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Vascular oxidative stress upregulates angiotensin II type I receptors via mechanisms involving nuclear factor kappa B

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Abstract

The association of oxidative stress with hypertension is well known. However, a causal role of oxidative stress in hypertension is unclear. Vascular angiotensin II type 1 receptor (AT1R) upregulation is a prominent contributor to pathogenesis of hypertension. However, the mechanisms causing this upregulation are unknown. Oxidative stress is an important regulator of protein expression via activation of transcription factors such as nuclear factor kappa B (NFκB). The present study was carried out to test the hypothesis that oxidative stress contributes to vascular AT1R upregulation via NFκB in human aortic smooth muscle cells (HASMC) and spontaneously hypertensive rats (SHR). HASMC exposed to oxidative stress exhibited a robust increase in AT1R mRNA in HASMC. Furthermore, oxidative stress failed to upregulate AT1Rs in the presence of either an antioxidant catalase or siRNA against p65 subunit of NFκB. To test the role of oxidative stress and NFκB in hypertension, prehypertensive SHR were treated with NFκB inhibitor pyrrolidine dithiocarbamate from 5 weeks to 11–12 weeks of age. At 11–12 weeks of age, SHR exhibited increased NFκB expression, AT1R upregulation and exaggerated Ang II-induced vasoconstriction as compared to age-matched Wistar Kyoto (WKY) rats. PDTC treatment of SHR lowered NFκB expression, normalized AT1R expression and Ang II-induced vasoconstriction. More importantly, PDTC treatment significantly attenuated hypertension development in SHR. In conclusion, vascular oxidative can upregulate AT1R, via mechanisms involving NFκB, and contribute to the development of hypertension.

Keywords

AT1 receptors; NFκB; oxidative stress; PDTC; vasoconstriction

Introduction

Oxidative stress has been identified as a strong underlying factor in hypertension (1–3). There is a strict balance between reactive oxygen species (ROS) production and ROS

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Declaration of interest

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neutralization by antioxidant mechanisms in the vasculature. An imbalance in this system by increased ROS production and/or reduced antioxidant mechanisms results in oxidative stress (4). Oxidative stress has been reported in vascular tissue of several models of experimental hypertension such as spontaneously hypertensive rats (SHR), stroke prone SHR and Dahl salt sensitive rats (5–8). However, whether oxidative stress is a cause or a consequence of hypertension remains to be established. Also, the mechanisms by which oxidative stress could contribute to hypertension are not completely understood.

A hallmark of hypertension is increased vascular resistance resulting from enhanced vasoconstriction (9,10) and/or impaired endothelium-dependant vasodilation (11–13). Ang II is a potent vasoconstrictor and an important modulator of vascular tone. Ang II elicits most of its vascular effects via angiotensin II type 1 (AT1) receptors (AT1R) which are predominant in both the large conduit and small resistance vessels (14). Primary vascular effects of AngII contributing to hypertension are vasoconstriction, vascular remodeling and resultant increase in vascular tone, which contributes to increasing total peripheral resistance. AT1R expression and signaling is upregulated in human and experimental hypertension (15–17). However, the cause of AT1R upregulation and the mechanism involved therein are elusive.

Several reports suggest oxidative stress as being an important regulator of gene transcription via modulation of several redox sensitive transcription factors like nuclear factor kappa B (NFκB), activator protein-1 and Sp1 (18–20). Redox modulation of these transcription factors has been associated with pathophysiological conditions such as atherosclerosis, diabetes, hypertension and cancer (21). Of particular interest is NFκB that has been implicated in several diseases. We and others have previously shown that oxidative stress activates NFκB via nuclear translocation (18,20,22,23). Furthermore, the AT1R gene promoter has consensus binding site for NFκB (24). Thus, NFκB could be a putative candidate in mediating AT1R upregulation and could play a role in the development of hypertension.

Therefore, the present studies were designed to determine whether oxidative stress can upregulate AT1Rs in human aortic smooth muscle cells (HASMC). We also wanted to ascertain whether early oxidative stress via NFκB activation causes vascular AT1R upregulation and contributes to the development of hypertension in SHR.

