast to most other known vasoconstriction agents such as endothelin-1, Ang II and norepinephrine, S1P enhances urine and electrolyte excretion despite the reduction in renal blood flow [Bischoff et al., 2001]. Thus, the fact that Ang II decreases SK1 activity in the cortex could suggest a counterbalance in the Ang II-induced vasoconstrictor effect.

On the other hand, the stimulatory effect of BK and Ang II on medullary and papillary SK1 activity can synergize or even mediate the BK-induced diuretic and natriuretic effects, while counterbalancing the antidiuretic effect of Ang II.

Besides the potential implication of the renal SK1/S1P pathway as modulator of renal function, SK1 could also play an important role as renoprotector. Of interest, it has been demonstrated that a specific agonist of S1P receptors ameliorated ischemic acute renal failure and has been proposed as a renoprotective agent for preventing and treating ischemic renal failure.

Endogenous SK1 activity has a housekeeping function as part of the sphingomyelin degradative pathway [Buehrer et al., 1993; Igarashi et al., 1997] assisting in clearing Sph and Cer, 2 molecules with proapoptotic functions [Igarashi et al., 1997; Hannun et al., 2000]. The enzymatic product of SK1, S1P, is known to be proliferative [Goodemote et al., 1995], a suppressor of apoptosis [Cuvillier et al., 1996; Goetzl et al., 1998], a modulator of cell motility [Sadahira et al., 1992; Bornfeldt et al., 1995] and an angiogenic vascular maturation agent [Ancellin et al., 2002], all functions involved in tissue preservation and regeneration.

It has been reported that acute ischemia followed by reperfusion induces an acute tubular injury and alters cortical Cer and Sph accumulation [Zager et al., 1997]. Since SK1 is responsible for transforming the proapoptotic molecules Cer and Sph in the antiapoptotic S1P, it seems reasonable to think that in the cellular types where SK1 is active, the enzyme clears both Sph and Cer, producing S1P and avoiding apoptosis. Thus, it seems likely that as the cellular SK1 is more active, the possibility that the cell becomes affected under conditions of stress is lower. We suggest that proximal convoluted tubular cells, where SK1 is not so active and distal convoluted cells, which are almost devoid of SK1, are more sensitive to injury, as is seen in ischemia and reperfusion.

The SK1/S1P pathway resembles cyclooxygenase/prostaglandins pathways in many aspects, such as the compartmentalized expression and activity of the enzymes, and also in the fact that the enzymatic products are lipid mediators that act in a paracrine and autocrine fashion. Moreover, both pathways exert regulation of kidney function and modulation of renal blood flow. Thus, we are tempted to speculate that SK1/S1P could be considered as a novel intrarenal system for protection against injury and also as a modulator of kidney function. In this respect, knowledge about the specific location and activity of SK1/S1P pathway can open a new field for the understanding of renal function and preservation. S1P receptor expression in the different kidney zones will also give information about the importance of this pathway in kidney physiology, and future experiments will focus on studying S1P receptor immunolocalization in the kidney.

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