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H_2O_2 Stimulates Cl^-/HCO_3^- Exchanger Activity Through Oxidation of Thiol Groups in Immortalized SHR Renal Proximal Tubular Epithelial Cells

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ABSTRACT

Cl^-/HCO_3^- exchanger and Na^+/H^+ exchanger 3 are the main transporters responsible for NaCl reabsorption in kidney proximal tubules (PT). It is well accepted that membrane exchangers can be regulated by reactive oxygen species (ROS). In the kidney, ROS are known to contribute to decreases in Na^+ excretion and consequently increase blood pressure. The present study investigated mechanisms by which H_2O_2-induced stimulation of Cl^-/HCO_3^- exchanger activity is enhanced in proximal tubular epithelial (PTE) cells immortalized from spontaneously hypertensive rats (SHR) as compared to normotensive Wistar Kyoto (WKY). H_2O_2 decreased K_m values for Cl^-/HCO_3^- exchanger activity in SHR PTE cells, but had no effect on the kinetic parameters in WKY cells. DTDP stimulated in a concentration-dependent manner Cl^-/HCO_3^- exchanger activity in both cell lines, but SHR PTE cells were 2.4-fold more responsive to this oxidant. In contrast, thimerosal had no effect on exchanger activity in both cell lines. The effects of H_2O_2 and DTDP upon the exchanger activity were blocked by DTT in WKY and SHR PTE cells. Similar to H_2O_2, DTDP decreased K_m values for Cl^-/HCO_3^- exchanger activity in SHR PTE cells. Basal content of free thiol groups was higher in WKY PTE cells than in SHR. Upon H_2O_2 treatment the free thiol groups decreased in both cell lines; however, this decrease was more pronounced in WKY cells. In conclusion, in SHR PTE cells H_2O_2 stimulates Cl^-/HCO_3^- exchanger activity via modification of thiol groups of intracellular and/or transmembrane protein. Furthermore, the thiol oxidation-dependent pathway also increases the HCO_3^- affinity in SHR PTE cells. J. Cell. Biochem. 112: 3660–3665, 2011.

KEY WORDS: CL^-/HCO_3^- EXCHANGER; SHR; HYPERTENSION; OXIDATIVE STRESS; THIOL GROUPS

Hypertension is a heterogeneous disease with a great variety of underlying causes involving both genetic and environmental factors, which together determine the manifestation and severity of the disease. Renal NaCl reabsorption regulation is essential in NaCl balance, and is important in the control of extracellular volume and blood pressure [Zeng et al., 2004]. Approximately, 50–70% of the filtered chloride is reabsorbed in the proximal tubule (PT) [Aronson and Giebisch, 1997] and the major mechanism responsible for this reabsorption is the apical Na^+/H^+ exchanger subtype 3 (NHE3) and the Cl^-/HCO_3^- exchanger, which are essential for intracellular pH (pH_i) and cell volume regulation [Petrovic et al., 2003].

Oxidative stress results from an increase in reactive oxygen species (ROS) production, a decrease in antioxidant defenses, or both. Several studies demonstrate a relationship between oxidative stress and hypertension (reviewed in [Paravicini and Touyz, 2008]). However, the exact mechanism by which ROS mediate increases in blood pressure remains unclear. It is recognized that ROS can either activate or inactivate membrane channels, transcription factors, metabolic enzymes, and also participate in the regulation of signaling pathways [Winterbourn and Hampton, 2008]. In the kidney, ROS are known to contribute to decreases in Na^+ excretion and subsequent increase in blood pressure. It has been previously reported that ROS stimulate NaCl absorption in the thick ascending limb of the loop of Henle, by stimulating Na/K/2Cl co-transporter and NHE [Ortiz and Garvin, 2002; Juncos and Garvin, 2005; Silva et al., 2006]. The effect of ROS in the renal PT is not well defined. We have previously shown that spontaneously hypertensive rats

Abbreviations: BCECF-AM, acetoxymethyl ester of 2′, 7′-bis (carboxyethyl)-5(6)-carboxyfluorescein; DTDP, 2,2′-dithiodipyridine; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NHE, Na^+/H^+ exchanger; PT, proximal tubule; PTE, proximal tubular epithelial; ROS, reactive oxygen species; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto rat.

