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## Accepted Manuscript

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Increased responsiveness to JNK1/2 mediates the enhanced H<sub>2</sub>O<sub>2</sub>-induced  
stimulation of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger activity in immortalized renal proximal  
tubular epithelial cells from the SHR

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**Running title:** H<sub>2</sub>O<sub>2</sub> and Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger activity

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## Abstract

We have previously demonstrated that exogenous  $\text{H}_2\text{O}_2$  stimulates  $\text{Cl}^-/\text{HCO}_3^-$  exchanger activity in immortalized renal proximal tubular epithelial (PTE) cells from both the Wistar-Kyoto (WKY) rat and the spontaneously hypertensive rat (SHR), this effect being more pronounced in SHR cells. The aim of the present study was to examine the mechanism of  $\text{H}_2\text{O}_2$ -induced stimulation of  $\text{Cl}^-/\text{HCO}_3^-$  exchanger activity in WKY and SHR cells. It is now reported that the SHR PTE cells were endowed with an enhanced capacity to produce  $\text{H}_2\text{O}_2$ , comparatively with WKY cells and this was accompanied by a decreased expression of SOD2, SOD3, and catalase in SHR PTE cells. The stimulatory effect of  $\text{H}_2\text{O}_2$  on the exchanger activity was blocked by SP600125 (JNK inhibitor), but not by U0126 (MEK1/2 inhibitor) or SB203580 (p38 inhibitor) in both cell lines. Basal JNK1 and JNK2 protein expression was higher in SHR PTE cells than in WKY PTE cells.  $\text{H}_2\text{O}_2$  had no effect on p-JNK1/2 in WKY PTE cells over time. By contrast,  $\text{H}_2\text{O}_2$  treatment resulted in a rapid and sustained increase in JNK1/2 phosphorylation in SHR PTE cells, which was completely abolished by apocynin. Treatment of SHR PTE cells with apocynin significantly decreased the  $\text{H}_2\text{O}_2$ -induced stimulation of  $\text{Cl}^-/\text{HCO}_3^-$  exchanger activity. It is concluded that  $\text{H}_2\text{O}_2$ -induced stimulation of  $\text{Cl}^-/\text{HCO}_3^-$  exchanger activity is regulated by JNK1/2, particularly by JNK2, in SHR PTE cells. The imbalance between oxidant and antioxidant mechanisms in SHR PTE cells enhances the response of JNK1/2 to  $\text{H}_2\text{O}_2$ , which contributes to their increased sensitivity to  $\text{H}_2\text{O}_2$ .

**Keywords:**  $\text{Cl}^-/\text{HCO}_3^-$  exchanger; Hypertension; Oxidative stress;  $\text{H}_2\text{O}_2$ ; JNK

## Abbreviations

BCECF-AM – acetoxymethyl ester of 2', 7'-bis (carboxyethyl)-5(6)-carboxyfluorescein

BSA – bovine serum albumin

DTT – dithiothreitol

EDTA – ethylenediamine tetraacetic acid

ERK – extracellular signal-regulated kinase

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

JNK – c-Jun-NH<sub>2</sub>-terminal kinase

MAPK – mitogen-activated protein kinase

NADPH – nicotinamide adenine dinucleotide phosphate, reduced form

NHE – Na<sup>+</sup>/H<sup>+</sup> exchanger

Nox – NADPH oxidase

PBS – phosphate buffer saline

PKA – protein kinase A

PKC – protein kinase C

PT – proximal tubule

PTE – proximal tubular epithelial

ROS – reactive oxygen species

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SOD – superoxide dismutase

SHR – spontaneously hypertensive rat

WKY – Wistar Kyoto rat

## 1. Introduction

Essential hypertension is a complex and multifactorial pathology with a wide variety of underlying causes. One reason for the development and maintenance of this form of hypertension is the reduced capacity of the kidneys to excrete water and salts in appropriate relation to the intake [1]. In parallel with sodium, chloride is reabsorbed as the main accompanying anion and NaCl reabsorption regulation is critical in renal NaCl balance, which in turn controls extracellular volume and blood pressure [2]. About 50-70% of the filtered chloride is reabsorbed in the proximal tubule (PT) [3]. The main mechanism of NaCl reabsorption in the PT is via apical  $\text{Na}^+/\text{H}^+$  exchanger subtype 3 (NHE3) that works in parallel with the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger that is essential for intracellular pH and cell volume regulation [4]. Our group recently demonstrated the presence of an apical  $\text{Cl}^-/\text{HCO}_3^-$  exchanger, the slc26a6 protein, in immortalized renal proximal tubular epithelial (PTE) cells from the spontaneously hypertensive rat (SHR) and Wistar Kyoto (WKY) rat [5]. An increase activity of the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger in immortalized PTE cells from SHR when compared with WKY cells was also reported [5].

Oxidative stress is the result of either an increase in reactive oxygen species (ROS) production, a decrease in antioxidant defences, or both. ROS are naturally produced as a result of oxygen metabolism and include free radicals, like superoxide anion ( $\text{O}_2^{\cdot-}$ ) and hydroxyl radical ( $\cdot\text{OH}$ ), and nonradicals, namely  $\text{H}_2\text{O}_2$  [6]. The association between oxidative stress and hypertension has been verified in several models of hypertension (reviewed in [6]), such as the SHR, which is a well characterized and extensively used genetic model for studying essential hypertension. However, the exact mechanism by which ROS mediate increases in blood pressure remains unclear. Animal studies have

shown that increased ROS production in the kidney decreased sodium excretion [7, 8]. In fact, it was observed that ROS stimulated NaCl absorption in the thick ascending limb of the loop of Henle, by enhancing Na/K/2Cl co-transport and stimulating Na<sup>+</sup>/H<sup>+</sup> exchanger present in this nephron segment [9-11]. The effect of ROS in renal proximal tubule is not well established. Indeed, superoxide has been reported to decrease renal proximal tubule transport by inhibiting NHE3 activity in the adult SHR [12, 13]. However, in the young (5 weeks), basal proximal tubular reabsorption is greater in the SHR than in the WKY rat which was shown to be due to increased basal angiotensin II effect [14], and NHE3 activity is increased especially at 6 weeks of age: expression may or may not be increased [15-18]. These studies support our findings of increased Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> and NHE3 activity as the renal proximal tubule cells were obtained from 4-8 weeks old rats [19]. However, a direct comparison between the micropuncture studies and our studies using immortalized renal proximal tubule cells may not be possible because the cells were obtained from the S1 segment while the micropuncture data were most likely obtained from the S2 segment.

