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A SUBPRESSOR DOSE OF ANGIOTENSIN II ELEVATES BLOOD PRESSURE IN A NORMOTENSIVE RAT MODEL BY OXIDATIVE STRESS

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Short Title: Angiotensin II induces oxidative stress in a normotensive rat

Summary

Oxidative stress is an imbalance between free radicals and antioxidants, and is an important etiological factor in the development of hypertension. Recent experimental evidence suggests that subpressor doses of angiotensin II elevates oxidative stress and blood pressure. We aimed to investigate the oxidative stress related mechanism by which a subpressor dose of angiotensin II induces hypertension in a normotensive rat model.

Normotensive male Wistar rats were infused with a subpressor dose of angiotensin II for 28 days. The control group was sham operated and infused with saline only.

Plasma angiotensin II and H₂O₂ levels, whole-blood glutathione peroxidase, and AT-1a, Cu/Zn SOD, and p22phox mRNA expression in the aorta was assessed.

Systolic and diastolic blood pressures were elevated in the experimental group.

There was no change in angiotensin II levels, but a significant increase in AT-1a mRNA expression was found in the experimental group. mRNA expression of p22phox was increased significantly and Cu/Zn SOD decreased significantly in the experimental group. There was no significant change to the H₂O₂ and GPx levels.

Angiotensin II manipulates the free radical-antioxidant balance in the vasculature by selectively increasing O₂⁻ production and decreasing SOD activity and causes an oxidative stress induced elevation in blood pressure in the Wistar rat.

Keywords: Angiotensin II; oxidative stress; subpressor; superoxide radical; hypertension

Introduction

Oxidative stress describes a state of potential damage caused by free radicals to biologically critical molecules, and has been linked with various disease states including hypertension (Sies 1997, Halliwell and Gutteridge 1999, Dalle-Donne *et al* 2006, Harrison *et al* 2007). The elevation of the reactive oxygen species (ROS) superoxide (O_2^-) and hydrogen peroxide (H_2O_2) disturbs the prooxidant-antioxidant balance and has been shown to be the major contributors to the pathophysiology of hypertension (Paravicini and Touyz 2008). The O_2^- radical has been shown to scavenge nitric oxide and reduce its bioavailability, thus reducing its vasodilatory role on the vasculature (McIntyre *et al* 1999, Bryan *et al* 2006). The relative increase of both O_2^- and H_2O_2 with resultant tissue oxidative stress has also been shown to decrease the natriuretic and diuretic properties of the kidney, thus reducing the organs ability to regulate blood pressure (Meng *et al* 2003). Both these species have also been shown to promote vascular smooth muscle cell (VSMC) growth, and contribute to the blood pressure amplifier consequence of vascular remodelling (Touyz 2003, Taniyama and Griendling 2003, Touyz 2004, Paravicini and Touyz 2008).

The octapeptide Angiotensin II (Ang II) plays an important physiological role in blood pressure regulation. Ang II acts to elevate blood pressure by its well established pressor effects, which broadly increase plasma volume and vascular resistance (Sealy and Laragh 1995). Compelling experimental evidence suggests that both the Ang II and the oxidative stress mechanisms are linked, where Ang II has been shown to stimulate the production of O_2^- in the vasculature of rats, via the activation of the membrane bound enzyme NADPH oxidase (Griendling *et al* 1994, Rajagopalan *et al* 1996, Fukui *et al* 1999, Zalba *et al* 2000). NADPH oxidase is a

multi-subunit enzyme that catalyses O_2^- production via the 1-electron reduction of O_2 using NADPH/NADH (Paravicini and Touyz 2008). The Ang II and oxidative stress relationship and its link to an elevated blood pressure in hypertension is outlined in the proposed scheme in Figure 1.

Previous studies have shown that infusion of subpressor doses of Ang II over different time frames in normotensive animal models induces hypertension and elevates markers of oxidative stress (Haas *et al* 1999, Reckelhoff *et al* 2000). Thus this study was undertaken to investigate the mechanism by which a chronic subpressor dose of Ang II induces hypertension in a normotensive rat model and determine the role of oxidative stress in the vascular compartment on the elevation of blood pressure.

Methods

Animal Protocol

Experimental animals were obtained from the University of Kwa-Zulu Natal Biomedical Resource Unit. The University of Kwa-Zulu Natal Animal Ethics Subcommittee approved all animal experiments for this study.

Drug Administration

Sixteen male Wistar rats (150g) were randomly divided into 2 groups (n=8) viz. the Control group (Wistar-Saline infused) and the Experimental group (Wistar-Ang II infused). The animals were anaesthetized using a combination of ketamine (80 mg/kg) and xylazine (10 mg/kg) and further subjected to an inhalant gaseous anaesthesia (0.5-2% halothane) to maintain anaesthesia. Using sterile techniques, an incision was made in the midscapular region, where a pocket was created with a haemostat and the osmotic mini-pumps (ALZET™ model 2004; Alza Corp) containing Ang II (Sigma A9525) dissolved in 0.9% saline (infusion rate 10ng/kg/min^{-1}) (Reckelhoff *et al* 2000) were implanted and the pocket subsequently sutured. These osmotic pumps are designed to deliver the predetermined dose for a period of 28 days. The control group was sham operated, and underwent the same surgical procedure, however their mini osmotic pumps contained 0.9% saline only.