Materials and methods

Human aortic smooth muscle cells

HASMCs (ATCC, Manassas, VA) were grown in manufacturer-recommended modified F12K medium (Gibco, Carlsbad, CA). For experiments involving combination of oxidants (L-buthionine sulfoximine (BSO) and hydrogen peroxide (H₂O₂)), serum starved cells were treated with BSO (400 μM) for 24 h. In the final 3 h of the BSO treatment, the media was supplemented with 50 μM H₂O₂. In experiments involving antioxidant treatment with catalase, the catalase (100 U/ml) was added 15 min prior to the oxidant treatment and maintained throughout the duration of the treatment. At the end of the treatment, the cells were used for the experiments described below.

Quantitative PCR for AT1R

HASMCs were treated with oxidants or oxidants+catalase as indicated in results. Total RNA was prepared from cells using the RNeasy mini kit (Qiagen, Valencia, CA) as per the manufacturer's recommendations. Total RNA (1 µg) was reverse transcribed to cDNA using Advantage RT for PCR kit (Clontech, Mountain View, CA). cDNA was used to perform qRT-PCR with specific primer probe assay mix directed towards human AT1R (HS00258937_m1, Applied Biosystems, Grand Island, NY) using 18s rRNA as internal control by RT-PCR machine (Applied Biosystems 7300). Briefly, 5 µL of cDNA was mixed with 25 µL of Taqman Gene expression master mix and 2.5 µL of AT1 assay mix or 18s rRNA assay mix, and the final volume made up to 50 µL with DEPC-treated water. The PCR reaction was performed on the Applied Biosystems 7300 Real-Time PCR System. The reaction was initiated by heating at 50 °C for 2 min and 95 °C for 10 min. The samples were amplified for 40 thermal cycles (95 °C for 15 s and 60 °C for 1 min). The relative fold expression to the control cells was calculated using the delta/ delta Ct method.

Detection of intracellular oxidative stress

Intracellular oxidative stress was assessed by measuring the levels of ROS using cell permeable fluorescent probe, dichlorofluorescein diacetate (CM-H2DCFDA) (Invitrogen, Carlsbad, CA). Treated cells were loaded with 5 µM of CMH2DCFDA in DMSO at 37 °C for 30 min. The excess dye was removed by washing the cells with PBS 2–3 times. The cells were incubated in PBS in dark for 15 additional minutes. The fluorescence intensity, as a result of CM-H2DCFDA oxidation by intracellular ROS, was recorded using a Synergy 2 microplate reader (Excitation: 492–495 nm/Emission: 517–527 nm). The cells were collected using a cell scraper and the protein concentration was calculated using bicinchoninic acid assay. The results were normalized using protein concentration.

siRNA transfection protocol

HASMC at 30–50% confluence were used for siRNA transfection. siRNA against p65 subunit of NFκB (cell signaling technology, Danvers, MA) was used to knock down p65 expression in HASMC. siRNA was diluted to achieve final concentration (200 nM) in the serum free media without growth factors and antibiotics/antimycotics. For each well, the siRNA solution was mixed with the transfecting reagent oligofectamine (4 µL) and the volume was adjusted to 200 µL. The siRNA–oligofectamine mixture was incubated for 20 mins at room temperature to allow complex formation and further diluted to 500 µL with serum free media. Cells were incubated with siRNA–oligofectamine mixture for 4 h. After 4 h, 1.5 ml of 1.5 ° complete growth media was added and cells were allowed to grow for 48 h, at the end of which treatment with oxidants was performed.

Animals

Studies were carried out in SHR and Wistar Kyoto (WKY) rats served as controls. These rats (Harlan, Indianapolis, IN) were fed standard rat chow diet, and had free access to water. For pyrrolidine dithiocarbamate (PDTC) treatment, 5-week-old SHR were treated with PDTC (Sigma Aldrich, St. Louis, MO) in drinking water at a dose of 105 mg/kg/day for a period of 6–7 weeks. Age-matched untreated SHR and WKY rats served as controls. All

experiments were performed in compliance with University of Houston guidelines and protocols for care and use of laboratory animals. The animal protocol is approved by the IACUC.