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proximal tubular epithelial (SHR PTE) cells have an enhanced sensitivity to H_2O_2-induced stimulation of Cl^-/HCO_3^- exchanger activity in comparison with Wistar Kyoto (WKY) PTE cells [Simao et al., 2008a; Simao et al., 2010], which is the result of increased responsiveness to JNK1/2 [Simao et al., 2010]. In addition to MAPK activation, ROS can also modulate ion transport by several other mechanisms, such as, posttranslational modifications of channel proteins (oxidation of aminoacids residues) [Matalon et al., 2003]. There is a growing interest in the role of thiol–disulfide oxidoreduction as a mechanism of redox regulation of cellular functions and gene expression. In fact, the reversible oxidation and reduction of thiol proteins is thought to be the main mechanism by which ROS influence cellular signal transduction pathways [Winterbourn and Hampton, 2008]. The aim of the present study was to investigate further mechanisms by which H_2O_2-induced stimulation of Cl^-/HCO_3^- exchanger activity is enhanced in PTE cells immortalized from SHR as compared to normotensive WKY.

MATERIALS AND METHODS

CELL CULTURE

Immortalized renal PTE cells were obtained from primary cultures from S1 segments of PTs of 4–8-week-old WKY and SHRs [Woost et al., 1996]. These cell lines formed polarized monolayers with apical microvilli, tight junctional complexes, and convolutions of the basolateral plasma membrane. Immortalized WKY and SHR cell lines express a proximal tubular phenotype and are morphologically and functionally similar to primary cultures [Woost et al., 1996]. Cells 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) supplemented with 100 U/ml penicillin G (Sigma), 0.25 μg/ml amphotericin B (Sigma), 100 μg/ml streptomycin (Sigma), 4 μg/ml dexamethasone (Sigma), 5 μg/ml transferrin (Sigma), 5 μg/ml insulin (Sigma), 5 ng/ml selenium (Sigma), 10 ng/ml epidermal growth factor (Sigma), 5% fetal bovine serum (Sigma), and 25 mM N-2-hydroxyethylpiperazine-N′-2-ethanosulfonic acid (HEPES; Sigma). For subculturing, the cells were dissociated with 0.10% trypsin-EDTA (Sigma), split 1:8 and cultured 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). The cell medium was changed every 2 days, and the cells reached confluence after 3–5 days of incubation. For approximately 2 h prior to each experiment, the cells were maintained in fetal bovine serum-free medium. Experiments were performed 4 days after the initial seeding; each cm^2 contained about 50 μg of cell protein.

pHi 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., 2004]. At day 4 after seeding SHR and WKY PTE cells were incubated at 37°C for 30 min with the membrane-permeant acetoxymethyl ester derivative of 2′,7′-bis (carboxyethyl)-5,6-carboxyfluorescein, BCECF-AM (10 μM). Cells were then washed twice with prewarmed modified Krebs–Hensleit buffer before initiation of the fluorescence recordings. The Krebs medium had the following composition (in mM): 115 NaCl, 25 $NaHCO_3$, 5.4 KCl, 2.8 $CaCl_2$, 1.2 $MgSO_4$, 0.3 NaH_2PO_4, 0.3 KH_2PO_4, 10 HEPES, 5 glucose, pH 7.4 (adjusted with Tris base). Cells were placed in the sample compartment of a dual-scanning microplate spectrofluorometer (Spectramax Gemini XS, Molecular Devices, Sunnyvale), and fluorescence was measured every 17 s alternating between 440 and 490 nm excitation and 535 nm emission, with a cutoff filter of 530 nm. The ratio of intracellular BCECF fluorescence at 490 and 440 nm was converted to pHi by comparison with values from an intracellular calibration curve using nigericin 10 μM in a high-K^+ solution (in mM:15 NaCl, 130 KCl, 0.3 KH_2PO_4, 0.3 NaH_2PO_4, 5 glucose, 1.2 $MgSO_4$, 2.8 $CaCl_2$, and 10 HEPES) with pH ranging from 6.6 to 7.8 [Thomas et al., 1979].

