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RESEARCH PAPER

Oxidative stress and α_1-adrenoceptor-mediated stimulation of the Cl^-/HCO_3^- exchanger in immortalized SHR proximal tubular epithelial cells

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Background and purpose: This study evaluated the signalling coupled to the α_1-adrenoceptor-induced stimulation of the Cl^-/HCO_3^- exchanger in hypertension.
Experimental approach: The Na^+-independent HCO_3^- transport system activity was assayed as the initial rate of pH_i recovery after an alkaline load (CO_2/HCO_3 removal) in immortalized renal proximal tubular epithelial cells from spontaneously hypertensive rat (SHR) and their normotensive control (Wistar Kyoto rat; WKY).
Key results: Noradrenaline increased Cl^-/HCO_3^- exchanger activity with EC_{50} values of 0.6 and 5.3 μM in SHR and WKY cells, respectively. These effects were abolished by prazosin, but not by yohimbine. Phenylephrine increased Cl^-/HCO_3^- exchanger activity in SHR and WKY cells (EC_{50} of 2.6 and 4.9 μM, respectively). Phenylephrine-mediated increase in Cl^-/HCO_3^- exchanger activity in WKY and SHR cells was inhibited by protein kinase C (PKC), MAPK/ERK kinase (MEK) and p38 mitogen-activated protein kinase (p38 MAPK) inhibitors. The expression of α_{1A}- and α_{1B}-adrenoceptors was identical in WKY and SHR cells. SHR cells generated more H_2O_2 than WKY cells. In SHR cells, the NADPH oxidase inhibitor apocynin reduced their increased ability to generate H_2O_2 and abolished their hypersensitivity to phenylephrine, but failed to affect basal Cl^-/HCO_3^- exchanger activity. H_2O_2-dependent stimulation of Cl^-/HCO_3^- exchange activity was significantly higher in SHR than in WKY cells.
Conclusions and implications: Differences between WKY and SHR cells on their sensitivity to α_1-adrenoceptor stimulation did not correlate with the abundance of α_{1A}- and α_{1B}-adrenoceptors and may be related to the increased generation of H_2O_2, which may amplify the response downstream of α_1-adrenoceptor activation.
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Keywords: Cl^-/HCO_3^- exchanger; α_1-adrenoceptor; H_2O_2; hypertension; SHR; WKY

Abbreviations: ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; NHE, Na^+/H^+ exchanger; PDBu, phorbol-12,13-dibutyrate; PTE, proximal tubular epithelial; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat

Introduction

A considerable part of filtered HCO_3^- is reabsorbed in the renal proximal tubules by the anion exchangers (Krapf and Alpern, 1993; Soleimani and Singh, 1995). The Na^+/HCO_3^- cotransporter, the Na^+-dependent Cl^-/HCO_3^- exchanger and the Na^+-independent Cl^-/HCO_3^- exchanger have been described in the kidney (Alpern, 1990; Hara *et al*., 2000; Soleimani and Burnham, 2000; Petrovic *et al*., 2003; Mount and Romero, 2004). These anion exchangers facilitate the reversible electroneutral exchange of Cl^- for HCO_3^- across the plasma membrane and regulate intracellular pH (pH_i), intracellular chloride concentration, bicarbonate metabolism and cell volume. After an intracellular acid load, the cell responds with stimulation of the Na^+/H^+ exchanger (NHE) (Gomes *et al*., 2001; Pedrosa *et al*., 2004a, b, c; Gomes and Soares-da-Silva, 2006), the Na^+/HCO_3^- cotransporter and the Na^+-dependent Cl^-/HCO_3^- exchanger to mediate the recovery of pH_i (Lazdunski *et al*., 1985; Dart and Vaughan-Jones, 1992; Gomes *et al*., 2001; Gomes and Soares-da-Silva, 2006). In contrast, the Na^+-independent Cl^-/HCO_3^- exchanger usually mediates the recovery from an intracellular alkalinization (Xu and Spitzer, 1994). Our group recently demonstrated the presence of an apical Cl^-/HCO_3^- exchanger in immortalized renal proximal tubular epithelial (PTE) cells from the spontaneously hypertensive rat (SHR) and Wistar Kyoto rat (WKY), which may correspond to the SLC26A6 protein (Pedrosa *et al*., 2004d).

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One proposal for the initiation and maintenance of hypertension centres on a reduced capacity of the kidney to excrete salt and water in proper relation to intake (Guyton *et al*., 1972). Renal sympathetic nerves and circulating catecholamines are involved in the regulation of Na^+ and water excretion in the kidney (Wilborn *et al*., 1998). The catecholamine noradrenaline is the major endogenous neurotransmitter in renal sympathetic nerves and mediates sympathetic regulation of blood pressure. Noradrenaline interacts with both the α- and β-adrenoceptors in the renal proximal tubules (DiBona, 1985; Wilborn *et al*., 1998; Kanagy, 2005). Studies have shown that renal nerves, acting through α-adrenoceptors, enhance proximal tubular sodium reabsorption in the kidney. These studies suggest that noradrenaline, acting via α- and/or β-adrenoceptors, may contribute to the development of hypertension (Baines and Ho, 1987; Gesek and Schoolwerth, 1990).

The present study evaluated the activity of the Cl^-/HCO_3^- exchanger in immortalized renal PTE cells from SHR and WKY, and its sensitivity to noradrenaline. We found that SHR PTE cells express an enhanced sensitivity to α_1-adrenoceptor-mediated stimulation of Cl^-/HCO_3^- exchanger activity. This enhanced sensitivity of the Cl^-/HCO_3^- exchanger to the α_1-adrenoceptor stimulation appeared not to be linked to differences in the signal-transduction pathway coupled to α_1-adrenoceptors, the activation of which involves PKC and p38 mitogen-activated protein kinase (MAPK) in both WKY and SHR cells; rather, this appeared to result from differences in hydrogen peroxide (H_2O_2) production.

Methods

Cell culture

Immortalized renal PTE cells from 4- to 8-week-old WKYs and SHRs (Woost *et al*., 1996) 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^{-1} penicillin G, 0.25 μg ml^{-1} amphotericin B, 100 μg ml^{-1} streptomycin (Sigma Chemical Company), 4 μg ml^{-1} dexamethasone (Sigma Chemical Company), 5 μg ml^{-1} transferrin (Sigma Chemical Company), 5 μg ml^{-1} insulin (Sigma Chemical Company), 5 ng ml^{-1} selenium (Sigma Chemical Company), 10 ng ml^{-1} epidermal growth factor (Sigma Chemical Company), 5% fetal bovine serum (Sigma Chemical Company) and 25 mM HEPES (Sigma Chemical Company).

For subculturing, the cells were dissociated with 0.10% trypsin–EDTA, split 1:8 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). For the measurement of α_1-adrenoceptor expression, the cells were seeded in six-well plastic culture clusters (Costar). The cell medium was changed every 2 days, and the cells reached confluence after 3–5 days of incubation. The cells were maintained in fetal bovine serum-free medium for 24 h before each experiment. 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.

pH_i measurements

In pH_i measurement experiments, WKY and SHR PTE cells were grown in 96-well plates. pH_i was measured as previously described (Pedrosa *et al*., 2004d). At days 4–5 after seeding SHR and WKY PTE cells cultured in 96-well plates, pH_i measurements were performed after loading the cells with 10 μM acetoxymethyl ester of 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein at 37 °C for 30 min. Cells were placed in the sample compartment of a dual-scanning microplate spectrofluorometer (Spectramax Gemini XS; Molecular Devices, Sunnyvale, CA, 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 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein 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 (Thomas *et al*., 1979).

