H$_2$O$_2$ Stimulation of the Cl$^-$/$\text{HCO}_3^-$ Exchanger by Angiotensin II and Angiotensin II Type 1 Receptor Distribution in Membrane Microdomains

Rui Pedrosa, Van Anthony M. Villar, Annabelle M. Pascua, Sónia Simão, Ulrich Hopfer, Pedro A. Jose, Patrício Soares-da-Silva

Abstract—The present study tested the hypothesis that angiotensin II (Ang II)–induced oxidative stress and Ang II–stimulated Cl$^-$/$\text{HCO}_3^-$ exchanger activity are increased and related to the differential membrane Ang II type 1 (AT$_1$) receptor and reduced nicotinamide-adenine dinucleotide phosphate oxidase expression in immortalized renal proximal tubular epithelial (PTE) cells from the spontaneously hypertensive rat (SHR) relative to its normotensive control (Wistar Kyoto rat [WKY]). The exposure of cells to Ang II increased Cl$^-$/$\text{HCO}_3^-$ exchanger activity with EC$_{50}$s of 0.10 and 12.2 nmol/L in SHR and WKY PTE cells, respectively. SHR PTE cells were found to overexpress nicotinamide-adenine dinucleotide phosphate oxidase 2 and 4 and were endowed with an enhanced ability to generate H$_2$O$_2$. The reduced nicotinamide-adenine dinucleotide phosphate oxidase inhibitor apocynin reduced the production of H$_2$O$_2$ in SHR PTE cells and abolished their hypersensitivity to Ang II. The expression of the glycosylated form of the AT$_1$ receptor in both lipid and nonlipid rafts were higher in SHR cells than in WKY PTE cells. Pretreatment with apocynin reduced the abundance of AT$_1$ receptors in both microdomains, mainly the glycosylated form of the AT$_1$ receptor in lipid rafts, in SHR cells but not in WKY PTE cells. In conclusion, differences between WKY and SHR PTE cells in their sensitivity to Ang II correlate with the higher H$_2$O$_2$ generation that provokes an enhanced expression of glycosylated and nonglycosylated AT$_1$ receptor forms in lipid rafts. (Hypertension. 2008;51:1332-1338.)

Key Words: Cl$^-$/$\text{HCO}_3^-$ exchanger ■ AT$_1$R ■ H$_2$O$_2$ ■ lipid rafts ■ hypertension ■ spontaneously hypertensive rats ■ Wistar Kyoto rats

An abnormal relationship between Na$^+$/H$^+$ exchanger (NHE) activity and natriuretic and antinatriuretic agents, such as dopamine or angiotensin II (Ang II), respectively, has been implicated in hypertension, but the link is less established for the anion exchangers. We have shown recently that D$_1$-like dopamine receptor stimulation inhibits the activity of the Cl$^-$/$\text{HCO}_3^-$ exchanger in immortalized renal proximal tubular epithelial (PTE) cells from normotensive Wistar Kyoto rats (WKYs) but not in PTE cells from the spontaneously hypertensive rat (SHR).$^3$ Most likely SHR cells have a defective transduction of the D$_1$ receptor signal downstream to the Cl$^-$/$\text{HCO}_3^-$ exchanger, as shown for the NHE3 and Na$^+$/K$^+$-ATPase.$^2$-$^4$ On the other hand, preliminary studies showed that immortalized WKY and SHR PTE cells responded to Ang II with stimulation of Cl$^-$/$\text{HCO}_3^-$ exchanger activity through AT$_1$ activation.$^5$ However, SHR PTE cells were endowed with an enhanced sensitivity to Ang II–induced stimulation of the Cl$^-$/$\text{HCO}_3^-$ exchanger activity.$^5$