Cl^-/HCO_3^- EXCHANGER ACTIVITY

Although there is no specific assay for Cl^-/HCO_3^- exchange-mediated activity, several findings strongly suggest that pH_i recovery after removal of CO_2/HCO_3 in the absence of Na^+ reflects the activity of the Cl^-/HCO_3^- exchanger. Thus, 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., 2004]. Briefly, cells were loaded in serum-free medium with 10 μM BCECF-AM, for 30 min at 37°C in 5% CO_2–95% air atmosphere. The cells were washed free of dye and loaded with Krebs–Hensleit solution (25 mM $NaHCO_3$) for 25 min (phase 1). Then, extracellular solution was replaced by a Krebs–Hensleit $NaHCO_3$-free solution for 10 min (phase 2). In the $NaHCO_3$-free bathing solution, $NaHCO_3$ was replaced by an equimolar concentration of choline. In experiments intended to evaluate the kinetic parameters, NaHCO3 in the Krebs–Hensleit solution (phase 1, 25 min) was replaced by an equimolar concentration of sodium gluconate. The test compounds were added to the extracellular fluid 40 min before starting the bicarbonate-dependent pH_i recovery. Cells were placed in the sample compartment of a dual-scanning microplate spectrofluorometer and fluorescence monitored every 17 s alternating between 440 and 490 nm excitation at 535 nm of emission, with a cutoff filter of 530 nm.

DETERMINATION OF CELL PROTEIN THIOLS WITH DTNB

WKY and SHR PTE cells were cultured in 96-well plates and treated with H_2O_2 (10 μM; 0–40 min). Next, 100 μM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was added to the wells. Following 40 min incubation at room temperature the absorbance of 2-nitro-5-thiobenzoate was measured at 412 nm and the amount of free thiol groups per cell was calculated using a cysteine calibration curve.

DRUGS

Hydrogen peroxide, DTDP, DTNB, DTT, L-cysteine, thimerosal were obtained from Sigma Chemical Company (St. Louis, MO). BCECF-AM and nigericin were obtained from Molecular Probes.

DATA ANALYSIS

Arithmetic means are given with standard error of the mean (SEM). Statistical analysis was performed by one-way analysis of variance

(ANOVA) followed by the Newman–Keuls test for multiple comparisons. A *P*-value less than 0.05 was assumed to denote a significant difference.