Until recently, the effects of ROS on Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger activity were not known. We previously showed that SHR PTE cells have an enhanced sensitivity to H<sub>2</sub>O<sub>2</sub>-induced stimulation of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger activity when compared with WKY PTE cells [20] while previous studies on the effect of ROS on proximal tubule transport involved superoxide [12, 13]. In an attempt to determine the mechanisms responsible for differences in H<sub>2</sub>O<sub>2</sub> sensitivity between renal PTE cells from hypertensive and normotensive rats, the present study examined the role of the major mitogen-activated protein kinase (MAPK) signalling pathways. MAPKs are serine/threonine-specific protein kinases that respond to extracellular stimuli, e.g., oxidative stress, and are known to regulate several cellular functions. MAPKs include the extracellular signal-

regulated kinases (ERK1/2), c-Jun-NH<sub>2</sub>-terminal kinases (JNK), and p38 MAPK [21]. The antioxidant capacity of both WKY and SHR PTE cells was also investigated. It is reported here that H<sub>2</sub>O<sub>2</sub>-induced stimulation of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger activity is regulated by JNK1/2 in SHR PTE cells. Additionally, the elevated intracellular H<sub>2</sub>O<sub>2</sub> levels caused by the imbalance between oxidant and antioxidant enzymes in SHR PTE cells may be the underlying cause for the JNK1/2 responsiveness in this cell line.

## 2. Material and methods

### 2.1 Cell culture

Immortalized renal PTE cells were obtained from primary cultures from S1 segments of proximal tubules of 4- to 8 week-old WKY and SHRs [19]. 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 [19]. Cells were maintained in a humidified atmosphere of 5% CO<sub>2</sub>-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-ethanesulfonic 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<sup>2</sup> growth areas (Costar, Badhoevedorp, The

Netherlands). For  $pH_i$  measurement experiments, cells were grown in 96 well plates (Costar) and for immunoblotting cells were grown in 6 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 hours 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  $\mu g$  of cell protein. In experiments with apocynin, cells were cultured until the day of experience in DMEM-F12 supplemented with 100  $\mu M$  apocynin.

## 2.2 $pH_i$ measurements

In intracellular pH ( $pH_i$ ) measurement experiments, WKY and SHR PTE cells were grown in 96 well plates.  $pH_i$  was measured as previously described [5]. At day 4 after seeding SHR and WKY PTE cells were incubated at 37 °C for 30 min with 10  $\mu M$  BCECF-AM. 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<sub>3</sub>, 5.4 KCl, 2.8 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub>, 0.3 NaH<sub>2</sub>PO<sub>4</sub>, 0.3 KH<sub>2</sub>PO<sub>4</sub>, 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 nm and 440 nm was converted to  $pH_i$  by comparison with values from an intracellular calibration curve using nigericin 10  $\mu M$  in a high-K<sup>+</sup> solution (in mM: 15 NaCl, 130 KCl, 0.3 KH<sub>2</sub>PO<sub>4</sub>, 0.3 NaH<sub>2</sub>PO<sub>4</sub>, 5 glucose, 1.2 MgSO<sub>4</sub>, 2.8 CaCl<sub>2</sub> and 10 HEPES) with pH ranging from 6.6 to 7.8 [22].

### 2.3 $\text{Cl}^-/\text{HCO}_3^-$ exchanger activity

Although there is no specific assay for  $\text{Cl}^-/\text{HCO}_3^-$  exchange-mediated activity, several findings strongly suggest that  $\text{pH}_i$  recovery after removal of  $\text{CO}_2/\text{HCO}_3^-$  in the absence of  $\text{Na}^+$  reflects the activity of the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger. Thus, the  $\text{Na}^+$ -independent  $\text{HCO}_3^-$  transport system activity was assayed as the initial rate of  $\text{pH}_i$  recovery after an alkaline load ( $\text{CO}_2/\text{HCO}_3^-$  removal), in the absence of  $\text{Na}^+$ , as previously described [5]. Briefly, cells were loaded in serum-free medium with 10  $\mu\text{M}$  BCECF-AM, the membrane-permeant acetoxymethyl ester derivative of 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein, for 30 min at 37°C in 5%  $\text{CO}_2$  - 95% air atmosphere. The cells were washed free of dye and loaded with Krebs-Hensleit solution (25 mM  $\text{NaHCO}_3$ ) for 25 min. Then, extracellular solution was replaced by a Krebs-Hensleit  $\text{NaHCO}_3$ -free solution for 10 min. In the  $\text{NaHCO}_3$ -free bathing solution,  $\text{NaHCO}_3$  was replaced by an equimolar concentration of choline. The test compounds were added to the extracellular fluid 40 min before starting the bicarbonate-dependent  $\text{pH}_i$  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.

### 2.4 Immunoblotting

WKY and SHR PTE cells cultured to 90% of confluence were washed twice with PBS and total cell protein extracted for phospho-JNK1/2, total-JNK1/2, SOD1, SOD2, SOD3, catalase and  $\beta$ -tubulin detection. For MAPK detection, cells were lysed by brief sonication in 2x SDS sample buffer (62.5 mM Tris-HCl, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue) supplemented with protease inhibitors (aprotinin and leupeptin 2  $\mu\text{g}/\text{ml}$  each). Extracts were heated at 95 °C for 5 minutes,

cooled on ice and loaded (10  $\mu$ l) onto a 10% SDS-PAGE gel. For detection of the remaining proteins, cells were lysed by brief sonication 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 PMSF, 1% NP-40 (IGEPAL), 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM NaF, aprotinin and leupeptin 1  $\mu\text{g mL}^{-1}$  each) and incubated on ice for 30 min. 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. Between 50 – 80  $\mu\text{g}$  of protein were mixed in 2x SDS sample buffer and boiled for 5 min. Proteins were electrotransferred onto nitrocellulose membranes and the transblot sheets were blocked with 5% of non-fat dry milk in Tris-HCl 25 mM pH 7.5, NaCl 150 mM, during 1h. For MAPK proteins, the transblot sheets were blocked with 5% of BSA (bovine serum albumin) in Tris-HCl 25 mM pH 7.5, NaCl 150 mM and 0.1% Tween 20. The membranes were incubated with the appropriate primary antibody overnight at 4°C (phospho-JNK1/2 mouse monoclonal antibody 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA; total-JNK1/2 rabbit polyclonal antibody 1:1000, Santa Cruz Biotechnology; SOD1 rabbit polyclonal antibody 1:1000, Santa Cruz Biotechnology; SOD2 goat polyclonal antibody 1:100, Santa Cruz Biotechnology; SOD3 goat polyclonal antibody 1:100, Santa Cruz Biotechnology, catalase rabbit polyclonal antibody 1:2000, Calbiochem, Nottingham, UK and  $\beta$ -tubulin polyclonal antibody 1:800, Santa Cruz Biotechnology). The immunoblots were subsequently washed and incubated with a fluorescent-labeled goat anti-rabbit (IRDye<sup>Tm</sup> 800, Rockland, Gilbertsville, PA), a goat anti-mouse (AlexaFluor 680, Molecular Probes, Paisley, UK) or a donkey anti-goat (IRDye<sup>Tm</sup> 800, Rockland) secondary antibody, for 60 min at room temperature and protected from light. The

membranes were washed and imaged by scanning at both 700 nm and 800 nm, with an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

## 2.5 Drugs

Anisomycin, apocynin,  $H_2O_2$ , SB203580, SP600125, U0126 were obtained from Sigma Chemical Company (St. Louis, MO). Acetoxymethyl ester of 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM) and nigericin were obtained from Molecular Probes.