Caveolae/lipid rafts are specialized membrane microdomains that coordinate and regulate a variety of signaling processes. A crosstalk among Ang II type 1 (AT$_1$) receptors, lipid rafts, and reactive oxygen species derived from reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase was postulated in vascular smooth muscle cells. Indeed, Ushio-Fukai et al.$^8$ have shown that transactivation of the epidermal growth factor receptor induced by Ang II is mediated through cSrc, a proximal target of reactive oxygen species derived from NADPH oxidase, and depends on AT$_1$ receptor trafficking through lipid rafts. In addition, renal H$_2$O$_2$ that influences the development of hypertension and renal function.$^7$-$^8$ is an indirect product of the NADPH oxidase activity, the expression of which is increased in the SHR.$^9$

The study hypothesizes that PTE cells from SHRs have an enhanced oxidative stress condition coupled with NADPH oxidase expression that provokes changes in the function and membrane distribution of AT$_1$ receptors. In line with this view, this study was designed to understand the relationships among the enhanced sensitivity of Ang II–induced stimulation of the Cl$^-$/$\text{HCO}_3^-$ exchanger, oxidative stress, and...
distribution of AT1 receptors in membrane lipid rafts in cultured, immortalized PTE cells from SHRs.

Methods

Cell Culture
Immortalized renal PTE cells from 4- to 8-week-old WKY rats and SHRs10 were maintained in a humidified atmosphere of 5% CO2-95% air at 37°C (for details please see the data supplement available online at http://hyper.ahajournals.org).

Intracellular pH Measurements
In intracellular pH (pHi) measurement experiments, WKY and SHR PTE cells were grown in 96-well plates. pH was measured as described previously.6

Cl-/HCO3- Exchanger Activity
The Na+-independent HCO3- transport system activity was assayed as the initial rate of pHi recovery after an alkaline load (CO2/HCO3 removal), in the absence of Na+, as described previously1 (for details please see the online data supplement).

AT1, Receptor and NADPH Oxidase Immunoblotting
WKY and SHR PTE cells cultured to 90% confluency were washed twice with PBS, and total cell protein was extracted for AT1 receptor NADPH oxidase (NOX) NOX2 and NOX4 detection (for protein extraction details please see the online data supplement). Proteins were subjected to SDS-PAGE (10% SDS polyacrylamide gel) and electrotransferred onto nitrocellulose membranes. The transblot extraction details please see the online data supplement). Proteins were incubated overnight at 4°C with polyclonal antibody rabbit anti-AT1 receptor (1:1000; Santa Cruz Biotechnology), goat polyclonal NOX4 (1:1000; Santa Cruz Biotechnology), goat polyclonal NOX2 (1:1000; Santa Cruz Biotechnology), and mouse monoclonal β-actin primary antibody (1:20 000; Laboratory Vision Corporation).

Membranes were incubated with appropriate fluorescently labeled secondary antibodies for 60 minutes at room temperature and protected from light, and an Odyssey Infrared Imaging System was used for detection. The immunoblots against the AT1 receptor were subsequently washed and incubated with fluorescent-labeled goat antirabbit (1:10 000; Santa Cruz Biotechnology), goat polyclonal NOX2 (1:1000; Santa Cruz Biotechnology), goat polyclonal NOX4 (1:1000; Santa Cruz Biotechnology), and mouse monoclonal β-actin primary antibody (1:20 000; Laboratory Vision Corporation).