Cl^-/HCO_3^- exchanger activity

The Na^+-independent HCO_3^- transport system activity was assayed as the initial rate of pH_i recovery after an alkaline load (CO_2/HCO_3 removal), in the absence of Na^+, as previously described (Pedrosa *et al*., 2004d; Fraga *et al*., 2006). In the Krebs HCO_3^--free medium used, sodium was replaced by an equimolar concentration of choline. The Krebs HCO_3^--free medium also contained pargyline (100 μM) and tolcapone (1 μM) to inhibit the enzymes monoamine oxidase and catechol-*O*-methyltransferase, respectively. The test compounds were added to the extracellular fluid 40 min before the start of the pH_i recovery period after the alkaline load.

Immunoblotting

WKY and SHR PTE cells cultured to 90% of confluence were washed twice with phosphate-buffered saline and total cell protein was extracted for α_{1A}- and α_{1B}-adrenoceptor detection. Briefly, to obtain total cell extract, 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, 0.25% sodium deoxycholate, 1 mM phenylmethylsulphonyl fluoride, 1% Nonidet P-40 (Igepal), 1 mM Na_3VO_4, 1 mM NaF, aprotinin and leupeptin 1 μg ml^{-1} each) and incubated on ice for 30 min. After centrifugation (16 000 *g*, 30 min, 4 °C), the supernatant was collected and protein concentration determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with BSA as standard. Sixty micrograms of protein was mixed in 2 × sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 2% 50 mM dithiothreitol, 0.1% w/v bromophenol blue) and boiled for 5 min. Proteins were subjected to SDS-polyacrylamide gel electrophoresis (10% SDS-polyacrylamide gel) and electrotransferred onto nitrocellulose membranes. The transblot sheets were blocked for 1 h with 5% of non-fat dry milk in 25 mM Tris-HCl pH 7.5

and 150 mM NaCl. The membranes were subsequently incubated overnight at 4 °C with appropriately diluted antibodies (goat polyclonal anti-α_{1A}-adrenoceptor and anti-α_{1B}-adrenoceptor, 1:400 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-β-actin primary antibody, 1:60 000, Santa Cruz Biotechnology). The membranes were subsequently washed and incubated with fluorescent-labelled donkey anti-goat (1:10 000; IRDye 800, Rockland, Gilbertsville, PA, USA) or a fluorescent-labelled goat anti-mouse secondary antibody (1:10 000; IRDye 680, LI-COR Biosciences, Lincoln, NE, USA), respectively, 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).

Measurement of H_2O_2

Hydrogen peroxide 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. Measurement of H_2O_2 was evaluated either directly by H_2O_2 released from the WKY and SHR monolayer cultured in 96-well plates or by H_2O_2 accumulated in the extracellular medium during 24 h after the 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

Arithmetic means are given with s.e.mean or geometric means with 95% confidence values. Statistical significance was determined using one-way ANOVA followed by Newman–Keuls test for multiple comparisons. A value of $P<0.05$ was assumed to denote a significant difference.

Drugs

Apocynin, chelerythrine chloride, L-(−)-noradrenaline (+)-bitartrate salt monohydrate, pargyline hydrochloride, (*R*)-(−)-phenylephrine hydrochloride, phorbol-12,13-dibutyrate (PDBu), prazosin hydrochloride, yohimbine hydrochloride, U 0126 and H_2O_2 were purchased from Sigma Chemical Company. PD 098059, SB 203580 and anisomycin were obtained from Research Biochemicals International (Natick, MA, USA). Acetoxymethyl ester of 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein nigericin and the Amplex Red Hydrogen Peroxide Assay kit were obtained from Molecular Probes Inc.. Tolcapone was kindly donated by the late Professor Mosé DaPrada (Hoffmann La Roche, Basel, Switzerland).

Results

The addition of noradrenaline before (40 min) and during (10 min) the HCO_3^--dependent recovery of pH_i increased the Cl^-/HCO_3^- exchanger activity in a concentration-dependent manner in WKY and SHR PTE cells (Figure 1). However, as shown in Figure 1a, the sensitivity of noradrenaline-

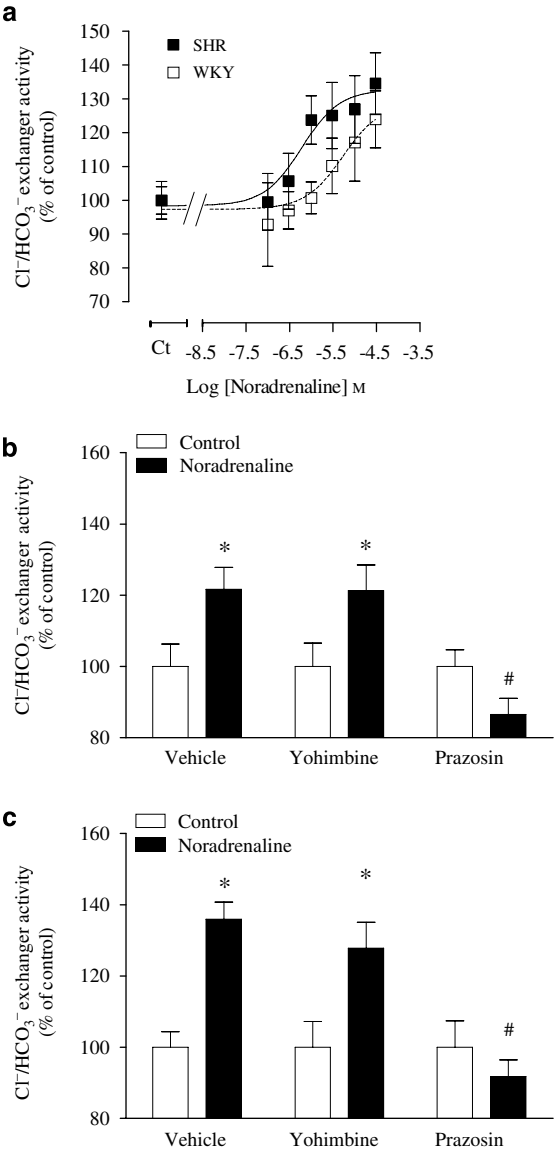

Figure 1 (**a**) Concentration-dependent effect of noradrenaline (40 min exposure) on Cl^-/HCO_3^- exchanger activity in WKY and SHR PTE cells. The absolute control values for Cl^-/HCO_3^- exchanger activity in pH units min^{-1} were 0.1532 ± 0.01 (WKY cells) and 0.2413 ± 0.01 (SHR cells). (**b** and **c**) Effect of noradrenaline (30 μM for 40 min exposure) on Cl^-/HCO_3^- exchanger activity in the absence and presence of yohimbine (100 nM) and prazosin (3 μM) in (**b**) WKY and (**c**) SHR PTE cells. The absolute control values for Cl^-/HCO_3^- exchanger activity, in pH units min^{-1}, in vehicle were 0.1492 ± 0.01 (WKY cells) and 0.2388 ± 0.01 (SHR cells). Symbols or columns represent the mean of 6–20 experiments per group; vertical lines indicate s.e.mean. Significantly different from corresponding control values (*$P<0.05$) and values for noradrenaline alone ($^{\#}P<0.05$). PTE, proximal tubular epithelial; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat.