## 2.6 Data analysis

Geometric means are given with 95% confidence limits and arithmetic means are given with S.E.M. 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.

## 3. Results

Exogenous  $H_2O_2$  (0.3 – 10  $\mu M$ ) was previously reported by our group to stimulate, in a concentration-dependent manner,  $Cl^-/HCO_3^-$  exchanger activity in both WKY and SHR PTE cells [20]. In the present series of experiments  $H_2O_2$  (40 min exposure; 10  $\mu M$ ) increased  $Cl^-/HCO_3^-$  exchanger activity by 120% and 170% in WKY and SHR PTE cells, respectively (**table 1**). As a first approach to examine mechanisms responsible for the differences in sensitivity to  $H_2O_2$ -induced stimulation of  $Cl^-/HCO_3^-$  exchanger activity between WKY and SHR PTE cells, we evaluated the antioxidant capacity of both cell lines. **Figure 1** shows the expression of the SOD family and catalase antioxidant enzymes. Although the expression of SOD1 was similar in SHR and WKY PTE cells, the expressions of SOD2 and SOD3 were decreased by 70% to

80% in SHR PTE cells (**figure 1**). Also, the expression of catalase was significantly lower (50% reduction) in SHR PTE cells than in WKY PTE cells (**figure 1**).

To further elucidate the mechanisms involved in H<sub>2</sub>O<sub>2</sub>-induced stimulation of the exchanger activity in both cell lines, we next tested the effects of MAPK inhibitors. As shown in **figure 2**, the stimulatory effect of H<sub>2</sub>O<sub>2</sub> (40 min exposure; 10  $\mu$ M) on the Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger activity in both WKY and SHR PTE cells was prevented by the JNK1/2 inhibitor (SP600125; 20  $\mu$ M), but not by the MEK1/2 inhibitor (U0126; 10  $\mu$ M) or the p38 inhibitor (SB203580; 10  $\mu$ M). To clarify the involvement of JNK in the regulation of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger activity, the effect of anisomycin (40 min exposure; 0.1  $\mu$ M), a potent JNK/SAPK and p38 activator, on Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger activity was tested in the presence of JNK1/2 inhibitor (SP600125; 20  $\mu$ M). As shown in **figure 3**, the stimulatory effect of anisomycin on the Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger activity was similar in WKY and SHR PTE cells and prevented by the JNK1/2 inhibitor.

Stimulation of p-JNK1/2 was also evaluated after treating WKY and SHR PTE cells with anisomycin and H<sub>2</sub>O<sub>2</sub>. Basal p-JNK1 and p-JNK2 protein expression was higher in SHR PTE cells than in WKY PTE cells, though differences attained statistical significance for JNK2 only (**figure 4**). Anisomycin-activated p-JNK1/2 was significantly (P<0.05) lower in WKY than in SHR PTE cells (**figure 5**). In both cell lines, anisomycin-activated p-JNK2 was significantly higher than activation of p-JNK1 (**figure 5**). Because H<sub>2</sub>O<sub>2</sub>-induced stimulation of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger activity in WKY and SHR PTE cells was only prevented by the JNK1/2 inhibitor, we next evaluated the activation of this MAPK following H<sub>2</sub>O<sub>2</sub> treatment (0, 5, 15, 30, 45 and 60 min exposure; 10  $\mu$ M) (**figure 6**). H<sub>2</sub>O<sub>2</sub> had no effect on JNK1/2 phosphorylation in WKY PTE cells (**figure 6**). By contrast, H<sub>2</sub>O<sub>2</sub> treatment in SHR PTE cells resulted in a rapid and sustained increase in JNK1/2 phosphorylation. Maximal effect on JNK1/2

phosphorylation in SHR PTE cells was obtained within 30 min of H<sub>2</sub>O<sub>2</sub> exposure (figure 6). Treatment of SHR PTE cells with apocynin, a NADPH oxidase inhibitor (100 μM, during the four days of culture) completely abolished the H<sub>2</sub>O<sub>2</sub>-induced JNK1/2 phosphorylation (figure 6). Treatment of SHR PTE cells with apocynin (100 μM, during the four days of culture) significantly decreased the H<sub>2</sub>O<sub>2</sub>-induced stimulation of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger activity in this cell line (figure 7).

#### 4. Discussion

The present study was designed to determine mechanisms responsible for differences between renal PTE cells from hypertensive and normotensive rats on their sensitivity to H<sub>2</sub>O<sub>2</sub>-induced stimulation of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger activity, namely the involvement of the major MAPK signalling pathways and the antioxidant capacity of these cells. It is reported here that the enhanced H<sub>2</sub>O<sub>2</sub>-induced stimulation of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger activity in SHR PTE cells is dependent on JNK1/2 activation. In addition, the elevated intracellular H<sub>2</sub>O<sub>2</sub> levels caused by an imbalance between oxidant and antioxidant enzymes in SHR PTE cells may be the underlying cause for the JNK1/2 responsiveness in this cell line.

It is well established that different levels of H<sub>2</sub>O<sub>2</sub> can induce different responses within a cell. We previously reported that exogenous H<sub>2</sub>O<sub>2</sub> stimulated, in a concentration-dependent manner, Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger activity in both WKY and SHR PTE cells, with SHR PTE cells more susceptible to this reactive oxygen specie [20] which is line with observations on the role of ROS in the regulation of ion transport in some cells [23, 24]. In fact, H<sub>2</sub>O<sub>2</sub> has been demonstrated to stimulate the activity of other transporters, namely NHE and Na/K/2Cl. NHE from Wistar [25] and Sprague-Dawley rat ventricular myocytes [26] were stimulated after exposure of cells to

micromolar  $\text{H}_2\text{O}_2$  concentrations. ROS also increased the activity of Na/K/2Cl and NHE from the thick ascending limb of the loop of Henle [9-11] although the effect on renal proximal tubule cells may vary depending on the age and possibly the location along the proximal tubule [12-14, 16-18]. Although ROS are mainly implicated in causing cell damage, recent studies suggest they also play a significant physiological role in numerous aspects of intracellular signal transduction and regulation, acting as second messengers [27-29], interacting with membrane-bound transport proteins [24].

The increased rate of  $\text{H}_2\text{O}_2$  production previously reported for immortalized SHR PTE cells [20] was accompanied by an increased expression of Nox2, as well as the associated protein p22<sup>phox</sup> [30]. The present study shows that expression of the main antioxidant enzymes were markedly decreased in SHR PTE cells than in WKY PTE cells. Taken together these results indicate that SHR PTE cells have an imbalance between oxidant and antioxidant mechanisms, favouring the oxidants, which may explain the increase in  $\text{H}_2\text{O}_2$  levels observed in SHR PTE cells. Similar results were previously obtained by other authors that reported increased oxidative stress in different tissues from the SHR, namely renal cortices [31, 32] and rostral ventrolateral medulla neurons [33].