AT1 Receptor Expression in Lipid and Nonlipid Rafts
To prepare lipid raft and nonlipid raft fractions, the cell pellets were subjected to sucrose gradient centrifugation using a detergent-free protocol, with slight modifications.11 The cell pellets were lysed in 1.5 mL of sodium carbonate (500 mmol/L; pH 11) in clear ultracentrifuge tubes, homogenized with 15 strokes of Dounce homogenizer, and sonicated using three 30-second bursts of the tip sonicator. One mL of the homogenate was diluted in 2 volumes of 80% sucrose, overlaid with 6 mL of 35% sucrose and 3 mL of 5% sucrose, and spun at 160 000g in a Beckman SW40 rotor at 4°C for 18 hours. The sucrose solutions were prepared in MBS (25 mmol/L of 2-[N-morpholino]ethanesulfonic acid; pH 6.7; 150 mmol/L NaCl). After centrifugation, twelve 1-mL fractions were collected and labeled as fractions 1 to 12 from top to bottom, representing the lipid raft (1 to 6) and nonlipid raft (6 to 12) fractions. Aliquots of each fraction were mixed with Laemmli buffer, boiled for 5 minutes, and subjected to immunoblotting using a rabbit polyclonal anti-AT1R antibody (Santa Cruz, Biotechnology). Immunoblotting for flotilltin-1, a specific lipid raft marker shown previously to be expressed in rat proximal tubule epithelial cells, was also performed to confirm the lipid and nonlipid raft fractions and for annexin 2, annexin 4, and annexin 5 (markers for intracellular membranes) to determine the presence of intracellular membrane proteins.

Measurement of H2O2
H2O2 was measured fluorometrically using the Amplex Red Hydrogen Peroxide Assay kit (Molecular Probes, Inc), as described previously.12

Results

The activity of the CI-/HCO3- exchanger was assayed as the initial rate of pHi recovery after an alkaline load (CO2/HCO3 removal) in the absence of sodium to avoid the contribution of other transporters, such as the NHE3, as reported previously.1 The addition of Ang II before (25 minutes) and during (10 minutes) the HCO3- dependent recovery of pHi increased the CI-/HCO3- exchanger activity in a concentration-dependent manner in WKY and SHR PTE cells (Figure S1). However, as observed in Figure S1, the sensitivity of Ang II–dependent stimulation of Cl-/HCO3- exchange activity was significantly higher in SHR than in WKY PTE cells. This is also evidenced by the 10-fold difference in EC50 values for the Ang II–induced stimulation of Cl-/HCO3- activity between SHR (0.10 mmol/L [0.09 to 0.12 mmol/L]) and WKY (12.2 mmol/L [11.3 to 13.2 mmol/L]) PTE cells. To investigate the type of Ang II receptor and the signal transduction pathway involved in the stimulation of the Cl-/HCO3- exchanger after exposure to Ang II, it was decided to use 2 different concentrations of Ang II that elicited similar increases in Cl-/HCO3- exchange activity in WKY and SHR PTE cells, ie, 30 and 1 mmol/L in WKY and SHR PTE cells, respectively. As shown in Figure S2, treatment of WKY PTE cells with 30 mmol/L Ang II increased CI-/HCO3- exchange activity to an extent similar to that observed with 1 mmol/L Ang II in SHR PTE cells. As depicted in Figure S3, the effect of Ang II (30 mmol/L in WKY PTE cells and 1 mmol/L in SHR PTE cells) on the activity of the Cl-/HCO3- exchanger was completely blocked by the AT1 receptor antagonist losartan (100 mmol/L) in both SHR and WKY PTE cells. By
contrast, the effect of Ang II was unaffected by the selective AT$_1$ receptor antagonist PD 123 319 (100 nmol/L; Figure S3).

To evaluate the contribution of protein kinase C and mitogen-activated protein kinase (MAPK), which are normally related with signal transduction coupled to Ang II receptors and also linked to Ang II–induced stimulation of sodium reabsorption in kidney and vascular tissues, we used specific inhibitors for protein kinase C, MAPK/extracellular signal–regulated kinase (MEK) kinases, and p38 MAPK. Pretreatment of cells for 30 minutes with the MAPK inhibitors PD 098059 (MEK 1 inhibitor) and U0126 (MEK 1/2 inhibitor) abolished the Ang II–induced stimulation of Cl$^-$/HCO$_3^-$ exchanger activity in both SHR and WKY PTE cells (Figure S4). A similar effect was obtained with the p38 MAPK inhibitor SB 203580, which completely abolished the Ang II–induced stimulation of Cl$^-$/HCO$_3^-$ exchanger activity (Figure S4). However, chelerythrine (1 μmol/L) did not affect the effect of Ang II in both WKY and SHR PTE cells (Figure S4).