RESULTS

The potential mechanisms responsible for the enhanced H_2O_2-induced stimulation of Cl^-/HCO_3^- exchanger activity in SHR PTE cells were explored. Figure 1 represents Cl^-/HCO_3^- exchanger activity as a function of extracellular HCO_3^- concentration (0–25 mM) in immortalized WKY and SHR PTE cells, in the absence and presence of H_2O_2 (10 μM; 40 min). In both WKY and SHR PTE cells, increases in extracellular HCO_3^- enhanced the activity of Cl^-/HCO_3^- exchanger (Fig. 1). The affinity for HCO_3^- was identical in WKY and SHR PTE cells, but V_{max} values were significantly higher in SHR PTE cells than in WKY PTE cells (Table I). Kinetic parameters (K_m and V_{max}) were not affected by H_2O_2 treatment in WKY PTE cells. In contrast, H_2O_2 significantly decreased K_m values in SHR PTE cells without affecting V_{max} values (Table I). Subsequently, the effect of oxidizing reagents on the Cl^-/HCO_3^- exchanger activity in WKY and SHR PTE cells was evaluated (Fig. 2). As can be observed, DTDP (a membrane permeable free thiol groups oxidizing reagent) stimulated, in a concentration-dependent manner, Cl^-/HCO_3^- exchanger activity in both WKY and SHR PTE cells (Fig. 2A). SHR PTE cells were more responsive to DTDP than WKY (178.9% ± 6.0 vs. 128.8% ± 8.8) (Fig. 2A). In contrast, thimerosal (a poorly membrane permeable free thiol groups oxidizing reagent) had no effect on exchanger activity in both cell lines (Fig. 2B). Table II shows that DTDP (100 μM; 40 min) increased V_{max} values in WKY PTE cells. On the contrary, DTDP had no effect on V_{max} but decreased K_m values in SHR PTE cells (Table II). As shown in Figure 3, the stimulatory effects of H_2O_2 (10 μM) and DTDP (100 μM) on Cl^-/HCO_3^- exchanger activity in WKY and SHR PTE cells were prevented by the reducing reagent DTT (100 μM). Figure 4 shows the concentration of free thiol groups in WKY and SHR PTE cells, in both basal conditions and after H_2O_2 treatment (10 μM; 0–40 min). As can be observed in the basal state, WKY PTE cells show a higher concentration of free thiol groups when compared with SHR PTE cells (482.2 ± 13.5 vs. 336.2 ± 20.8 μM) (Fig. 4A). After H_2O_2 exposure, the concentration of free thiol groups decreased in both cell lines, but this decrease was more pronounced in WKY PTE cells (32% vs. 20% reduction upon 40 min H_2O_2 exposure) (Fig. 4B).

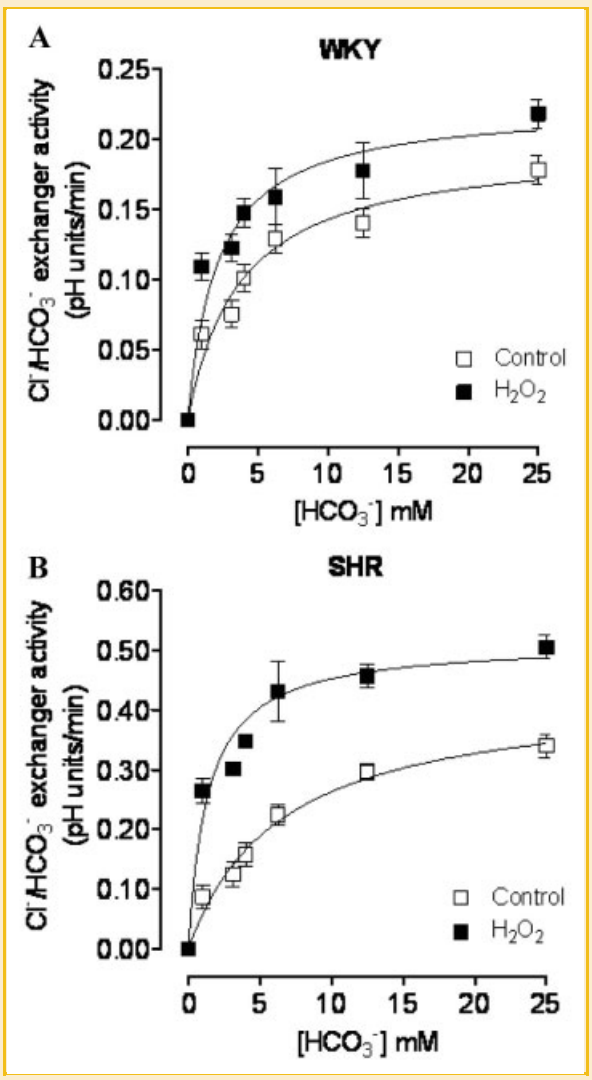

Fig. 1. A: Cl^-/HCO_3^- exchanger activity as a function of extracellular HCO_3^- concentration in immortalized WKY and in (B) SHR PTE cells, in the absence and presence of H_2O_2 (10 μM; 40 min). Symbols represent mean of three independent determinations; vertical lines show SEM.