dependent stimulation of Cl^-/HCO_3^- exchange activity was significantly higher in SHR than that in WKY PTE cells. This was also evidenced by the 10-fold difference in EC_{50} values for the noradrenaline-induced stimulation of Cl^-/HCO_3^- activity between SHR (geometric means [95% confidence limits]: 0.6 [0.5, 0.8] μM) and WKY (5.3 [4.3, 6.4] μM) PTE cells. The effect of noradrenaline (30 μM) in WKY and SHR PTE cells on the activity of Cl^-/HCO_3^- exchanger was completely blocked by the α_1-adrenoceptor antagonist prazosin (3 μM) but not by the α_2-adrenoceptor antagonist yohimbine (0.1 μM; Figures 1b and c).

As shown in Figure 2a, the sensitivity of SHR PTE cells to phenylephrine, a selective α_1-adrenoceptor agonist, was also markedly different from that observed for WKY PTE cells. In fact, the phenylephrine-dependent stimulation of Cl^-/HCO_3^- exchange activity was significantly higher in SHR PTE cells than that in WKY PTE cells. This was also evidenced by the significant twofold difference in EC_{50} values for the phenylephrine-induced stimulation of Cl^-/HCO_3^- activity between SHR (2.6 [1.9, 3.4] μM) and WKY (4.9 [2.7, 8.8] μM) PTE cells. As already shown for noradrenaline, the effect of phenylephrine (30 μM) in WKY and SHR PTE cells on the activity of Cl^-/HCO_3^- exchanger was completely blocked by the α_1-adrenoceptor antagonist prazosin (3 μM) but not by the α_2-adrenoceptor antagonist yohimbine (0.1 μM; Figures 2b and c). The expression of α_{1A}- and α_{1B}-adrenoceptors was evaluated in immortalized WKY and SHR PTE cells by immunoblot analysis. As shown in Figure 3, the level of expression of α_{1A}- and α_{1B}-adrenoceptors in WKY PTE cells was similar to that in SHR PTE cells.

There is evidence suggesting that second messenger pathways involved in noradrenaline-induced stimulation of sodium and bicarbonate transport across proximal tubules involve stimulation of PKC (Gesek *et al*., 1989; Gesek and Strandhoy, 1990; Chan *et al*., 2000; Liu and Gesek, 2001; Hutchinson and Bengtsson, 2005). To evaluate whether this was the case in the phenylephrine-induced stimulation of the Cl^-/HCO_3^- exchanger in WKY and SHR PTE cells, the effect of PDBu, an activator of classical and novel PKCs, was examined. Treatment of WKY and SHR PTE cells with PDBu (0.1 μM, 40 min) increased Cl^-/HCO_3^- exchanger activity in both WKY and SHR PTE cells (Figure 4). Chelerythrine (1 μM) antagonized the effect of PDBu (100 nM) and that of phenylephrine in both WKY and SHR PTE cells (Figures 4a and b).

To evaluate the contribution of MAPK, which is normally related to signal transduction coupled to α_1-adrenoceptors and also linked to noradrenaline-induced stimulation of sodium reabsorption in the kidney and vascular tissues (Liu and Gesek, 2001; Hutchinson and Bengtsson, 2005; Scarparo *et al*., 2006), we used specific inhibitors for MAPK/extracellular signal-regulated kinase kinase (MEK) and p38 MAPK. Pretreatment of cells for 15 min with the MEK 1 inhibitor PD 098059 abolished the phenylephrine-induced stimulation of Cl^-/HCO_3^- exchanger activity in WKY PTE cells, but not in SHR PTE cells (Figure 5). As SHR PTE cells only express ERK 2, but not ERK 1 (Parenti *et al*., 2000), the effect of U 0126, a MEK 1/2 inhibitor, was also tested. A different effect from PD 098059 was obtained with U 0126 and the p38 MAPK inhibitor, SB 203580, which completely abolished the phenylephrine-induced stimulation of Cl^-/HCO_3^- exchanger activity in both WKY and SHR PTE cells (Figures 5 and 6). Anisomycin, an activator of p38 MAPK, induced an increase in Cl^-/HCO_3^- exchanger activity similar to that observed with phenylephrine in both WKY and SHR PTE cells. This effect was completely abolished by SB 203580 (Figure 6).

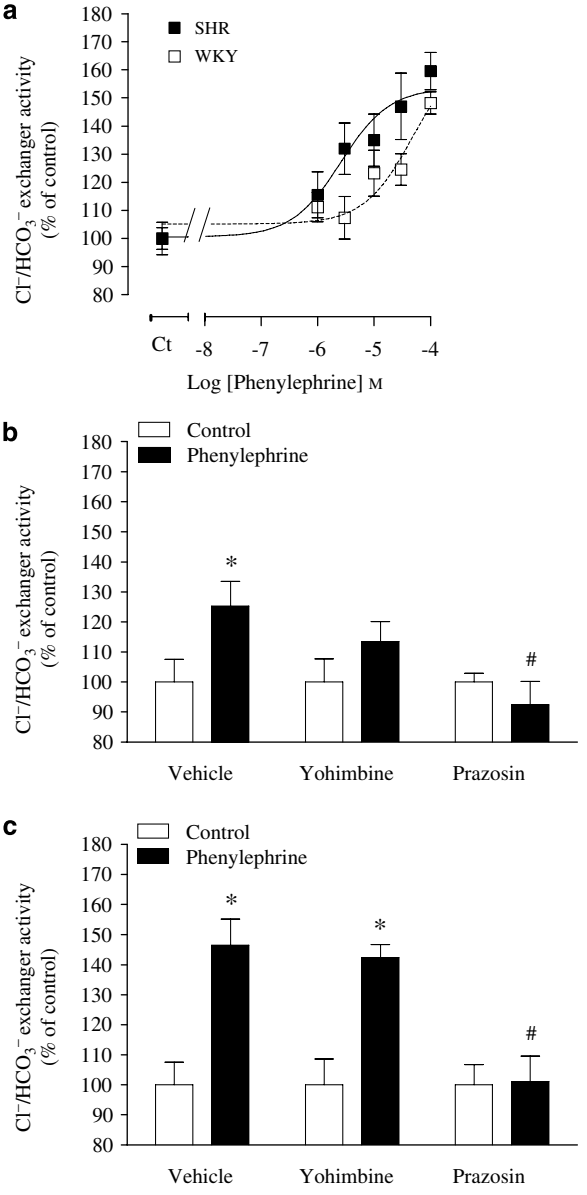

Figure 2 (**a**) Concentration-dependent effect of phenylephrine (40 min exposure) on Cl^-/HCO_3^- exchanger activity in WKY and SHR PTE cells. The absolute control values for Cl^-/HCO_3^- exchanger activity in pH units min^{-1} were 0.2238 ± 0.02 (WKY cells) and 0.3419 ± 0.01 (SHR cells). (**b** and **c**) Effect of phenylephrine (30 μM for 40 min exposure) on Cl^-/HCO_3^- exchanger activity in the absence and presence of yohimbine (100 nM) and prazosin (3 μM) in (**b**) WKY and (**c**) SHR PTE cells. The absolute control values for Cl^-/HCO_3^- exchanger activity, in pH units min^{-1}, in vehicle were 0.2054 ± 0.01 (WKY cells) and 0.2988 ± 0.01 (SHR cells). Symbols or columns represent the mean of 6–8 experiments per group; vertical lines indicate s.e.mean. Significantly different from corresponding control values (*$P<0.05$) and values for phenylephrine alone ($^{\#}P<0.05$). PTE, proximal tubular epithelial; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat.