Because recent studies have demonstrated a role for renal H$_2$O$_2$ in the development of hypertension and renal dysfunction, particularly in the SHR model, it was felt worthwhile to measure H$_2$O$_2$ generation and evaluate the involvement of H$_2$O$_2$ in the regulation of Cl$^-$/HCO$_3^-$ exchanger activity by Ang II in WKY and SHR PTE cells. The SHR PTE cells had an increased rate of H$_2$O$_2$ production (50.7 ± 0.4 versus 11.3 ± 0.1 nmol/L per minute) when compared with WKY PTE cells (Figure 1A). Treatment of cells with apocynin (100 μmol/L), an inhibitor of the NADPH oxidase complex, for 4 days after seeding reduced the extracellular levels of H$_2$O$_2$ in a concentration-dependent manner in SHR PTE cells but not in WKY cells (Figure 1B).

NOXs are transmembrane proteins that transfer electrons across biological membranes to reduce oxygen to superoxide. To date, 7 members of the NOX family have been discovered. The NOX enzyme family has been identified as a major source of reactive oxygen species that include superoxide anion, hydrogen peroxide, and hydroxyl radical. As shown in Figure 2A, the level of expression of NOX2 was significantly higher in SHR than in WKY PTE cells. As shown in Figure 2B, the level of expression of the NOX4 was also significantly higher in SHR than in WKY PTE cells.

Because enhanced H$_2$O$_2$ generation in SHR cells may be related to Ang II receptor activity, eventually resulting from the presence of angiotensinogen in the serum or the constitutively activity of the AT$_1$ receptors, we treated WKY and SHR PTE cells for 4 days after seeding with losartan (100 nmol/L) or PD 123 319 (100 nmol/L); the antagonists did not change the levels of H$_2$O$_2$ generation in both types of cells (data not shown). The treatment with apocynin (100 μmol/L) for 4 days after seeding also did not change the basal Cl$^-$/HCO$_3^-$ exchanger activity in both WKY (0.084 ± 0.004 versus 0.085 ± 0.006 pH units per minute) and SHR (0.239 ± 0.008 versus 0.240 ± 0.020 pH units per minute) PTE cells. However, treatment of SHR PTE cells with apocynin (100 μmol/L) completely blocked the ability of Ang II to stimulate Cl$^-$/HCO$_3^-$ exchanger activity with no changes in WKY PTE cells (Figure 3A and 3B). To confirm the relationship among the NADPH oxidase, oxidative stress, and enhanced response to angiotensin II–mediated stimulation of Cl$^-$/HCO$_3^-$ exchanger activity in SHR cells, we decided to test a lower concentration of apocynin. As shown in Figure 3B, treatment of SHR PTE cells with apocynin (10 μmol/L) during 4 days after seeding reduced the sensitivity of these cells to Ang II–mediated stimulation of Cl$^-$/HCO$_3^-$ exchanger activity, to an extent identical to that observed in WKY PTE cells.