DISCUSSION

We have recently shown that SHR PTE cells display enhanced sensitivity to H_2O_2-induced stimulation of Cl^-/HCO_3^- exchanger activity and that this effect is regulated, at least in part, by JNK1/2, in contrast to WKY PTE cells [Simao et al., 2008a; Simao et al., 2010]. The present study was designed to determine additional mechanisms responsible for differences between renal PTE cells from hypertensive and normotensive rats on their sensitivity to H_2O_2-induced stimulation of Cl^-/HCO_3^- exchanger activity. We have found that H_2O_2 increased Cl^-/HCO_3^- exchanger activity through modification of thiol groups of intracellular and/or transmembrane protein in SHR PTE cells. In addition, the "oxidized conformation" of the exchanger enhanced the affinity for HCO_3^- in SHR PTE cells only.

TABLE I. Kinetic Parameters for Cl^-/HCO_3^- Exchanger Activity in Immortalized WKY and SHR PTE Cells in the Absence and Presence of H_2O_2 (10 μM; 40 min)

	K_m (mM)		V_{max} (pH units/min)	
Treatment	WKY	SHR	WKY	SHR
Control	3.744 ± 0.866	6.440 ± 1.308	0.196 ± 0.014	0.432 ± 0.032*
H_2O_2	2.156 ± 0.781	1.361 ± 0.340**	0.224 ± 0.018	0.514 ± 0.026*

Values are means ± SEM of 5–12 experiments per group.
*Significantly different from corresponding WKY values ($P < 0.05$).
**Significantly different from corresponding control values ($P < 0.05$).

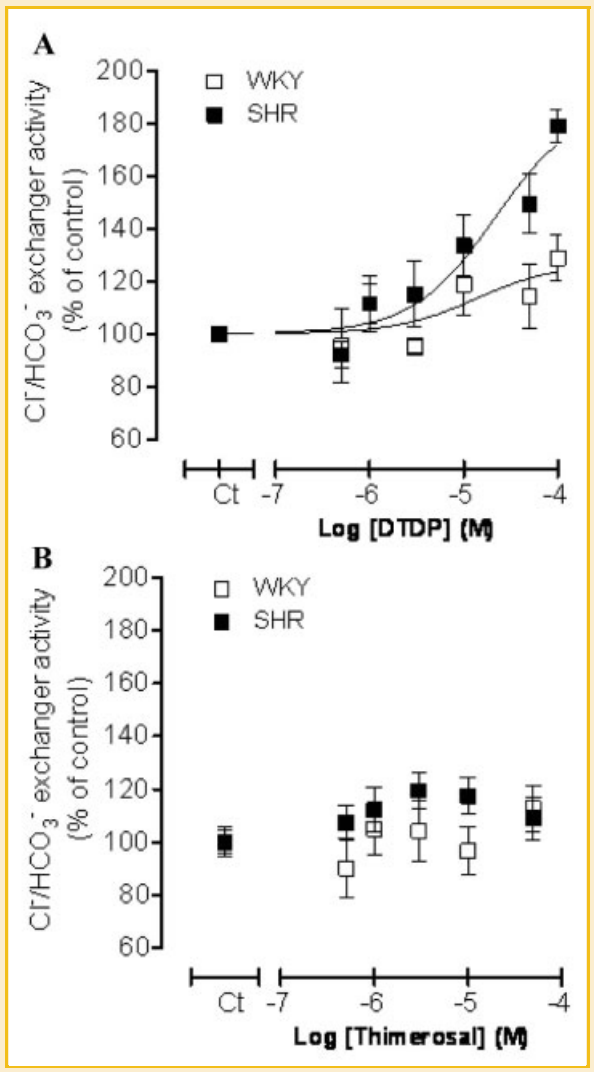

Fig. 2. A: Concentration-dependent effect of DTDP and (B) thimerosal (40 min exposure) on Cl^-/HCO_3^- exchanger activity in the presence of 25 mM HCO_3^- in WKY and SHR PTE cells. Symbols represent the mean of 4–8 experiments per group; vertical lines indicate SEM.