Differences in the oxidative environment between the two cell lines may explain the enhanced sensitivity of SHR PTE cells to  $\text{H}_2\text{O}_2$ -induced stimulation of  $\text{Cl}^-/\text{HCO}_3^-$  exchanger activity. However, we also tested the role of the major MAPK, to better elucidate the mechanism involved in this stimulation. Interestingly, only the JNK1/2 inhibitor (SP600125) prevented the stimulatory effect of  $\text{H}_2\text{O}_2$  on the  $\text{Cl}^-/\text{HCO}_3^-$  exchanger activity in WKY and SHR PTE cells. The  $\text{Cl}^-/\text{HCO}_3^-$  exchanger was previously shown to be an effector protein for PKA, PKC and p38 MAPK in both WKY and SHR PTE cells [34]. In the present study, the involvement of JNK1/2 in the

regulation of  $\text{Cl}^-/\text{HCO}_3^-$  exchanger activity in both cell lines was confirmed when anisomycin (a JNK/SAPK and p38 activator) stimulated the exchanger activity and p-JNK1/2 and these effects were prevented by the JNK1/2 inhibitor SP600125. Additionally, treatment with  $\text{H}_2\text{O}_2$  induced JNK1/2 phosphorylation in SHR PTE cells, while in WKY PTE cells no effect was observed. It should be underscored that the abundance of JNK2, but not that of JNK1, in SHR PTE cells was higher than in WKY PTE cells. In addition, JNK2 phosphorylation by both anisomycin and  $\text{H}_2\text{O}_2$  was higher in SHR PTE cells than in WKY PTE cells. These, therefore, suggest that JNK2 is likely the main MAPK promoting the enhanced  $\text{H}_2\text{O}_2$  response in SHR PTE cells. Taken together, these results suggest that JNK1/2, particularly JNK2, is involved in the regulation of  $\text{Cl}^-/\text{HCO}_3^-$  by  $\text{H}_2\text{O}_2$  in SHR PTE cells. Recently, SP600125 has been found to be a non-specific kinase inhibitor [35]. According to these authors, in addition to JNK, at least more 13 protein kinases were inhibited with similar or greater potency by SP600125. Therefore, the effect of SP600125 upon  $\text{H}_2\text{O}_2$ -induced stimulation of  $\text{Cl}^-/\text{HCO}_3^-$  exchanger activity in WKY cells might be a non-specific effect of this compound. It is suggested that, in WKY cells, JNK does not regulate  $\text{Cl}^-/\text{HCO}_3^-$  exchanger activity after exposure to exogenous  $\text{H}_2\text{O}_2$ ; the exogenous  $\text{H}_2\text{O}_2$ -induced stimulation of  $\text{Cl}^-/\text{HCO}_3^-$  exchanger activity in WKY cells necessarily involves the activation of other mechanisms. This data is in agreement with the fact that treatment with exogenous  $\text{H}_2\text{O}_2$  had no effect on JNK phosphorylation in WKY cells. The involvement of other protein kinases in the regulation of exchangers by ROS has been previously reported.  $\text{H}_2\text{O}_2$ -induced stimulation of NHE in neonatal Wistar rat ventricular myocytes is regulated by ERK1/2 [25] and the  $\text{H}_2\text{O}_2$ -induced stimulation of NHE in adult Sprague-Dawley rat ventricular myocytes involves both ERK1/2 and PKC [26].

The results presented here suggest that intracellular  $H_2O_2$  plays a crucial role in promoting JNK1/2 responsiveness in SHR PTE cells, since apocynin prevented  $H_2O_2$ -induced JNK1/2 activation in this cell line. JNKs, also known as stress-activated protein kinases (SAPKs), respond to extracellular stimuli, namely oxidative stress and its activation by  $H_2O_2$  has been demonstrated in numerous cell lines [25, 36, 37]. Several cell types are known to produce small amounts of ROS following stimulation with hormones, growth factors and cytokines. For example, various studies showed that different antioxidants, including a NADPH oxidase inhibitor, were effective in preventing angiotensin II-induced JNK phosphorylation, suggesting the involvement of reactive oxygen species in MAPK activation [38, 39]. We previously showed that the increased intracellular  $H_2O_2$  in SHR PTE cells was attenuated when cells were cultured in medium supplemented with apocynin (a NADPH oxidase inhibitor) while in WKY PTE cells no effect was observed [20]. In the present study, a significant reduction in  $H_2O_2$ -induced stimulation of  $Cl^-/HCO_3^-$  exchanger activity in SHR PTE cells in the presence of apocynin was observed. This result suggests that intracellular  $H_2O_2$  might amplify the effect of exogenous  $H_2O_2$  on  $Cl^-/HCO_3^-$  exchanger activity in SHR PTE cells. Comparable effects were recently observed for other proteins when SHR PTE cells were grown in the presence of apocynin. Intracellular  $H_2O_2$  also amplified the response downstream of  $\alpha_1$ -adrenoceptor activation [20],  $\alpha_2$ -adrenoceptor activation [30], and angiotensin AT1 receptor activation [40] in SHR PTE cells. Increased production of  $H_2O_2$  in SHR PTE cells also potentiated the response of aldosterone-induced NHE 1 activation in this cell line [41].

Slc26 transporters are regulated by post-translational changes and macromolecular complex formation. At present, we can not determine whether regulation of  $Cl^-/HCO_3^-$

exchanger activity by JNK1/2 in SHR PTE cells is the result of a direct interaction between these two proteins or if JNK1/2 induces the activation of other molecules.

It is concluded that  $\text{H}_2\text{O}_2$ -induced stimulation of  $\text{Cl}^-/\text{HCO}_3^-$  exchanger activity is dependent on JNK1/2 activation, particularly JNK2, in SHR PTE cells. The elevated intracellular  $\text{H}_2\text{O}_2$  levels caused by the imbalance between oxidant and antioxidant mechanisms in SHR PTE cells may be the underlying cause for the JNK1/2 increased responsiveness in this cell line.

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## Figure Legends

**Figure 1.** Protein expression of SOD1, SOD2, SOD3 and catalase in WKY and SHR PTE cells. Representative immunoblots are depicted on top of the bar graph. Columns represent mean of 4 independent immunoblots; vertical lines indicate SEM. Values are normalized to the level of  $\beta$ -tubulin expression in each condition and expressed as % of WKY PTE cells. SOD1 ~ 20 kDa; SOD2 ~ 25 kDa; SOD3 ~ 30 kDa; catalase ~ 60 kDa;  $\beta$ -tubulin ~ 55 kDa.

**Figure 2.** Effect of  $\text{H}_2\text{O}_2$  (40 min exposure; 10  $\mu\text{M}$ ) on  $\text{Cl}^-/\text{HCO}_3^-$  exchanger activity in (A) WKY and (B) SHR PTE cells in the absence and presence of U0126 (10  $\mu\text{M}$ ), SB203580 (10  $\mu\text{M}$ ) and SP600125 (20  $\mu\text{M}$ ). The absolute control values for  $\text{Cl}^-/\text{HCO}_3^-$  exchanger activity in pH units/min were  $0.1660 \pm 0.01$  (WKY cells) and  $0.2322 \pm 0.01$  (SHR cells) and were significantly different from each other. 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  $\text{H}_2\text{O}_2$  alone (#  $P < 0.05$ ).