![Figure 1](http://hyper.ahajournals.org/)
The distribution and abundance of AT$_1$ receptors in membrane microdomains were evaluated in WKY and SHR PTE cells using sucrose gradient fractionation followed by Western blotting using uniform amounts of proteins in corresponding fractions. Flotillin-1, a specific lipid raft protein marker$^{17,18}$ shown previously to be expressed in rat membrane microdomains,$^{11}$ was used to distinguish lipid from nonlipid rafts. In both cell lines, 2 distinct bands corresponding with the native, nonglycosylated ($\approx$45 kDa) and glycosylated ($\approx$60 kDa; preliminary data) forms of AT$_1$ receptors were observed in both lipid (fractions 1 to 6) and nonlipid rafts (fractions 7 to 12; Figure 4). Apart from the difference in the distribution of the AT$_1$ receptor in lipid and nonlipid rafts, the most significant difference was the greater abundance of the glycosylated form of the AT$_1$ receptor in both lipid and nonlipid rafts in SHR PTE cells compared with WKY PTE cells. Pretreatment with apocynin (10 µmol/L) drastically reduced the AT$_1$ receptor abundance in both microdomains in SHR PTE cells but not in WKY cells PTE (Figure 4). This effect was more marked in lipid rafts, particularly with the glycosylated form of the AT$_1$ receptor. Ang II (1 nmol/L) slightly increased the amount of glycosylated AT$_1$ receptor in lipid rafts in SHR PTE cells but not in WKY PTE cells. Pretreatment with apocynin (10 µmol/L) abolished the Ang II–induced increased abundance of the glycosylated AT$_1$ receptor form in lipid rafts in SHR PTE cells (Figure 4).

To determine the inclusion of intracellular membranes in the various fractions obtained after ultracentrifugation, the fractions were also probed for annexin 2, annexin 4, and annexin 5, which all belong to a family of calcium ion (Ca$^{2+}$)–dependent, phospholipid-binding proteins involved in vesicle trafficking.$^{19}$ Annexins are generally regarded as cytosolic proteins$^{19}$ and, as such, are commonly used as markers for intracellular membranes. No bands were observed after probing for annexin 2 and annexin 4; however, bands corresponding with annexin 5 were observed mostly in nonlipid rafts (Figure S5). These data suggest that the signal bands observed in the lipid raft fractions are mainly surface...
membrane lipid raft proteins and are unadulterated by proteins found in intracellular membranes.

The AT1 receptor transcripts and AT1 receptor expression were also evaluated in total cell extract from immortalized WKY and SHR PTE cells by RT-PCR and by immunoblot analysis, respectively. As shown in Figures S6 and S7, both WKY and SHR PTE cells were also evaluated in total cell extract from immortalized cells. The results presented here clearly establish a connection between MEK and p38 MAPK in the signal transduction pathways coupled to Ang II–induced stimulation of Cl–/HCO3− exchanger activity in both WKY and SHR PTE cells. In conclusion, the signal transduction pathway coupled to Ang II–induced stimulation of Cl–/HCO3− exchanger activity in SHR PTE cells appears to be similar to that in WKY PTE cells.

Because of the finding that the signal transduction pathway associated with Ang II–induced stimulation of Cl–/HCO3− exchanger activity was similar in WKY and SHR PTE cells, it was hypothesized that oxidative stress, which has been clearly implicated in hypertension,7–9 could be involved in such differences in the response to Ang II in WKY and SHR PTE cells. To test this possibility, the generation of H2O2, a marker of oxidative stress, was measured in WKY and SHR PTE cells. SHR PTE cells were found to be endowed with an increased rate of H2O2 production, which was 5-fold that of WKY cells. As a result of this enhanced ability to generate H2O2, SHR PTE cells accumulated greater amounts of H2O2 in the extracellular medium. One of the mechanisms involved in the enhanced generation of H2O2 in SHR PTE cells could be the overexpression of NADPH oxidase, as has been found in the SHR.9 In fact, the abundance of NOX2 and NOX4 was found markedly increased in SHR PTE cells. The finding that immortalized renal PTE cells from the SHR overexpress NOX2 and NOX4 is in line with the finding that increased NOX2 and NOX4 mRNA and protein were found in the kidney of SHRs.9,20,21 The view that apocynin, an inhibitor of NADPH oxidase subunit assembly, clearly reduced the extracellular levels of H2O2 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 H2O2 in the extracellular medium also abolished the enhanced sensitivity in Ang II–mediated stimulation of Cl–/HCO3− exchanger activity. It should be emphasized that, after treatment with apocynin, WKY PTE cells were endowed with identical sensitivity to Ang II in terms of the AT1 receptor–mediated stimulation of Cl–/HCO3− exchanger activity. This would agree with the view that the enhanced sensitivity to Ang II–mediated stimulation of Cl–/HCO3− exchanger activity in SHR cells is a consequence of the oxidative stress condition, resulting from increases in the generation of H2O2.