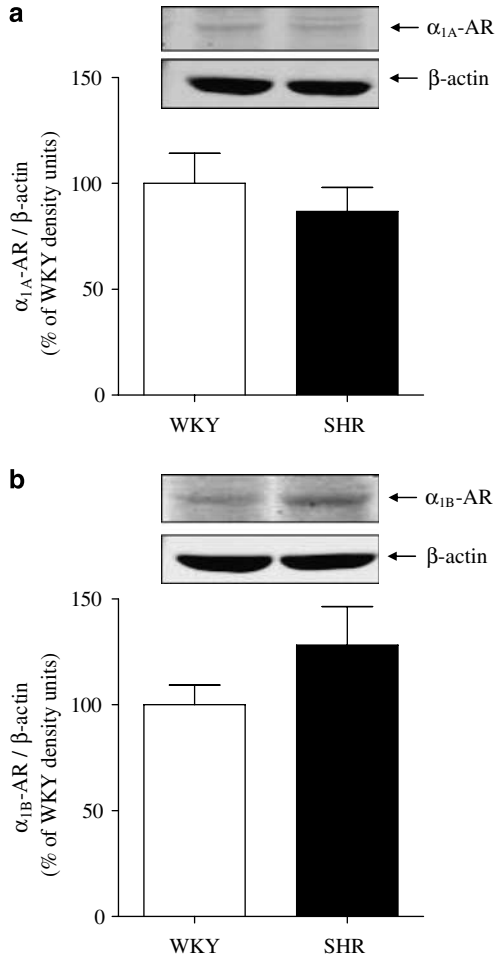

Figure 3 Expression of (**a**) α_{1A}-adrenoceptors and (**b**) α_{1B}-adrenoceptors in WKY and SHR PTE cells. Representative immunoblots are shown above the bar graphs. Columns represent mean of four independent immunoblots; vertical lines show s.e.mean. α_{1A}-adrenoceptors ~52 kDa; α_{1B}-adrenoceptors ~60 kDa; β-actin ~40 kDa. PTE, proximal tubular epithelial; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat.

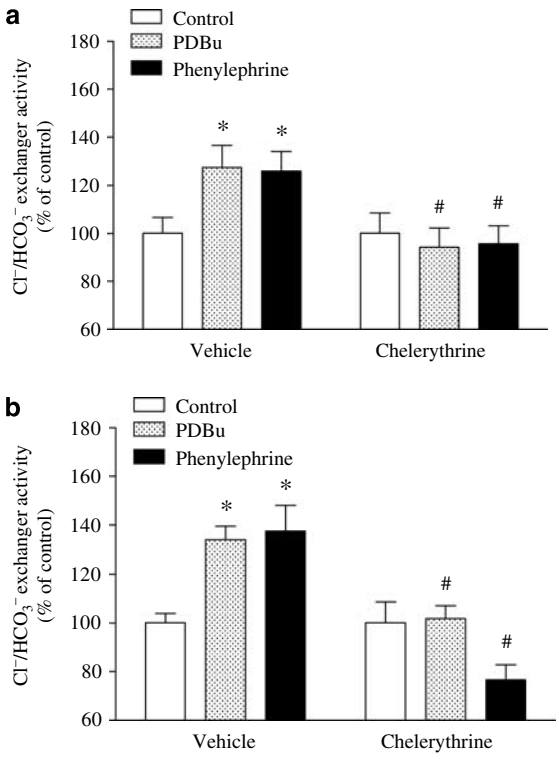

Figure 4 Effect of PDBu (0.1 μM) and phenylephrine (30 μM) for 40 min on Cl^-/HCO_3^- exchanger activity in (**a**) WKY and (**b**) SHR PTE cells in the absence and presence of chelerythrine (1 μM). The absolute control values for Cl^-/HCO_3^- exchanger activity, in pH units min^{-1}, in vehicle were 0.1957 ± 0.01 (WKY cells) and 0.2612 ± 0.01 (SHR cells). Each column represents the mean of 7–15 experiments per group; vertical lines indicate s.e.mean. Significantly different from corresponding control values ($^*P<0.05$) and values for PDBu or phenylephrine alone ($^\#P<0.05$). PDBu, phorbol-12,13-dibutyrate; PTE, proximal tubular epithelial; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat.

These results suggest that stimulation of α_1-adrenoceptors may lead to simultaneous activation of PKC, ERK 1/2 kinases and p38 MAPK transduction pathways in WKY PTE cells and PKC, ERK 2 and p38 MAPK transduction pathways in SHR PTE cells, with a common point in the cascade of events, as either chelerythrine or SB 203580 completely prevented the effects of phenylephrine. To confirm the involvement of both PKC and p38 MAPK in the stimulation of Cl^-/HCO_3^- exchanger activity evoked by α_1-adrenoceptor stimulation and to clarify the sequence of events in more detail, we performed complementary studies involving stimulation of PKC and p38 MAPK. To promote PKC activation, cells were incubated in the presence of PDBu (0.1 μM) and its effects on Cl^-/HCO_3^- exchanger activity were evaluated in the absence and presence of PKC, MEK and p38 MAPK inhibitors. Under these experimental conditions, the effects of PDBu in both WKY and SHR PTE cells were abolished by the PKC inhibitor chelerythrine and the p38 MAPK inhibitor SB 203580, but not by the MEK inhibitor PD 098059 (Figure 7). To promote p38 MAPK activation, cells were incubated in the presence of anisomycin (0.1 μM) and its effects on Cl^-/HCO_3^- exchanger activity were evaluated in the absence and presence of PKC, MEK and p38 MAPK inhibitors. Under these experimental conditions, the effect of anisomycin in both WKY and SHR PTE cells was abolished by the p38 MAPK inhibitor SB 203580, but not by the PKC inhibitor chelerythrine and the MEK 1 inhibitor PD 098059 (Figure 8). The Cl^-/HCO_3^- exchanger in SHR PTE cells responded to PKC and p38 MAPK stimulation similarly to that observed in WKY PTE cells (Figures 7 and 8).