Blood pressure determination and sample preparation

Blood pressure was measured as previously reported, using aortic catheterization as detailed below (25). Briefly, rats were anesthetized with inactin (100 mg/kg ip) and tracheotomy was performed to facilitate breathing. To measure blood pressure, the descending aorta was catheterized with PE-50 tubing, connected to a Grass pressure transducer PT300, and blood pressure was recorded using a data acquisition system (PolyView, Grass Ins, Warwick, RI). After 30–45 min of stabilization period, the systolic and diastolic blood pressures were recorded. At the end of the blood pressure measurement, femoral artery and aorta were excised and flash-frozen using liquid nitrogen and stored at -80°C for further analysis.

Markers of oxidative stress

Protein carbonyls (addition of aldehydes and ketones) were determined using a protein oxidation detection assay kit (Millipore, Temecula, CA) according to the manufacturer's protocol. Protein carbonyl is a marker of oxidative stress (26) and has been used in our previous studies (27). Superoxide dismutase (SOD) activity was measured in the femoral artery homogenate using the SOD assay kit from Cayman Chemical (Ann Arbor, MI). H_2O_2 levels were determined in femoral artery homogenates (30 microgram protein) using amplex red H_2O_2 kit (Molecular Probes, Eugene, OR) as described by Rush et al. (28). Nitrotyrosine levels were measured in femoral homogenate by western blotting using anti-nitrotyrosine antibody (Millipore).

NF κ B p65 levels

Nuclear fractions from freshly collected aortic tissue were isolated as per the manufacturer's instructions using NE-PER nuclear and cytosolic extraction reagents (Thermo Scientific, Rockford, IL). Levels of NF κ B in the nuclear fractions of aortic tissue were determined by western blotting using specific NF κ B p65 antibody (catalog number-4764s, Cell Signaling Technology, Danvers, MA).

Immunoblotting for AT1R

Portions of isolated femoral artery samples were homogenized in lysis buffer containing 0.25 mol/L of sucrose, 50 mmol/L of dithiothreitol, 3 mmol/L of HEPES (pH 7.9), 0.5 mmol/L of EGTA, 0.4 mmol/L of PMSF, protease inhibitor mixture and 1% Triton X-100. Samples were centrifuged, and supernatants were solubilized in Laemmli buffer. Equal amount of protein was loaded and western blotting was performed using primary antibody directed against the AT1R (Sc-1173, Santa Cruz Biotechnology, Santa Cruz, CA). Alpha-actin was used as loading control (Sc-58669, Santa Cruz Biotechnology). The density (arbitrary units) of the bands was quantified by Kodak Imaging software (Rochester, NY).

Vascular function studies

The vascular function studies were performed in mesenteric artery rings. For mesenteric preparations, a midline abdominal incision was made, and the mesenteric artery was removed and immediately placed in ice-cold Krebs–Henseleit buffer (mM: NaCl 118.4, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25.0, and glucose 10.0 [pH 7.4]). The arteries were cleaned of adherent tissue and cut into rings under a dissecting microscope. Each ring was fixed under a resting tension of 0.6 g in a 10-mL organ bath filled with Krebs–Henseleit buffer (37 °C) and continuously aerated with a 95% O₂/5% CO₂ gas mixture, and the rings were allowed to equilibrate for 90 min before the start of the experiments. Isometric tension change in response to angiotensin II (10⁻⁷M) was measured with a digital force isometric transducer (Harvard Apparatus, Holliston, MA) connected to a data acquisition system (AD Instruments, Colorado Springs, CO).

Statistical analysis

Results are expressed as means± SEMs. All data were subjected to statistical analyses using GraphPad Prism 4 (San Diego, CA). Student's *t*-test or one way ANOVA followed by the *post-hoc* Newman–Keuls test was performed to determine the significance of differences between different groups. Statistical significance was set at *p*<0.05.