### Discussion

The data reported here show that SHR PTE cells were 10 times more sensitive to Ang II than WKY PTE cells in stimulating Cl–/HCO3− exchanger activity. Despite differences in sensitivity to Ang II between SHR and WKY PTE cells, the results obtained indicated that Ang II–induced stimulation of Cl–/HCO3− exchanger activity was mediated through the activation of losartan-sensitive AT1 receptors coupled to MEK and MAPK pathways in both WKY and SHR PTE cells. The enhanced sensitivity to Ang II–induced stimulation of the Cl–/HCO3− exchanger activity through the AT1 receptor in SHR PTE cells was associated with the higher H2O2 generation that may have provoked an enhanced expression of glycosylated and nonglycosylated AT1 receptor forms in lipid rafts.

To elucidate the mechanism involved in the enhanced sensitivity to Ang II–mediated stimulation on the Cl–/HCO3− exchanger, we considered 3 possibilities. First, differences in the expression of AT1 receptors.

### Figure 4. Distribution of the native nonglycosylated (~45 kDa) and glycosylated (~60 kDa) forms of AT1 receptors in rat membrane microdomains (lipid raft: fractions 1 to 6; nonlipid rafts: fractions 7 to 12) before and after Ang II (1 nmol/L) stimulation in the presence or in the absence of apocynin (Apo; 10 μmol/L) pretreatment (4 days after seeding) in WKY and SHR PTE cells. The distribution of flotillin-1, a marker of lipid rafts, is also shown. These experiments were performed thrice with similar results.
Next we evaluated whether the enhanced sensitivity to Ang II in SHR PTE cells was linked to differences in the expression of AT1 receptors. The distribution of AT1 receptors in lipid and nonlipid rafts was clearly distinct in WKY and SHR PTE cells. SHR PTE cells were found to be endowed with more AT1 receptors in both microdomains than WKY PTE cells, with particular emphasis in lipid rafts. Moreover, differences between WKY and SHR PTE cells were more pronounced in the heavy bands that correspond with the glycosylated form of the AT1 receptor, which correspond with the active form of the receptor (data not shown). These differences are in agreement with the view that SHR cells have an enhanced sensitivity to the Ang II–induced stimulation of Cl-/HCO3- exchanger activity.

Although differences in the distribution of the glycosylated and the nonglycosylated forms of the AT1 receptor in lipid and nonlipid rafts, per se, may explain the enhanced response of SHR PTE cells to Ang II–induced stimulation of Cl-/HCO3- exchanger activity, the relationship between that response and the higher H2O2 generation is not linear. To evaluate the relationship among oxidative stress, the enhanced response to Ang II, and differences in the distribution of the glycosylated and the nonglycosylated forms of the AT1 receptor in lipid and nonlipid rafts, we felt that it was worthwhile to evaluate the effect of apocynin on the distribution of AT1 receptors. In SHR but not in WKY PTE cells, a drastic reduction in the expression of AT1 receptors in both microdomains, especially in the glycosylated forms of the receptor in lipid rafts, was observed when cells were grown in the presence of apocynin. This result is in agreement with the view that apocynin changed neither the H2O2 generation nor the presence of apocynin. This result is in agreement with the view that apocynin changed neither the H2O2 generation nor the Ang II–induced increases in the sensitivity to Ang II on the stimulation of the Cl-/HCO3- exchanger.

It is concluded that SHR PTE cells were 10 times more sensitive to Ang II than WKY PTE cells in stimulating Cl-/HCO3- exchanger activity. In both cell lines, the Ang II–mediated stimulation of Cl-/HCO3- exchanger activity proceeds through a molecular pathway that involves MEK and p38 MAPK downstream AT1 receptor activation. Differences between WKY and SHR PTE cells in their sensitivity to Ang II correlate with the higher H2O2 generation that provokes an enhanced expression of the glycosylated and nonglycosylated AT1 receptor form in lipid rafts.