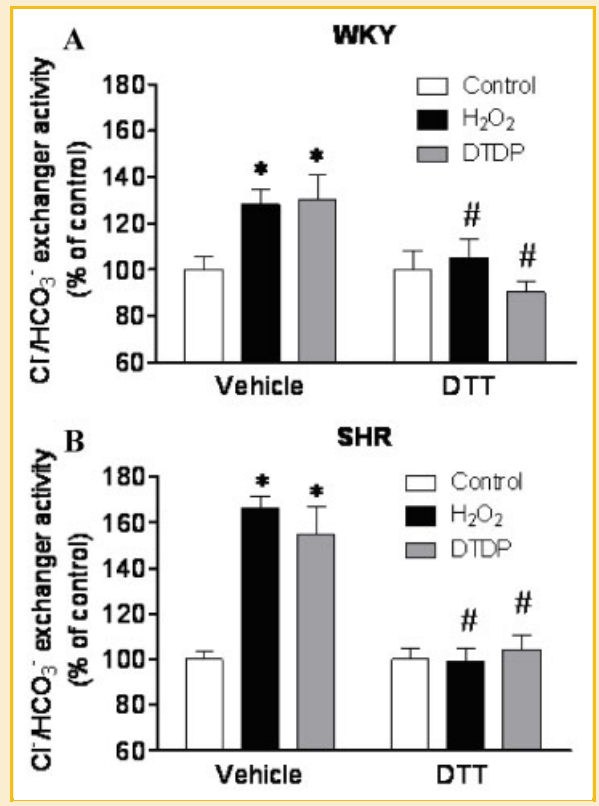

Fig. 3. A: Effect of H_2O_2 (10 μM) and DTDP (100 μM) on Cl^-/HCO_3^- exchanger activity in the presence of 25 mM HCO_3^- in WKY and in (B) SHR PTE cells, in the absence and presence of DTT (100 μM; 15 min pre-incubation). Each column represents the mean of 5–8 experiments per group; vertical lines indicate SEM. Significantly different from corresponding control values ($^*P < 0.05$) and values for H_2O_2 and DTDP alone ($\#P < 0.05$).

With the purpose of better understanding the mechanisms underlying the stimulation of Cl^-/HCO_3^- exchanger activity induced by H_2O_2 in both WKY and SHR PTE cells, the kinetic parameters of the exchanger in the presence of H_2O_2 were evaluated. H_2O_2 treatment was found to increase the affinity of the exchanger for HCO_3^- in SHR PTE cells, but had no effect on the kinetic parameters of WKY cells. This result suggests that in the presence of H_2O_2 the Cl^-/HCO_3^- exchange is facilitated in SHR PTE cells, possibly due to an alteration in the conformation of the protein. In order to explore this hypothesis we tested the effect of specific thiol oxidizing reagents upon the activity of the Cl^-/HCO_3^- exchanger. Cysteine residues of proteins are especially susceptible to ROS attack and, given the important role that disulfides play in protein structure and stability, alterations of reactive cysteine thiol groups may change protein function and modulate enzyme activity. In the present study the effects of H_2O_2 were mimicked by DTDP, i.e., stimulated in a concentration-dependent manner the Cl^-/HCO_3^- exchanger activity in WKY and SHR PTE cells [Simao et al., 2008a; Simao et al., 2010]. In contrast, thimerosal had no effect on Cl^-/HCO_3^- exchanger activity in both cell lines. Interestingly, the effects of H_2O_2 and DTDP were equally prevented by the reducing reagent DTT. Altogether, these results suggest that one of the mechanisms by which H_2O_2 regulates Cl^-/HCO_3^- exchanger activity might be through the modification of cysteine residues of the exchanger. This induces a stimulation of the exchanger activity in both WKY and SHR PTE cells. DTDP, as well as H_2O_2, is a small molecule which rapidly diffuses across biological membranes,

TABLE II. Kinetic Parameters for Cl^-/HCO_3^- Exchanger Activity in Immortalized WKY and SHR PTE Cells in the Absence and Presence of DTDP (100 μM; 40 min)

	K_m (mM)		V_{max} (pH units/min)	
Treatment	WKY	SHR	WKY	SHR
Control	2.228 ± 0.552	4.634 ± 0.768	0.177 ± 0.011	0.441 ± 0.024*
DTDP	3.057 ± 0.567	2.729 ± 0.531**	0.219 ± 0.013**	0.431 ± 0.025*

Values are means ± SEM of 5–12 experiments per group.
*Significantly different from corresponding WKY values ($P < 0.05$).
**Significantly different from corresponding control values ($P < 0.05$).