Because recent studies have demonstrated a role for renal H_2O_2 in the development of hypertension and renal dysfunction (Schnackenberg *et al.*, 1998; Vaziri *et al.*, 2000; Minuz *et al.*, 2002; Makino *et al.*, 2003), particularly in the SHR model (Adler and Huang, 2004; de Cavanagh *et al.*, 2006; Sullivan *et al.*, 2006), it seemed reasonable to measure H_2O_2 generation and evaluate the involvement of H_2O_2 in the regulation of Cl^-/HCO_3^- exchanger activity by phenylephrine in WKY and SHR PTE cells. The SHR PTE cells had an increased rate of H_2O_2 production (50.7 ± 0.4 versus 11.3 ± 0.1 nmol min^{-1}) when compared with WKY PTE cells (Figure 9a). Treatment of cells with apocynin (100 μM), an inhibitor of the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase complex, for 4 days after

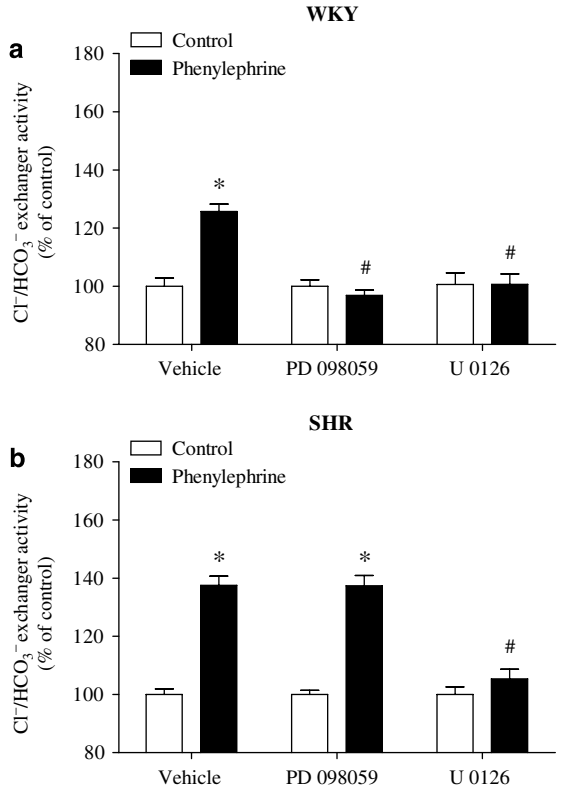

Figure 5 Effect of phenylephrine (30 μM) for 40 min on Cl^-/HCO_3^- exchanger activity in (**a**) WKY and (**b**) SHR PTE cells in the absence and presence of PD 098059 (10 μM) and U 0126 (10 μM). The absolute control values for Cl^-/HCO_3^- exchanger activity, in pH units min^{-1}, in vehicle were 0.2157 ± 0.02 (WKY cells) and 0.2912 ± 0.02 (SHR cells). Each column represents the mean of 7–15 experiments per group; vertical lines indicate s.e.mean. Significantly different from corresponding control values (*$P<0.05$) and values for phenylephrine alone ($^{\#}P<0.05$). PTE, proximal tubular epithelial; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat.

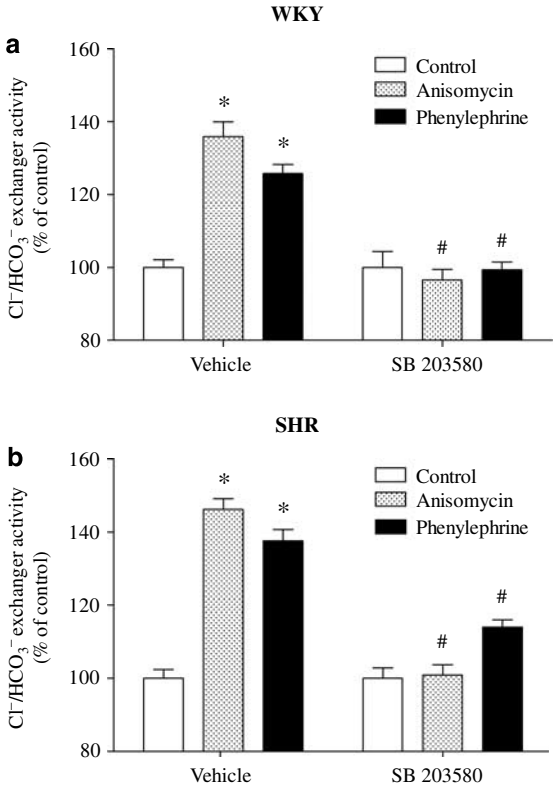

Figure 6 Effect of anisomycin (0.1 μM) and phenylephrine (30 μM) for 40 min on Cl^-/HCO_3^- exchanger activity in the presence or absence of SB 203580 (10 μM) in (**a**) WKY and (**b**) SHR PTE cells. The absolute control values for Cl^-/HCO_3^- exchanger activity, in pH units min^{-1}, in vehicle were 0.2067 ± 0.02 (WKY cells) and 0.3012 ± 0.02 (SHR cells). Each column represents the mean of 4–13 experiments per group; vertical lines indicate s.e.mean. Significantly different from corresponding control values (*$P<0.05$) and values for anisomycin or phenylephrine alone ($^{\#}P<0.05$). PTE, proximal tubular epithelial; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat.

seeding reduced the extracellular levels of H_2O_2 in SHR PTE cells, but not in WKY PTE cells (Figure 9b). The treatment with apocynin (100 μM) for 4 days after seeding did not change the basal Cl^-/HCO_3^- exchanger activity in both WKY (0.084 ± 0.004 versus 0.085 ± 0.006 pH units min^{-1}) and SHR (0.239 ± 0.008 versus 0.240 ± 0.020 pH units min^{-1}) PTE cells. However, treatment of SHR PTE cells with apocynin (100 μM) almost completely blocked the ability of phenylephrine to stimulate Cl^-/HCO_3^- exchanger activity with no effects in WKY PTE cells (Figure 10).

The effect of exogenous H_2O_2 on the Cl^-/HCO_3^- exchanger activity in WKY and SHR PTE cells was also evaluated (Figure 11). As can be observed, H_2O_2 stimulated the Cl^-/HCO_3^- exchanger activity in both WKY and SHR PTE cells, SHR PTE cells being much more sensitive to H_2O_2 (Figure 11).

Discussion

The present study was designed to evaluate the effects of noradrenaline and the signal-transduction pathway coupled to noradrenaline-induced stimulation of the Cl^-/HCO_3^- exchanger in immortalized renal PTE cells from WKY and SHR. The data reported here show that SHR PTE cells were 4 and 10 times more sensitive to phenylephrine and noradrenaline, respectively, than WKY PTE cells in stimulating Cl^-/HCO_3^- exchanger activity. Despite differences in sensitivity to phenylephrine and noradrenaline between SHR and WKY PTE cells, the results obtained indicate that noradrenaline- and phenylephrine-induced stimulation of Cl^-/HCO_3^- exchanger activity was mediated through the activation of prazosin-sensitive α_1-adrenoceptors coupled to PKC, ERK 2 and p38 MAPK pathways in both WKY and SHR PTE cells. However, the Cl^-/HCO_3^- exchanger in SHR PTE cells responded to PKC and p38 MAPK stimulation similarly to that observed in WKY PTE cells. The enhanced sensitivity to phenylephrine-induced stimulation of the Cl^-/HCO_3^- exchanger activity through the α_1-adrenoceptors in SHR PTE cells was associated with the higher H_2O_2 generation.