**Figure 3.** Effect of anisomycin (40 min exposure; 0.1  $\mu\text{M}$ ) on  $\text{Cl}^-/\text{HCO}_3^-$  exchanger activity in (A) WKY and (B) SHR PTE cells in the absence and presence of SP600125 (20  $\mu\text{M}$ ). The absolute control values for  $\text{Cl}^-/\text{HCO}_3^-$  exchanger activity in pH units/min were  $0.2121 \pm 0.01$  (WKY cells) and  $0.2499 \pm 0.02$  (SHR cells) and were significantly different from each other. Each column represents the mean of 3-8 experiments per group; vertical lines indicate SEM. Significantly different from corresponding control values (\*  $P < 0.05$ ) and values for anisomycin alone (#  $P < 0.05$ ).

**Figure 4.** Basal protein expression of p-JNK1 and p-JNK2 in WKY and SHR PTE cells. Values are normalized to the level of t-JNK1 and t-JNK2 expression in each condition and expressed as % of WKY PTE cells. The graph represents the mean of 3 experiments per group; vertical lines indicate SEM. Significantly different from corresponding values in WKY PTE cells (\* P<0.05).

**Figure 5.** Effect of anisomycin (40 min exposure; 0.1  $\mu$ M) on p-JNK1/2 expression in WKY and SHR PTE cells. Values are normalized to the level of t-JNK1/2 expression in each condition and expressed as % of control. Representative immunoblots are depicted on the top of the bar graph. Each graph represents the mean of 4 experiments per group; vertical lines indicate SEM. Significantly different from corresponding control values (\* P<0.05) and corresponding values in WKY PTE cells (# P<0.05). Ct – control, Aniso – anisomycin. p- and t-JNK1/2 ~ 54/46 kDa.

**Figure 6.** Effect of H<sub>2</sub>O<sub>2</sub> (10  $\mu$ M; 0, 5, 15, 30, 45, 60 min exposure) on p-JNK1/2 protein expression in WKY and SHR PTE cells, in the absence and presence of apocynin (100  $\mu$ M; during 4 days of culture). Each symbol represents the mean of 3 experiments per group; vertical lines indicate SEM. Values are normalized to the level of t-JNK1/2 expression in each condition and expressed as % of control. Significantly different from corresponding control values (\* P<0.05).

**Figure 7.** Effect of H<sub>2</sub>O<sub>2</sub> (10  $\mu$ M for 40 min) on Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger activity in SHR PTE cells in the absence and presence of apocynin (100  $\mu$ M; during 4 days after seeding). The absolute control values for Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger activity in pH units/min, in SHR cells were 0.2574 $\pm$ 0.01 (vehicle) and 0.2663 $\pm$ 0.01 (apocynin). Each column

represents the mean of 8-15 experiments per group; vertical lines indicate SEM.

Significantly different from corresponding control values (\*  $P < 0.05$ ) and values for

H<sub>2</sub>O<sub>2</sub> alone (#  $P < 0.05$ ).

**Table 1.**  $\text{Cl}^-/\text{HCO}_3^-$  exchanger activity in WKY and SHR PTE cells in the absence and in the presence of  $\text{H}_2\text{O}_2$  (40 min exposure; 10  $\mu\text{M}$ ).

Treatment	$\text{Cl}^-/\text{HCO}_3^-$ exchanger activity (% of control)	
	WKY	SHR
Control	$100.00 \pm 8.99$ (n=6)	$100.00 \pm 6.95$ (n=7)
$\text{H}_2\text{O}_2$	$120.52 \pm 3.88$ (n=12) *	$170.57 \pm 8.76$ (n=13) * #

The absolute control values for  $\text{Cl}^-/\text{HCO}_3^-$  exchanger activity in pH units/min were  $0.1725 \pm 0.02$  (WKY cells) and  $0.2432 \pm 0.01$  (SHR cells) and were significantly different from each other. Values are means  $\pm$  SEM of 4-13 experiments per group. Significantly different from control values (\*  $P < 0.05$ ) and from WKY values (#  $P < 0.05$ ).