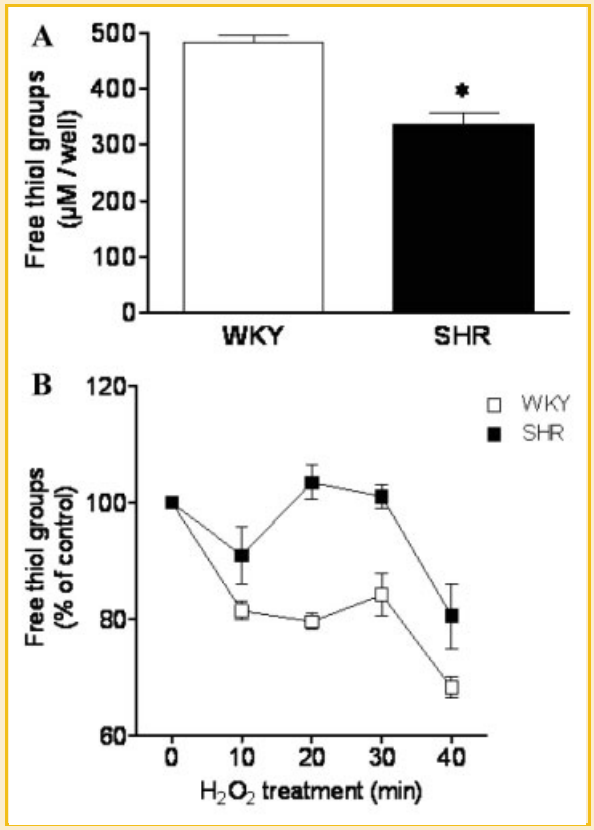

Fig. 4. A: Content of free thiol groups in WKY and SHR PTE cells in basal conditions and (B) after treatment with H_2O_2 (10 μM; 40 min). Columns represent the mean of three experiments per group; vertical lines indicate SEM. Significantly different from WKY values ($^*P < 0.05$).

whereas thimerosal has little membrane permeability. It is suggested that intracellular and/or transmembrane cysteine residues in the Cl^-/HCO_3^- exchanger may be the preferred targets of ROS attack, rather than the extracellular ones. In fact, thiol modifications occur selectively on cysteines that are accessible on the protein and that are more reactive.

Similar to that previously reported for H_2O_2 [Simao et al., 2008a; Simao et al., 2010], in the present study SHR PTE cells were also found to have an enhanced sensitivity to DTDP-induce stimulation of Cl^-/HCO_3^- exchanger activity than WKY cells. This suggests that Cl^-/HCO_3^- exchanger is more oxidized in SHR PTE cells than in WKY. At present, it is not known which protein encodes the Cl^-/HCO_3^- exchanger in WKY and SHR PTE cells. Assuming that the Cl^-/HCO_3^- exchanger is encoded by the same protein in both cell lines, they will be identical in terms of aminoacids composition, including cysteine residues content. As such, one plausible explanation for the differences in sensitivity to H_2O_2-induced Cl^-/HCO_3^- exchanger oxidation lies on the possibility that the exchanger may adopt different conformations in WKY and SHR PTE cells. The conformation adopted by Cl^-/HCO_3^- exchanger in SHR PTE cells, after H_2O_2 exposure, increases the exchanger activity. It is known that reversible adducts confer different charge, structure, and activity on the modified protein. On the other hand, irreversible oxidation is normally related to protein degradation and re-synthesis [Ying et al., 2007]. In the current study we also found that DTDP treatment increased the affinity of the transporter for HCO_3^- in SHR PTE cells. This data reinforces our view that an "oxidized conformation" of the exchanger, involving the modification of thiol groups, would favor the Cl^-/HCO_3^- exchange in SHR PTE cells by increasing the affinity for the substrate. Altogether these data show that the effects of H_2O_2 in SHR PTE cells are mimicked by DTDP. As a result, it is suggested that H_2O_2-induced stimulation of Cl^-/HCO_3^- exchanger occur though modification of thiol groups from the exchanger that increases its affinity for HCO_3^- in SHR PTE cells. In WKY PTE cells we found that thiol groups of Cl^-/HCO_3^- exchanger were also modified; however, we can not determine the exact mechanism by which H_2O_2 enhances the stimulation of Cl^-/HCO_3^- exchanger in these cell lines. The increases in V_{max} values induced by DTDP in WKY PTE cells are probably causal effects of this oxidant and were not reproduced by H_2O_2 and thus do not account to explain the H_2O_2-induced stimulation of Cl^-/HCO_3^- exchanger activity in WKY cells.