The results presented here show that noradrenaline stimulated Cl^-/HCO_3^- exchanger activity, in a concentration-dependent manner, in both WKY and SHR PTE cells. SHR PTE cells showed an enhanced sensitivity to noradrenaline-induced stimulation of the Cl^-/HCO_3^- exchanger activity compared with that in WKY PTE cells. This type of

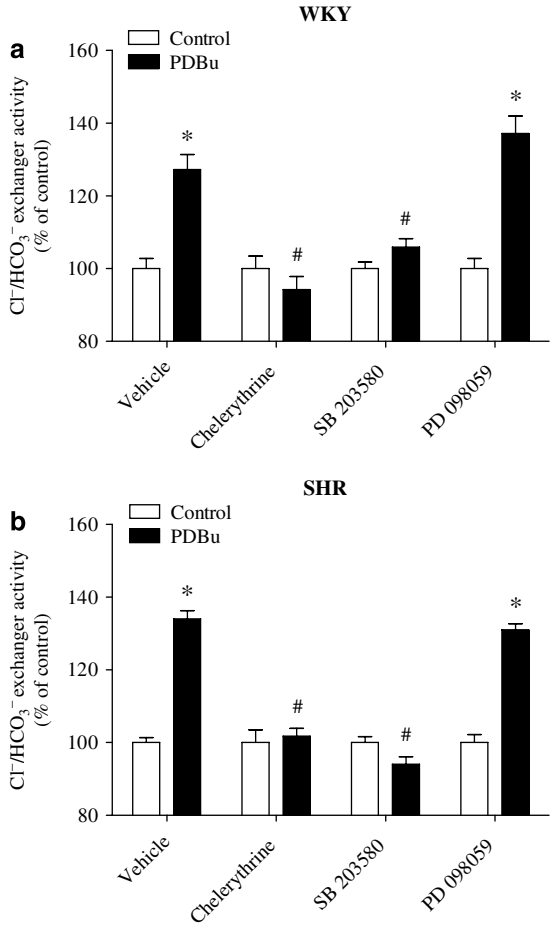

Figure 7 Effect of PDBu (0.1 μM) for 40 min on Cl^-/HCO_3^- exchanger activity in (**a**) WKY and (**b**) SHR PTE cells in the absence and presence of chelerythrine (1 μM), SB 203580 (10 μM) or PD 098059 (10 μM). The absolute control values for Cl^-/HCO_3^- exchanger activity, in pH units min^{-1}, in vehicle were 0.1625 ± 0.01 (WKY cells) and 0.2720 ± 0.01 (SHR cells). Each column represents the mean of 7–15 experiments per group; vertical lines indicate s.e.mean. Significantly different from corresponding control values (*$P<0.05$) and values for PDBu alone ($^{\#}P<0.05$). PDBu, phorbol-12,13-dibutyrate; PTE, proximal tubular epithelial; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat.

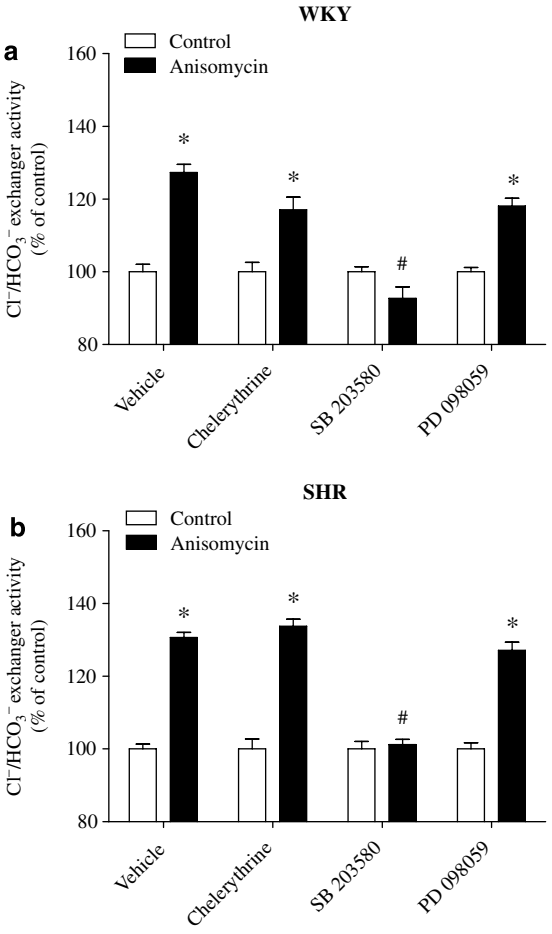

Figure 8 Effect of anisomycin (0.1 μM) for 40 min on Cl^-/HCO_3^- exchanger activity in (**a**) WKY and (**b**) SHR PTE cells in the absence and presence of chelerythrine (1 μM), SB 203580 (10 μM) or PD 098059 (10 μM). The absolute control values for Cl^-/HCO_3^- exchanger activity, in pH units min^{-1}, in vehicle were 0.1846 ± 0.01 (WKY cells) and 0.2624 ± 0.01 (SHR cells). Each column represents the mean of 7–15 experiments per group; vertical lines indicate s.e.mean. Significantly different from corresponding control values (*$P<0.05$) and values for anisomycin alone ($^{\#}P<0.05$). PTE, proximal tubular epithelial; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat.

response is consistent with previous experiments with another anti-natriuretic substance, angiotensin II, upon the Cl^-/HCO_3^- exchanger (Pedrosa and Soares-da-Silva, 2006). The effect of noradrenaline on Cl^-/HCO_3^- exchanger activity in WKY and SHR PTE cells was abolished by prazosin (α_1-adrenoceptor antagonist), but not by yohimbine (α_2-adrenoceptor antagonist). In addition, phenylephrine (α_1-adrenoceptor agonist) stimulated the exchanger activity in a concentration-dependent manner in WKY and SHR PTE cells. SHR PTE cells have an enhanced sensitivity to phenylephrine comparatively to WKY PTE cells. The effect of phenylephrine upon Cl^-/HCO_3^- exchanger activity was prevented by prazosin but not by yohimbine in WKY and SHR PTE cells. Taken together, these results indicate that noradrenaline-induced stimulation of the Cl^-/HCO_3^- exchanger activity occurs through the α_1-adrenoceptor. Major mechanisms intervening in renal proximal tubular NaCl absorption, pH_i and cell volume regulation have been suggested to occur through the concerted action of the Cl^-/HCO_3^- and NHEs. This fits well with previous studies that demonstrated that activation of the α_1-adrenoceptor also increases NHE activity in the proximal tubule (Gesek *et al*., 1989; Gesek and Schoolwerth, 1990; Liu *et al*., 1997). The observation that α_{1A}- and α_{1B}-adrenoceptor expression was identical in WKY and SHR PTE cells excludes the possibility that the enhanced sensitivity to phenylephrine and noradrenaline in stimulating Cl^-/HCO_3^- exchanger activity in SHR PTE cells might be related to differences in the density of α_1-adrenoceptors. This result is in agreement with previous reports that detected no significant changes in the expression of α_1-adrenoceptors in renal tissues from SHR and WKY (Yamada *et al*., 1986; Jeffries *et al*., 1988).