**Perspectives**

Woost et al.10 in a clone of immortalized cells used in the current studies, demonstrated that SHR cells have an enhanced Ang II–dependent activation of NHE. However, a sustained activation of the NHE is only possible in the presence of a parallel increase of an acidifying pathway, such as the Cl-/HCO3- exchanger, as demonstrated in the current report. This is also in agreement with the view that Ang II leads to enhanced proximal tubular sodium reabsorption in proximal tubular cells of SHRs.10,22–24

Our study also confirms the view that H2O2 influences renal ion transport.7,8 The enhanced sensitivity to Ang II–mediated stimulation of Cl-/HCO3- exchanger activity that is associated with increased oxidative stress results from the enhanced generation of H2O2 by NADPH oxidase in SHR cells. Indeed, H2O2 causes the uncoupling of dopamine D3–like receptors from G proteins in renal proximal tubules of Sprague-Dawley rats.7 This is particularly relevant in renal proximal tubules of the SHRs, which have a defective transduction of the D3 receptor signal downstream to the Cl-/HCO3- exchanger, NHE3, and Na+–K+-ATPase.2,24 All together it is likely that oxidative stress, NADPH oxidase, Ang II, and the uncoupling of D3 receptor are involved in the development of hypertension in SHRs. This hypothesis needs to be confirmed by future studies “in vivo.”

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**Disclosures**

None.

**References**


H$_2$O$_2$, stimulation of Cl/HCO$_3^-$ exchanger by angiotensin II and AT$_1$ receptor distribution in membrane microdomains

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Running title: Oxidative stress and AT1 receptor distribution

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Expanded Methods

Cell culture

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-ethansulfonic acid (HEPES; Sigma). For subculturing, the cells were dissociated with 0.10% trypsin-EDTA, split 1:4 and subcultured in Costar plates with 21-cm² growth areas (Costar, Badhoevedorp, The Netherlands). For pHᵢ measurement experiments, cells were grown in 96 well plates (Costar) or glass coverslips. For the measurement of SLC26A6 expression the cells were seeded in 6 wells plastic culture clusters (Costar). The cell medium was changed every 2 days, and the cells reached confluence after 3-5 days of incubation. For 24 hours prior to each experiment, the cells were maintained in fetal bovine serum-free medium. Experiments were generally performed 1-2 days after cells reached confluence and 4-5 days after the initial seeding; each cm² contained about 50 µg of cell protein.
**Cl-/HCO₃⁻ exchanger activity**

In the Krebs HCO₃⁻-free medium used, sodium was replaced by an equimolar concentration of choline. Though there is no specific assay for Cl-/HCO₃⁻ exchange-mediated activity, several findings strongly suggest that pHi recovery after removal of CO₂/HCO₃⁻ in the absence of Na⁺ reflects the activity of the Cl-/HCO₃⁻ exchanger. In brief, the pHi recovery after removal of CO₂/HCO₃⁻ in the absence of Na⁺ is markedly dependent on Cl⁻ (pHi recovery reduction: 99.5% and 83.3% in SHR and WKY cells, respectively), which clearly excludes the participation of NHE and Na⁺-HCO₃⁻ co-transporter (NBC) in the recovery process ¹. Moreover, the Cl-/HCO₃⁻ exchanger activity was exclusively observed on the apical side of the cells, which excludes the contribution of the NBC that is expressed in basal cell side ². The pHi recovery in these experimental conditions is strongly inhibited by DIDS ¹, ³, which fits well the view that NHE does not participate in the pHi recovery after the alkaline load.