Results

Studies performed in HASMC

Effect of oxidative stress on AT1R messenger RNA levels—Treatment with L-buthionine sulfoximine, a glutathione synthesis inhibitor, at a concentration of 100 and 400 μM for 24 h caused a modest increase in AT1 messenger RNA (mRNA), however it did not achieve statistical significance (Figure 1). Further, H₂O₂ (50 μM for 3 h) failed to increase AT1R mRNA in HASMCs. However, a robust increase of more than two-fold was seen when an oxidative insult was provided by combining these two oxidants (BSO (400 μM, 24 h)+H₂O₂ (50 μM, 3 h)) (Figure 1). Hence, for all further experiments this combination of oxidants was used to expose the cells to oxidative insult.

Effect of catalase on oxidative stress and AT1R upregulation

The oxidant treatment markedly increased the oxidative stress in HASMCs as evidenced by significantly high CM-DCFDA fluorescence (Figure 2). Concomitant treatment with catalase (100 U/ml) prevented the increase in oxidative stress whereas catalase by itself had no effect (Figure 2). Additionally, concomitant treatment with antioxidant catalase prevented the oxidant-induced increase in AT1R mRNA. These results suggest an important role of oxidative stress in upregulating AT1Rs (Figure 3).

Role of NFκB in oxidative stress-mediated AT1R upregulation

The effect of oxidant treatment on AT1R in the presence or absence of p65 siRNA was determined using qRT-PCR (Figure 4). Oxidant treatment caused a robust increase in AT1R mRNA in the absence of p65 siRNA. However, in the cells where p65 expression was

knocked down using siRNA, oxidant treatment failed to increase the AT1R mRNA (Figure 4).

Studies performed after pyrrolidine dithiocarbamate treatment in 11–12-week-old animals

Effect of PDTC treatment on hypertension development and oxidative stress in SHR—SHR at 5 weeks of age were treated with pyrrolidine dithiocarbamate (PDTC) for

a period of 5–6 weeks. Untreated SHR displayed significantly higher mean arterial pressure as compared to WKY rats (94 ± 9 mmHg, WKY rats versus 177 ± 2.7 mmHg, SHR, $p < 0.05$, Figure 3A). The mean arterial pressure of PDTC-treated SHR (SHR-PDTC) was significantly lower compared to untreated SHR (177 ± 2.7 mmHg, SHR versus 146 ± 2.8 mmHg, SHR-PDTC, Figure 5A). Untreated SHR exhibited enhanced oxidative stress in comparison to WKY rats as evidenced by elevated levels of vascular H_2O_2 levels. PDTC treatment lowered the hydrogen peroxide levels in SHR (Figure 5B).

Effect of PDTC treatment on NF κ B expression in SHR

Untreated SHR had increased expression of p65 subunit of NF κ B in the vascular homogenate in comparison with WKY rats. PDTC treatment decreased the p65 overexpression in SHR as determined by western blotting (Figure 6).

Effect of PDTC treatment on AT1R expression and function in SHR

Untreated SHR showed increased vascular angiotensin II type 1 receptor (AT1R) density compared to WKY rats (Figure 7A). PDTC treatment normalized the AT1R expression in SHR-PDTC. The AT1R upregulation in SHR was also associated with enhanced angiotensin II-induced vasoconstriction compared to WKY rats which was decreased in PDTC-treated SHR (Figure 7B).

Discussion

Our results indicate that vascular oxidative stress contributes to upregulation of AT1R expression in HASMCs and in SHR. Furthermore, results from *in vivo* and cell culture studies also suggest an important role of NF κ B in mediating oxidative stress-induced AT1R upregulation.