The α_1-adrenoceptors initiate their physiological effects by activating PL (PLC) in the cell membrane, resulting in

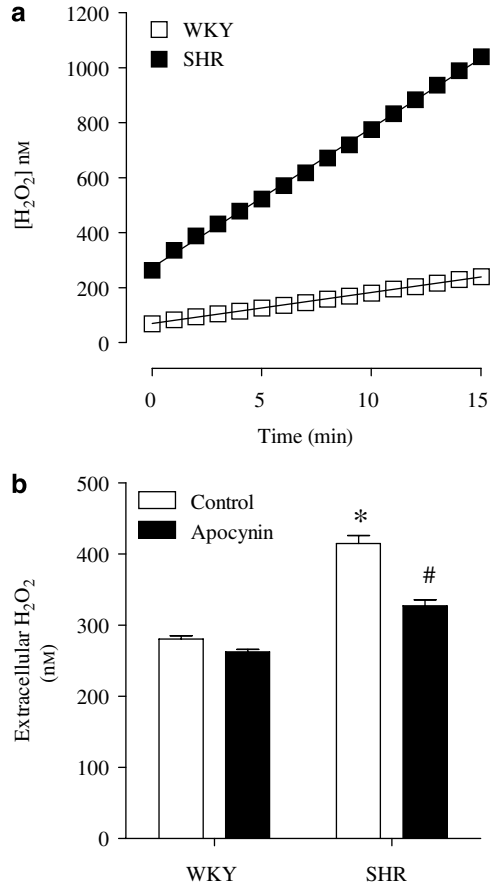

Figure 9 (**a**) Rate of H_2O_2 (nmol min^{-1}) released from WKY and SHR PTE cells in confluent monolayers (4 days after seeding). (**b**) Levels of extracellular H_2O_2 (nM) in WKY and SHR PTE cells in control cell culture conditions and in the presence of apocynin (100 μM; 4 days after seeding). Each column represents the mean of 6–16 experiments per group and vertical lines show s.e.mean. Significantly different from values for WKY (*$P<0.05$) or for control ($^{\#}P<0.05$). PTE, proximal tubular epithelial; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat.

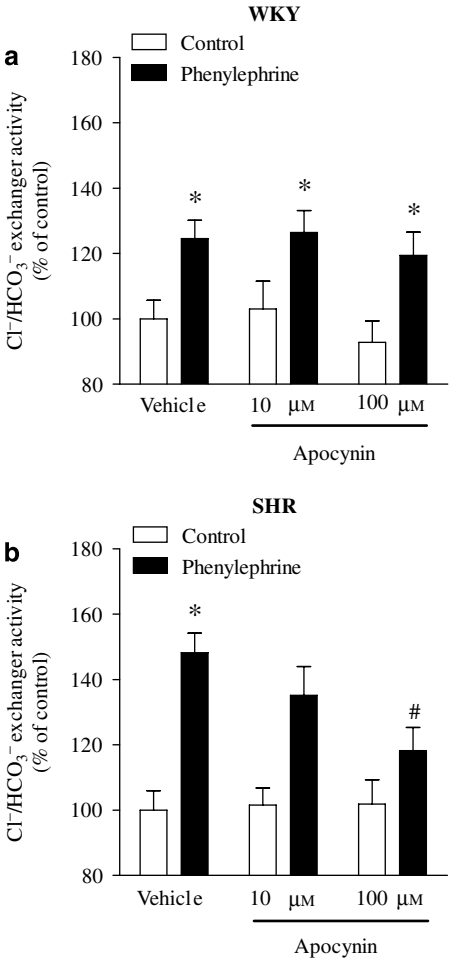

Figure 10 Effect of apocynin (10 and 100 μM; during 4 days after seeding) on phenylephrine (30 μM for 40 min) on Cl^-/HCO_3^- exchanger activity in (**a**) WKY and (**b**) SHR PTE cells. The absolute control values for Cl^-/HCO_3^- exchanger activity, in pH units min^{-1}, for vehicle were 0.2238 ± 0.02 (WKY cells) and 0.3419 ± 0.01 (SHR cells). Each column represents the mean of 4–8 experiments per group; vertical lines indicate s.e.mean. Significantly different from values for control (*$P<0.05$) or for vehicle ($^{\#}P<0.05$). PTE, proximal tubular epithelial; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat.

production of inositol 1,4,5-trisphosphate, which mobilizes intracellular Ca^{2+} and diacylglycerol, which in turn activates PKC (Theroux *et al*., 1996). More recently, α_1-adrenoceptors were found to activate a variety of other effectors, such as the MAPK pathways, in various cell types (Michelotti *et al*., 2000; Piascik and Perez, 2001). Downstream to PKC activation, other mechanisms involved in signal transduction coupled to α_1-adrenoceptor include activation of both the ERK and p38 MAPK pathways (Alexandrov *et al*., 1998; Snabaitis *et al*., 2000; Markou and Lazou, 2002). We examined some of these signalling pathways activated by α_1-adrenoceptors in WKY and SHR PTE cells. Previously, studies with mouse PTE cells demonstrated that α_1-adrenoceptor activation of NHE1 (NHE isoform 1) is also regulated by PKC, whereas NHE3 (NHE isoform 3) is controlled by MAPK (Liu and Gesek, 2001). In renal proximal tubules, the relationship between α_1-adrenoceptor and MAPK is also a well-established process (Liu and Gesek, 2001). However, the link between α_1-adrenoceptor-induced stimulation of sodium-reabsorptive mechanisms and MAPK is not so well established, although there is evidence suggesting that α_1-adrenoceptors activate distinct signalling pathways to regulate specific NHE isoforms localized on opposite membranes in polarized renal epithelial cells (Liu and Gesek, 2001). α_1-Adrenoceptor activation of NHE1 is regulated by PKC, whereas NHE3 is controlled by MAPK and serves to separately regulate pH_i, Na^+ absorption and Na^+ excretion in PTE cells (Liu and Gesek, 2001).

The data obtained in the present study provide evidence that activation of PKC, ERK 1/2 and p38 MAPK is required for α_1-adrenoceptor-induced stimulation of Cl^-/HCO_3^- exchanger in WKY PTE cells. On the other hand, in SHR cells only PKC, ERK 2 and p38 MAPK are required for α_1-adrenoceptor-induced stimulation of Cl^-/HCO_3^- exchanger. To evaluate the contribution of MAPK in the signal-transduction pathway coupled to phenylephrine-induced stimulation of Cl^-/HCO_3^- exchanger activity, specific MEK and p38 MAPK

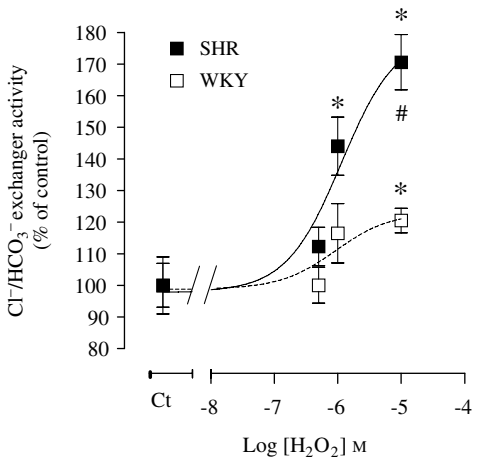

Figure 11 Concentration-dependent effect of H_2O_2 (40 min exposure) on Cl^-/HCO_3^- exchanger activity in WKY and SHR PTE cells. The absolute control values for Cl^-/HCO_3^- exchanger activity in pH units min^{-1} were 0.1725 ± 0.02 (WKY cells) and 0.2432 ± 0.01 (SHR cells). Symbols or columns represent the mean of 4–13 experiments per group; vertical lines indicate s.e.mean. Significantly different from corresponding control values ($^*P<0.05$) and corresponding values in SHR PTE cells ($^{\#}P<0.05$). H_2O_2, hydrogen peroxide; PTE, proximal tubular epithelial; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat.