**Fig. 1**

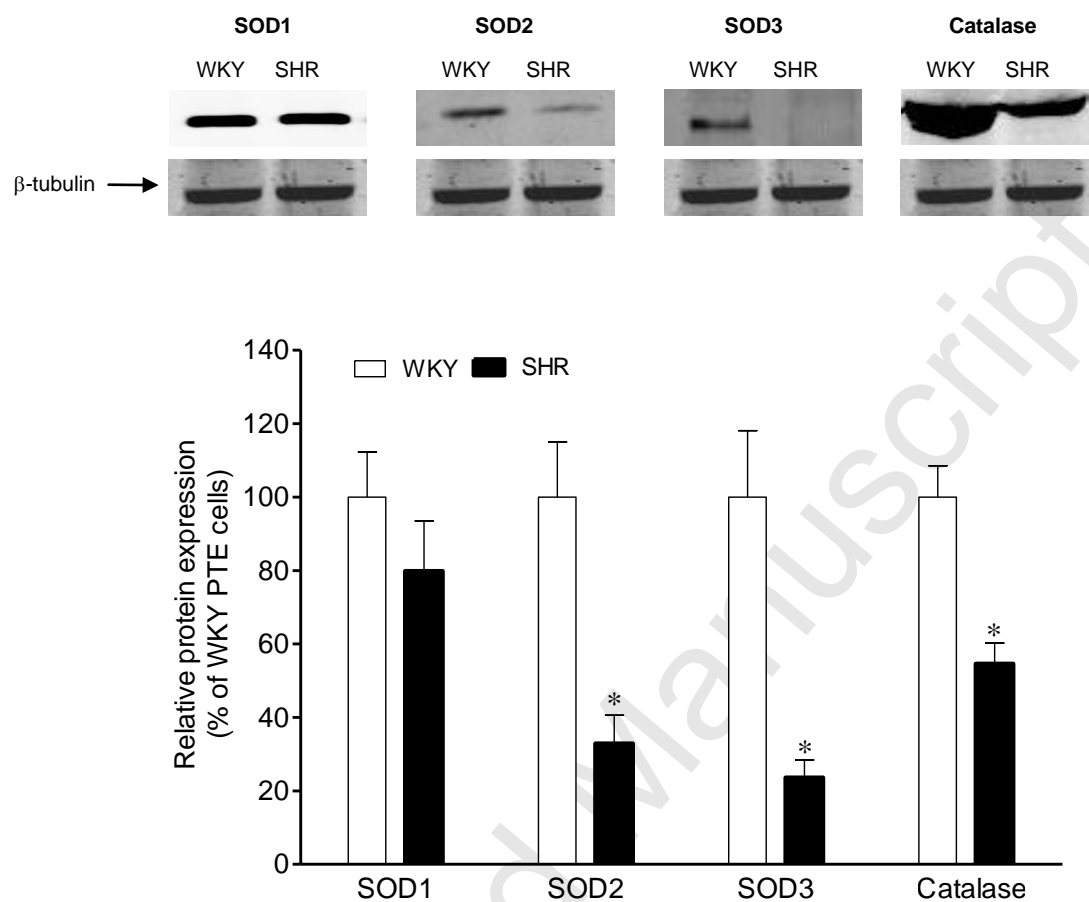


Fig. 2

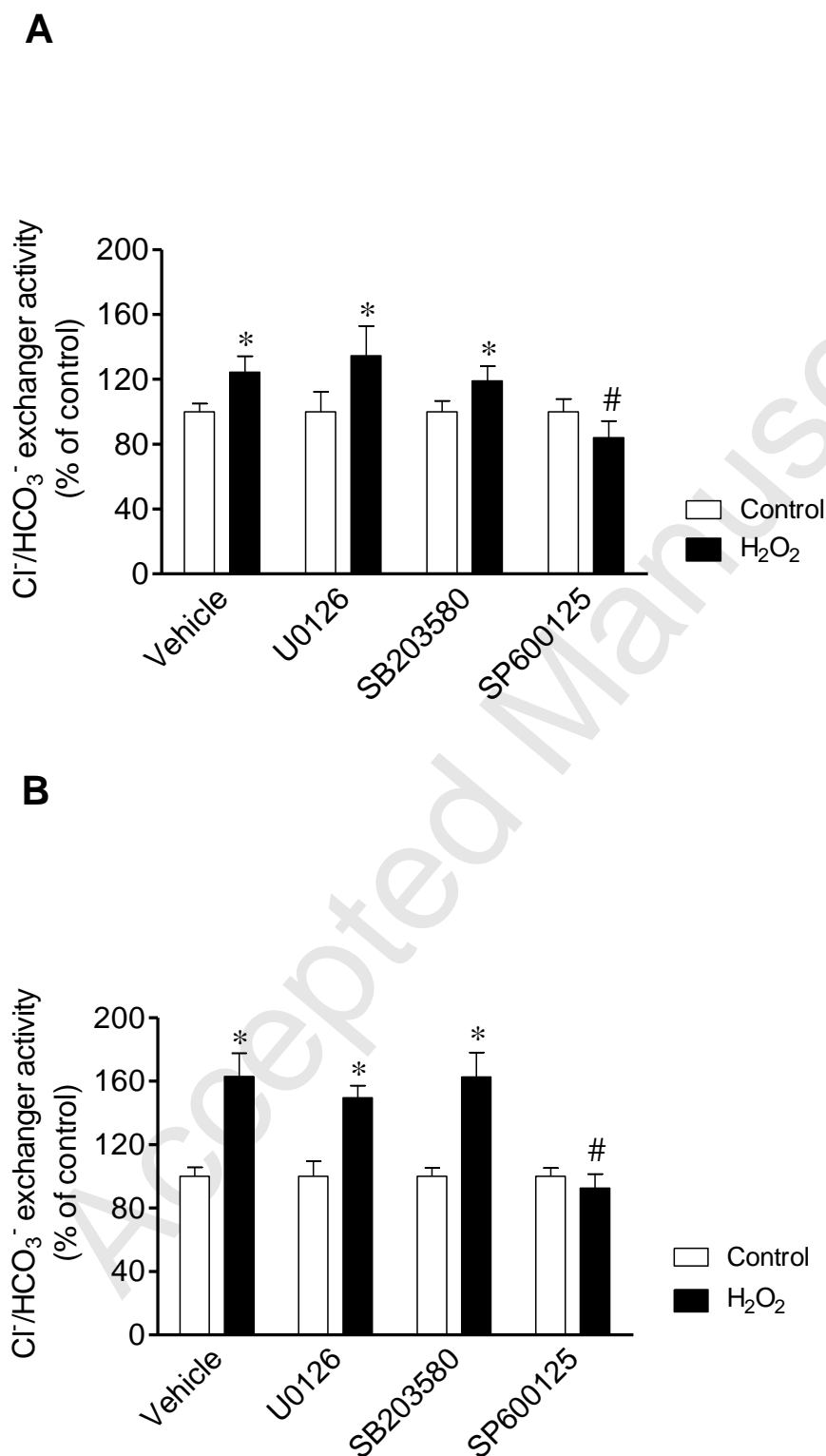
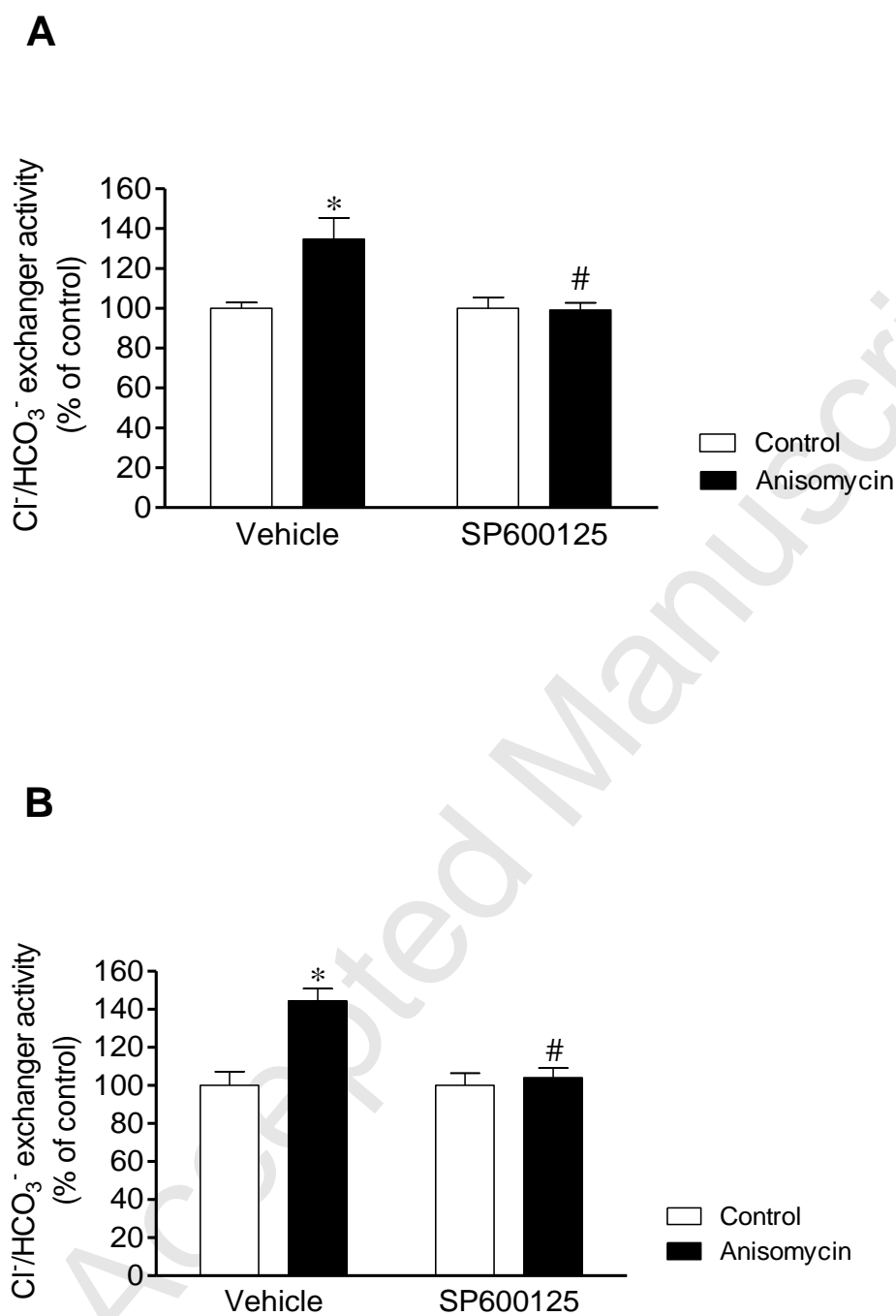