HASMCs were exposed to oxidative insult by incubating cells with BSO and H_2O_2 , either alone or in combination. These two oxidants were chosen since they act via two different mechanisms of action. BSO is a pro-oxidant that acts by depleting glutathione, a major endogenous antioxidant, whereas H_2O_2 is a direct oxidant. Cells exposed to BSO at a concentration of 100 and 400 μ M for 24 h exhibited moderate increase in AT1R mRNA. Cells incubated with 50 μ M of H_2O_2 for 3 h showed no increase in AT1R message levels. This, we anticipate, may be due to the ability of the endogenous antioxidant system to neutralize H_2O_2 . However, when the cells were treated with a combination of BSO and H_2O_2 , we observed a robust increase in AT1R mRNA. Thus, our results suggest that an attenuation of endogenous antioxidant capacity combined with an exogenous oxidative insult is responsible for a robust increase in AT1R mRNA levels. This is interesting because in SHR animals we also observed attenuated antioxidant capacity combined with increased

oxidative stress. Furthermore, the oxidative stress and AT1R upregulation seen with oxidant treatment in HASMCs were abolished with concomitant treatment with antioxidant enzyme catalase (100 U/ml). These results support a pivotal role of oxidative stress in upregulation of AT1Rs. Interestingly, oxidative stress has also been shown to play a role in AngII-induced AT1R upregulation in neuronal cell line (29). To study the involvement of NFκB in mediating AT1R upregulation, we adopted a siRNA approach. Oxidant treatment was unable to upregulate AT1Rs in cells transfected with siRNA against p65 subunit of NFκB. These data indicate that NFκB mediates the oxidative stress-induced AT1R upregulation. However, whether oxidative stress directly upregulates AT1Rs by increasing its transcription or indirectly via an intermediate molecule remains to be established. As an example, Mitra and colleagues have recently shown that AT1R upregulation in neuronal cell line occurs by sequential activation of NFκB and Elk-1 (30). Further experiments are required to completely elucidate the mechanisms of AT1R upregulation and the involvement of NFκB therein.

Oxidative stress has been associated with experimental hypertension as well as human hypertension and antioxidant supplementation lowers blood pressure (1,31–34). Additionally, AT1R plays a major role in pathogenesis and maintenance of high blood pressure. Several studies have shown that AT1R expression and signaling is upregulated in human and experimental hypertension (15–17). In several models of hypertension, oxidative stress and AT1R upregulation co-exist. We hypothesized that oxidative stress may play a causal role in hypertension development by upregulating AT1R via NFκB. To test this hypothesis, we treated prehypertensive 5-week-old SHR with an antioxidant pyrrolidine dithiocarbamate (PDTC), which has recognized NFκB inhibitory activity (35). PDTC treatment significantly attenuated the development of hypertension in SHR as indicated by 31mm Hg lower mean arterial pressure in PDTC-treated SHR compared to untreated SHR. It is worth mentioning that Rodriguez-Iturbe and colleagues have also shown that chronic treatment of SHR from 7 to 25 weeks of age with PDTC attenuates the development of hypertension (36). We also found a significant lowering of oxidative stress in SHR with PDTC treatment as evidenced by lower vascular hydrogen peroxide levels. PDTC treatment also reduced the protein expression of p65 subunit that was elevated in SHR as compared to WKY rats. This is in concurrence with previous studies, which have used PDTC in this model of hypertension (35,36). We report here the first instance of chronic PDTC treatment lowering oxidative stress and attenuating the increase in NFκB abundance in the vasculature.

The AT1R expression was also upregulated in adult control SHR as compared to WKY rats. The increase in AT1R expression was functionally relevant as evidenced by increased vasoconstriction in response to Ang II of SHR mesenteric artery rings in comparison to WKY rats. PDTC treatment normalized the AT1R upregulation seen in vasculature of SHR. PDTC treatment also attenuated the exaggerated vasoconstriction seen in SHR. Interestingly, the AT1R gene promoter has been demonstrated to have a consensus binding site for NFκB (24). Furthermore, Cowling and colleagues have shown that AT1R upregulation in response to tumor necrosis factor α and Interleukin 1β in cardiac fibroblasts involves NFκB (37). These results, in combination, strongly suggest a role of oxidative stress in upregulation of AT1R via NFκB activation.