inhibitors were used. The p38 MAPK inhibitor SB 203580 blocked the stimulation of Cl^-/HCO_3^- exchanger activity by phenylephrine in both WKY and SHR PTE cells. The finding that anisomycin, an activator of p38 MAPK, stimulated Cl^-/HCO_3^- exchanger activity in both WKY and SHR PTE cells, in a SB 203580-sensitive manner, supports the involvement of p38 MAPK in events downstream of α_1-adrenoceptor activation in which the Cl^-/HCO_3^- exchanger is the effector protein. The results presented here also clearly establish a connection between MEK and p38 MAPK in the signal-transduction pathways coupled to α_1-adrenoceptor-induced stimulation of Cl^-/HCO_3^- exchanger activity in both WKY and SHR PTE cells. In WKY PTE cells, the specific MEK 1 inhibitor PD 098059 blunted the stimulation of Cl^-/HCO_3^- exchanger activity induced by phenylephrine, but it was devoid of effects in SHR PTE cells. By contrast, the MEK 1/2 inhibitor U 0126 blunted the stimulation of Cl^-/HCO_3^- exchanger activity induced by phenylephrine in both WKY and SHR PTE cells. This fits well the evidence that SHR PTE cells only express ERK 2, whereas WKY PTE cells express both ERK 1 and ERK 2 (Parenti *et al*., 2000).

Transduction mechanisms set into motion during activation of α_1-adrenoceptor in WKY and SHR PTE cells involve the activation of both PKC and p38 MAPK pathways in a single sequence of events with PKC activation occurring before p38 MAPK activation, which most likely includes phosphorylation of p38 MAPK by PKC. Both anisomycin and PDBu were able to stimulate Cl^-/HCO_3^- exchanger activity to the same extent, with these effects being prevented by specific inhibitors of p38 MAPK (SB 203580) and PKC (chelerythrine). Similarly, p38 MAPK and PKC inhibition by SB 203580 and chelerythrine prevented the stimulation of Cl^-/HCO_3^- exchanger activity by the α_1-adrenoceptor agonist phenylephrine. This suggests the involvement of both kinases in the signal-transduction pathway following α_1-adrenoceptor activation, but does not constitute evidence that stimulation of α_1-adrenoceptor may lead to simultaneous activation of both p38 MAPK and PKC transduction pathways. In fact, the most likely possibility consists of a single sequence of events with PKC activation before p38 MAPK activation in the signalling cascade downstream to stimulation of α_1-adrenoceptors. This view is compatible with the finding that p38 MAPK inhibition by SB 203580 abolished the inhibitory effects of anisomycin, phenylephrine and PDBu, whereas PKC inhibition by chelerythrine abolished the effects of PDBu and phenylephrine, but not those elicited by anisomycin. The observation that the Cl^-/HCO_3^- exchanger in SHR PTE cells responded to PKC and p38 MAPK stimulation similarly to that observed in WKY PTE cells suggests that differences in sensitivity to phenylephrine and noradrenaline between SHR and WKY PTE cells may not be related to differences for the activation of these pathways in WKY and SHR PTE cells.

From the finding that the signal-transduction pathway associated with phenylephrine-induced stimulation of Cl^-/HCO_3^- exchanger activity is similar in WKY and SHR PTE cells, with the exception of the involvement of MEK 1, and that differences in the level of expression of α_1-adrenoceptors do not explain the differences in the sensitivity to phenylephrine, it was hypothesized that oxidative stress, which has been clearly implicated in hypertension (Makino *et al*., 2003; Adler and Huang, 2004; Asghar *et al*., 2006; de Cavanagh *et al*., 2006), could be involved in such differences in the response to phenylephrine in WKY and SHR PTE cells. To test this possibility, 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 be endowed with an increased rate of H_2O_2 production, which was fivefold than that in WKY cells. As a result of this enhanced ability to generate H_2O_2, SHR PTE cells accumulated greater amounts of H_2O_2 in the extracellular medium. One of the mechanisms that may be involved in the enhanced generation of H_2O_2 in SHR PTE cells could be the overexpression of NADPH oxidase, as has been found in the SHR (Adler and Huang, 2004). The view that apocynin, an inhibitor of NADPH oxidase, clearly reduced the extracellular levels of H_2O_2 in SHR PTE cells, but not in WKY PTE cells, implicates this enzyme in the enhanced oxidative stress in SHR PTE cells. In SHR PTE cells, treatment with apocynin, apart from decreasing the levels of H_2O_2 in the extracellular medium, also abolished the enhanced sensitivity in phenylephrine-mediated stimulation of Cl^-/HCO_3^- exchanger activity. It should be emphasized that, after treatment with 100 μM apocynin, WKY PTE cells exhibited the same sensitivity to phenylephrine in terms of the α_1-adrenoceptor-mediated stimulation of Cl^-/HCO_3^- exchanger activity. On the other hand, stimulation of Cl^-/HCO_3^- exchanger activity by H_2O_2 was significantly higher in SHR PTE cells than that in WKY PTE cells. Altogether, this would agree with the view that the enhanced sensitivity to phenylephrine-mediated stimulation of Cl^-/HCO_3^- exchanger activity in SHR cells is a consequence of the oxidative stress condition, resulting from increases in the generation of H_2O_2. The treatment with apocynin (100 μM) for 4 days after seeding did not change the basal Cl^-/HCO_3^-

exchanger activity in both WKY and SHR PTE cells, suggesting that apocynin only modifies responses after stimulus, but not the basal activities. In addition, we demonstrated that SHR PTE cells overexpressed the Cl^-/HCO_3^- exchanger SLC26A6 (sevenfold more than WKY PTE cells) (Pedrosa and Soares-da-Silva, 2006), which may explain the fact that inhibition of NADPH oxidase had no effect on the enhanced basal exchanger activity in SHR PTE cells. One aspect that it is under evaluation in our laboratory is the elucidation of the H_2O_2-sensitive mechanism responsible for the enhanced phenylephrine-induced stimulation of Cl^-/HCO_3^- exchanger activity in SHR PTE cells.

In conclusion, SHR PTE cells were 10 and 4 times more sensitive to noradrenaline and phenylephrine than WKY PTE cells in stimulating Cl^-/HCO_3^- exchanger activity, respectively. In both cell lines, the phenylephrine-mediated stimulation of Cl^-/HCO_3^- exchanger activity proceeds through a molecular pathway that involves PKC and p38 MAPK downstream α_1-adrenoceptor activation. Differences between WKY and SHR PTE cells in their sensitivity to phenylephrine do not correlate with the expression of the α_{1A}- and α_{1B}-adrenoceptors and may be related to the increased generation of H_2O_2, which may amplify the phenylephrine response downstream of α_1-adrenoceptor activation.

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Conflict of interest

The authors state no conflict of interest.

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