Fig. 3



**Fig. 4**

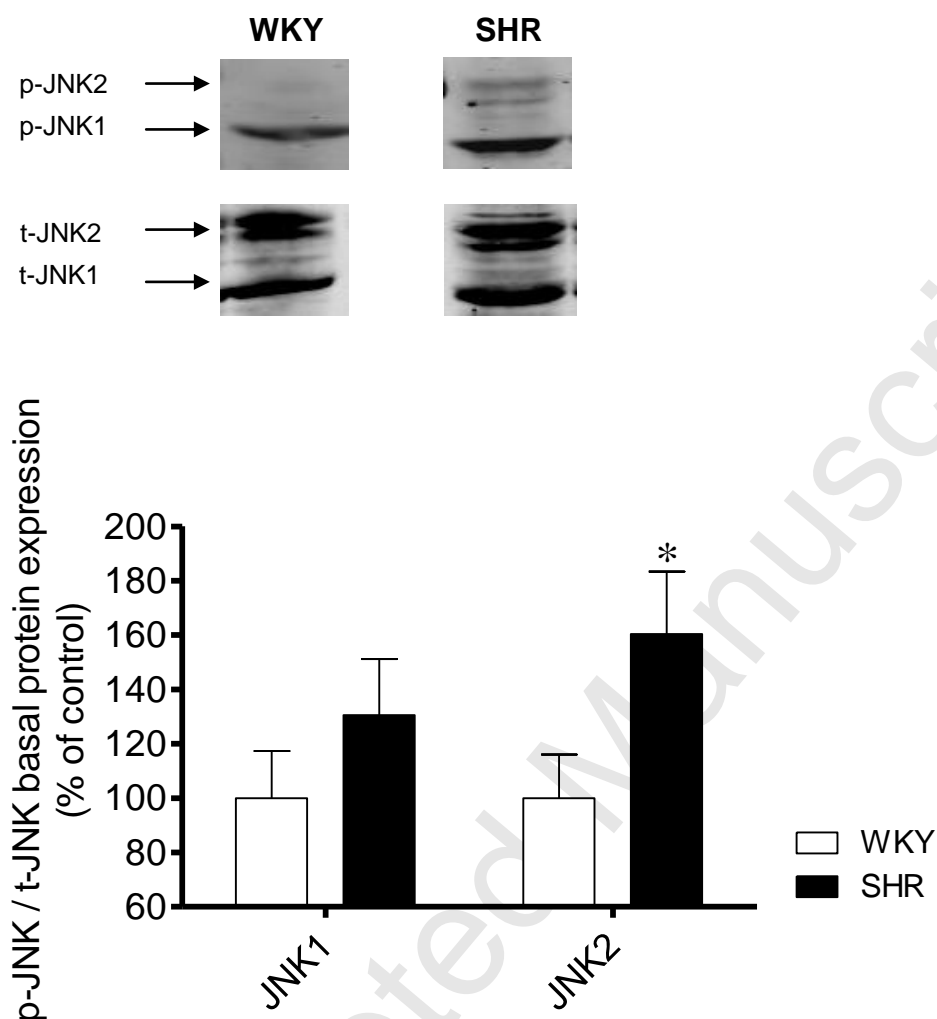


Fig. 5

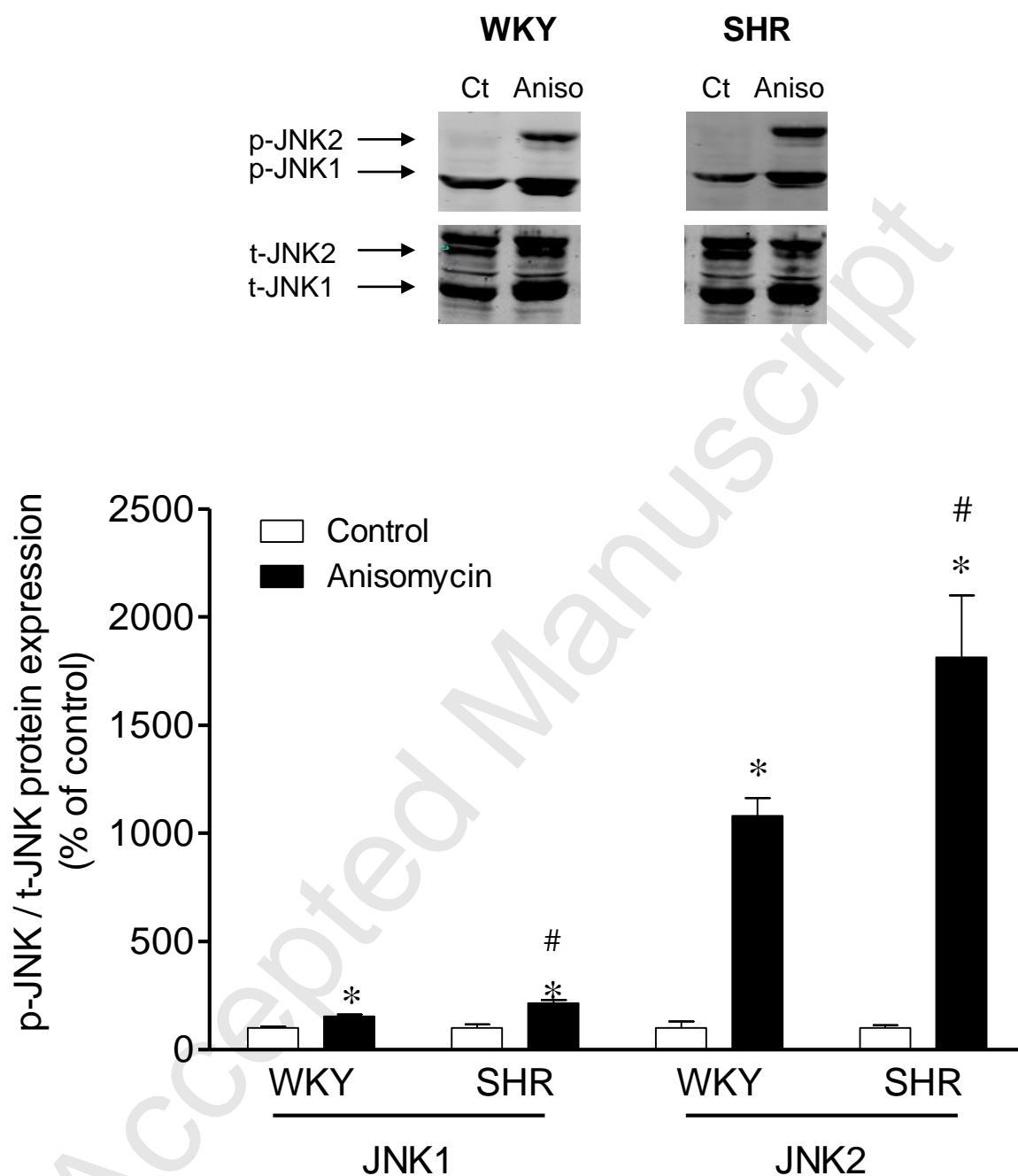


Fig. 6

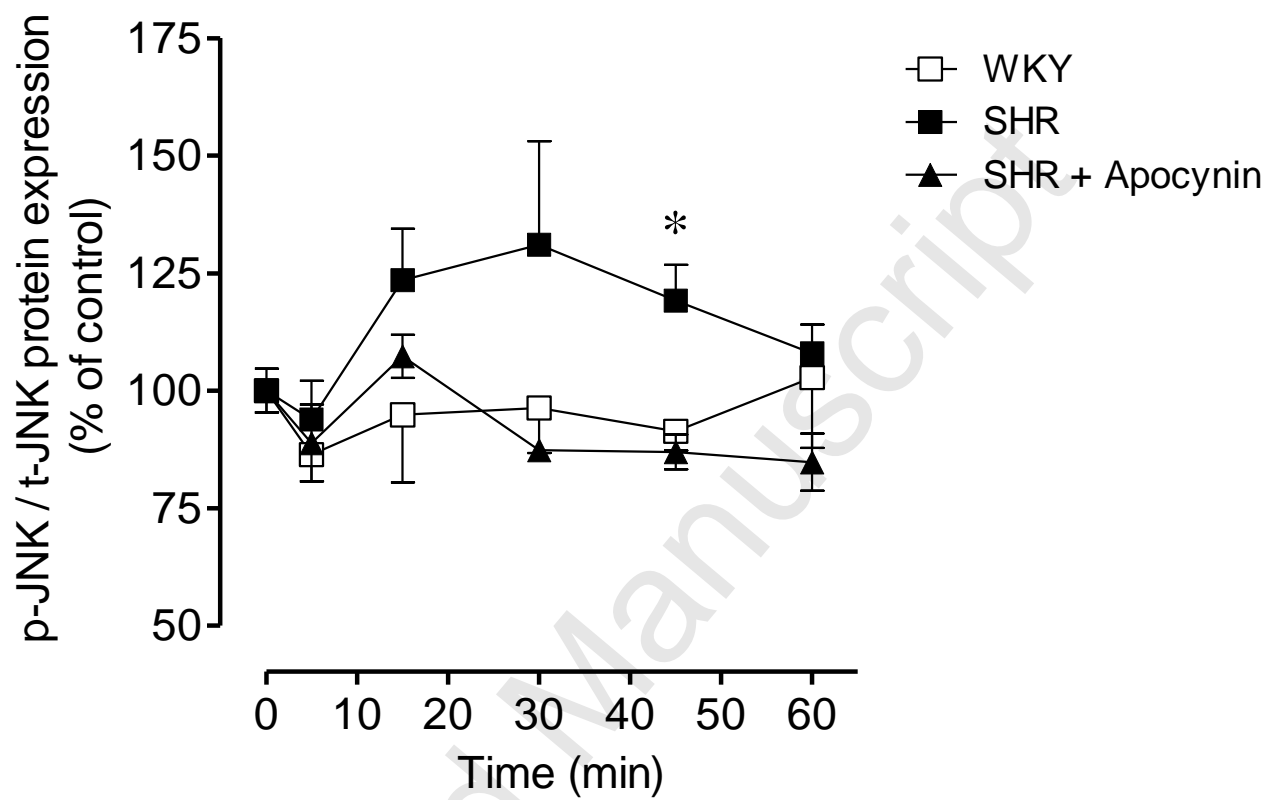
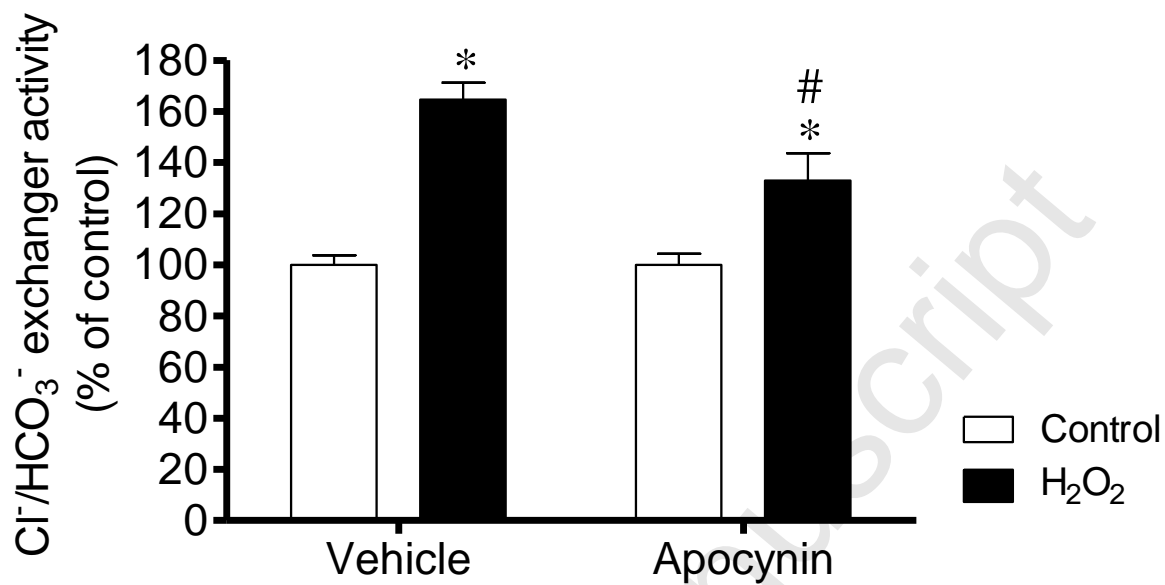


Fig. 7



$\text{H}_2\text{O}_2$ -induced stimulation of  $\text{Cl}^-/\text{HCO}_3^-$  exchanger activity is regulated by JNK1/2 in SHR cells. The imbalance between oxidant-antioxidant mechanisms in SHR cells enhances the response of JNK1/2 to  $\text{H}_2\text{O}_2$ , which contributes to their increased sensitivity.