This study provides insight towards the importance of vascular oxidative stress in context of regulation of AT1R expression. However, some limitations exist. PDTC is a nonspecific inhibitor of NF κ B, which reduced total protein expression of p65 subunit in our studies. It is difficult to ascertain whether the antioxidant action of PDTC contributed to lower NF κ B protein expression or whether it was the direct NF κ B inhibitory action of PDTC. However, our cell culture studies support a direct role of NF κ B in upregulating AT1Rs. Secondly, the blood pressure in SHR after PDTC treatment, although significantly lower than untreated SHR, was still higher in comparison to WKY rats. This is not unexpected as it underlines the notion of hypertension being a multifactorial disorder with contributions from pathophysiological mediators such as increased sympathetic tone, endothelial dysfunction, renal sodium retention.

Conclusion

In conclusion, we report here that oxidative stress, in cell culture and in SHR, is associated with increased NF κ B activation and AT1R upregulation. NF κ B blockade by PDTC treatment during this early phase of hypertension development significantly attenuates rise in blood pressure possibly via lowering oxidative stress and subsequent inhibition of NF κ B activity. The normalization of AT1R upregulation and prevention of exaggerated AngII-induced vasoconstriction as a result of NF κ B blockade appear to be important mediators of PDTC's effect on hypertension.

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Figure 1.

AT1R mRNA levels in HASMC determined using qRT-PCR following treatment with oxidants BSO and H₂O₂ either alone or in combination as indicated. Bars represent mean $\pm$ SEM. Data ($n=3-5$) were analyzed using one-way ANOVA followed by the *post-hoc* Newman-Keuls test. ** $p<0.05$ versus control.

Figure 2.

Intracellular oxidative stress determined using CM-H₂DCFDA fluorescence in control HASMCs, HASMCs treated with oxidants (BSO + H₂O₂), catalase (100U/mL)+oxidants or catalase (100U/mL) alone. Bars represent mean±SEM. Data ($n=3$) were analyzed using one-way ANOVA followed by the *post-hoc* Newman–Keuls test. ** $p<0.05$ versus control.

Figure 3.

AT1R mRNA levels determined using qRT-PCR in control HASMCs, HASMCs treated with oxidants (BSO+H₂O₂) or oxidants+ catalase (100 U/ml) indicated. Bars represent mean $\pm$ SEM. Data ($n=3$) were analyzed using one-way ANOVA followed by the *posthoc* Newman–Keuls test. *** $p<0.05$ versus control.

Figure 4.

AT1R mRNA levels determined using qRT-PCR in control HASMCs and HASMCs treated with oxidants (BSO+H₂O₂) either in the presence or absence of siRNA against NFκB p65 subunit. Bars represent mean±SEM. Data ($n=3-5$) was analyzed using one-way ANOVA followed by the *post-hoc* Newman-Keuls test. * $p<0.05$ versus control.

Figure 5.

Blood pressure and oxidative stress after PDTC treatment in adult untreated WKY rats (WKY), untreated SHR (SHR) and PDTC-treated SHR (SHR-PDTC). (A) Mean arterial pressure and (B) vascular hydrogen peroxide levels. Bars represent mean±SEM. Data ($n=5-6$ rats) were analyzed using one-way ANOVA followed by the *post-hoc* Newman-Keuls test. * $p<0.05$ versus WKY. # $p<0.05$ versus SHR.

Figure 6.

(A) NFκB p65 subunit expression after PDTC treatment in adult untreated WKY rats (WKY), untreated SHR (SHR) and PDTC treated SHR (SHR-PDTC). Bars represent mean $\pm$ SEM. Data ($n=5-6$ rats) were analyzed using one-way ANOVA followed by the *post-hoc* Newman-Keuls test. * $p<0.05$ versus WKY. # $p<0.05$ versus SHR. (B) Representative blot showing p65 subunit expression in SHR (Lane 1), SHR-PDTC (Lane 2) and WKY (Lane 3).

Figure 7.

AT1R expression and function after PDTC treatment in adult untreated WKY rats (WKY), untreated SHR (SHR) and PDTC-treated SHR (SHR-PDTC). (A) AT1R expression in vascular homogenate. (B) Angiotensin II-induced contractions in isolated mesenteric artery rings. Bars represent mean±SEM. Data ($n=5-6$ rats) were analyzed using one-way ANOVA followed by the *post-hoc* Newman–Keuls test. * $p<0.05$ versus WKY. # $p<0.05$ versus SHR.