In the present study, a lower content of free thiol groups was detected, i.e., cysteine residues in the reduced form, in SHR than in WKY PTE cells. This correlates well with previous data that SHR PTE cells have increased levels of H_2O_2 production due to an imbalance between oxidant and antioxidant enzymes [Simao et al., 2008a; Simao et al., 2008b; Simao et al., 2010]. As a result, the thiol groups of proteins in SHR cells would be mostly in the oxidized form (and less in the "free" form). Since the thiol groups in SHR cells are less in the "free" form they will not be so available to react with exogenous H_2O_2. Therefore and as expected, H_2O_2 treatment reduced the free thiol groups in both cell lines, but this reduction was less pronounced in SHR PTE cells. Since the thiol groups in WKY cells are more available than in SHR cells they will react more rapidly with exogenous H_2O_2 (10 min exposure). SHR PTE cells require a prolonged exposure (40 min) to exogenous H_2O_2 in order to decrease their free thiol groups, possible because the free thiol groups in these cells are less accessible or less exposed to the extracelular environment. In a study similar to that reported here, the effect of H_2O_2 on the calcium-permeable TRPC6 (transient receptor potential channel subtype 6) was evaluated. The authors concluded that 10 μM H_2O_2 activated TRPC6 channels through modification of thiol groups of intracellular proteins, through enhancement of the sensitivity of the channels to diacylglycerol and through promotion of TRPC6 protein trafficking to the cell membrane [Graham et al., 2010]. Furthermore, a recent study evaluated the effect of a reactive nitrogen species (RNS), peroxynitrite ($ONOO^-$), upon the activity of the sarco-endoplasmic reticulum calcium Ca^{2+} ATPase (SERCA) and concluded that $ONOO^-$ directly activated SERCA by S-glutathiolation. According to these authors the modification of SERCA is blocked by irreversible oxidation of the relevant cysteine thiols during atherosclerosis [Adachi et al., 2004]. Currently, it is well recognized that apart from ROS/RNS deleterious effects they also have a role as second messengers, particularly H_2O_2. The tenuous line separating the two different roles for ROS/RNS is related to concentration as well as time of exposure. Another aspect to consider is the type of ROS/RNS in question, since different ROS/RNS can induce different cellular responses. Here, we show for the first time a possible mechanism by

which low micromolar H_2O_2 concentrations induce stimulation of Cl^-/HCO_3^- exchanger activity in an experimental model of essential hypertension, and it is suggested that this exacerbated stimulation may contribute to the increased NaCl reabsorption in the SHR.

In conclusion, in SHR PTE cells H_2O_2 stimulates Cl^-/HCO_3^- exchanger activity via modification of thiol groups of intracellular and/or transmembrane proteins. Moreover, oxidation of thiol groups from Cl^-/HCO_3^- exchanger increases the affinity for HCO_3^- in SHR PTE cells.

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