**Protein extraction**

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, 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 (16,000g, 30 min, 4°C), the supernatant was collected and protein concentration determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin as standard. Twenty micrograms of protein were mixed in 6x 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.
RT-PCR

WKY and SHR PTE cells were grown to 90% confluence and total RNA was extracted using RNeasy RNA Extraction kit (Qiagen) and quantified using Smartspec spectrophotometer (Bio-Rad). Uniform amounts (1 μg) of RNA were used for semi-quantitative RT-PCR, which was carried out using Superscript III One-step RT-PCR Kit (Invitrogen) at the following conditions: 55º C for 30 min, 40 cycles of 95º C for 30 sec, 55º C for 30 sec, 68º C for 1 min, and one cycle of 68º C for 3 min. Primers used included TCAACCTCTACGCCAGTGTG and CAGCACATCCAGGAAAGTGA for AT1R (467 bp) and AGAAAATCTGGCACCACACC and CTCCTTAATGTCACGCACGA for β-actin (388 bp). The amplicons were resolved in 2% AGE and the AT1R band densities were quantified and normalized for those of β-actin.

References

Figure S1. Concentration-dependent effect of angiotensin II (incubation time: 25 min) on Cl⁻/HCO₃⁻ exchanger activity in WKY and SHR PTE cells. Symbols represent the means of 6-16 experiments per group; vertical lines indicate SEM. Ct = control
Figure S2

Figure S2. Assessment of (A and B) intracellular pH recovery and (C and D) Cl⁻/HCO₃⁻ exchanger activity under V_{max} conditions as the initial rate of pH_{i} recovery after an alkaline load (CO₂/HCO₃⁻ removal) in absence or in presence of angiotensin II (WKY = 30 nM; SHR = 1 nM) in WKY and SHR PTE cells. Traces represent means of 7-11 experiments per group. Columns represent mean of 6-8 independent determinations; vertical lines show SEM. Significantly different from corresponding control values (* P<0.05) using the Student’s t test.
Figure S3. Effect of angiotensin II (WKY = 30 nM; SHR = 1 nM) for 25 min on Cl⁻/HCO₃⁻ exchanger activity, in the absence and the presence of PD 123319 (100 nM) and Losartan (100 nM) in WKY or SHR PTE cells, respectively. Each column represents the mean of 6-8 experiments per group; vertical lines indicate SEM. Significantly different from corresponding values for vehicle (* P<0.05) using the Newman-Keuls test.
Figure S4. Effect of angiotensin II (WKY = 30 nM; SHR = 1 nM) for 25 min on Cl⁻/HCO₃⁻ exchanger activity in WKY and SHR PTE cells in the absence and the presence of U0126 (10 µM), PD 98059 (10 µM), SB 203580 (10 µM) or chelerythrin (1 µM). Each column represents the mean of 12-16 experiments per group; vertical lines indicate SEM. Significantly different from corresponding control values (* P<0.05) or from corresponding values for the vehicle (# P < 0.05) using the Newman-Keuls test.
Figure S5

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**SHR**

**WKY**

**Figure S5.** Distribution of annexin 5 in rat membrane microdomains (lipid and non-lipid rafts) in WKY and SHR PTE cells. These experiments were performed twice with similar results.
Figure S6. Expression of AT1 receptors and β-actin in total cell extracts from WKY and SHR PTE cells. Values are expressed as mean ± SEM (n=3/group). Representative immunobLOTS are depicted above the bar graphs. Columns represent mean of 4 independent immunoblots; vertical lines show S.E.M. AT1 ~ 45 kDa; β-actin ~ 40 kDa.
Figure S7

**Figure S7.** Basal mRNA expression of AT$_1$ receptor (AT$_1$R) in WKY and SHR PTE cells. Rat PTE cells were grown to 90% confluence and total RNA was extracted and used for semi-quantitative RT-PCR of AT$_1$R. The amplicons were resolved in 2% AGE and the AT$_1$R band densities were quantified and normalized for those of β-actin. Values are expressed as mean ± SEM (n=3/group).