Blood Pressure Measurements

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) was measured weekly using the non-invasive tail cuff method as previously described by Somova *et al* 1998 (II TC Model 31 NIBP). Animals were trained and acclimatized to the blood

pressure measuring equipment for a period of 1 week prior to the commencement of the experimentation.

Termination

After 28 days all groups were sacrificed by exsanguination. Briefly, animals were first sedated by placing them in a gas chamber containing 5% halothane. Whole blood was collected by cardiac puncture in EDTA coated test tubes. Plasma was processed, aliquoted and snap frozen (in liquid nitrogen) for analysis. Ascending aorta segments were also collected for mRNA analysis and also snap frozen. All collected tissue was stored at -70°C for further analyses.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from the ascending aorta segments using a modified Trizol (Invitrogen) protocol (Perou *et al* 1999). RNA pellets were dissolved in diethylpyrocarbonate-treated water and the concentration was determined spectrophotometrically at 260 nm. cDNA synthesis was performed on 4ng total RNA, using the Bio-Rad iScript cDNA synthesis kit according to the manufacturer's protocol (Bio-Rad, Hercules, CA,USA).

Real-Time Polymerase Chain Reaction (PCR) Protocol for AT1-a, p22phox,

Cu/Zn SOD and GAPDH

Each real-time PCR reaction was performed in a total volume of 10 µl LightCycler mastermix. Mastermix was made up of 4.8 µl water, 1.2 µl MgCl₂ (3 mmol/µl), 0.5 µl reverse primer, 0.5 µl of forward primer, 1 µl Fast Start SYBR Green I and 2 µl sample cDNA for AT1-a; 5.05 µl water, 1.2 µl MgCl₂ (3 mmol/µl), 0.5 µl reverse

primer, 0.25 μ l of forward primer, 1 μ l Fast Start SYBR Green I and 2 μ l sample cDNA for p22phox; 4.8 μ l water, 1.2 μ l $MgCl_2$ (3 mmol/ μ l), 0.5 μ l reverse primer, 0.5 μ l of forward primer, 1 μ l Fast Start SYBR Green I and 2 μ l sample cDNA for Cu/Zn SOD and 4.8 μ l water, 1.2 μ l $MgCl_2$ (3 mmol/ μ l), 0.5 μ l reverse primer, 0.5 μ l of forward primer, 1 μ l Fast Start SYBR Green I and 2 μ l sample cDNA for GAPDH. Respective primer sequences are listed in Table 1.

All reactions were run in a Roche Lightcycler Ver. 1.5 with 1 cycle of 95°C (10 minutes), followed by 45 cycles of 95°C (6 seconds), 65°C (6 seconds) and 72°C (6 seconds) for AT-1a; 1 cycle of 95°C (10 minutes), followed by 45 cycles of 95°C (6 seconds), 58°C (10 seconds) and 72°C (6 seconds) for p22phox; 1 cycle of 95°C (10 minutes), followed by 40 cycles of 95°C (6 seconds), 58°C (10 seconds) and 72°C (6 seconds) for Cu/Zn SOD and 1 cycle of 95°C (10 minutes), followed by 45 cycles of 95°C (6 seconds), 65°C (6 seconds) and 72°C (6 seconds) for GAPDH.

Each sample was run in duplicate. Relative mRNA copy numbers were calculated by generating standard curves using serial dilutions of a known concentration of cDNA. AT1-a, p22phox, Cu/Zn SOD and GAPDH mRNA levels were calculated as number of molecules per μ g of cDNA.

Gene expression data for each of the groups mRNA was normalized with GAPDH, by expressing data as a ratio of each of AT1-a, p22phox and Cu/Zn SOD respectively to GAPDH mRNA expression.

Standard Curve

A dilution series of p22phox, Cu/Zn SOD and AT1a and GAPDH amplicon were used as a standard template for reactions ranging from 1011 to 1020 copies per PCR

reaction. Quantitative analysis of the data was done employing LightCycler analysis software (Version 3.3).

Blood and Plasma Analysis

Plasma Angiotensin II

An aliquot of plasma underwent solid phase extraction according to the manufacturers instructions using a C-18 Sep Column (BACHEM). The eluted fraction was assayed using a competitive enzyme immunoassay kit obtained from BACHEM.

Plasma Hydrogen Peroxide

Plasma H₂O₂ levels was quantified colorimetrically using a commercially available kit from Assay Designs – Stressgen. The principle is based on the reaction of xylenol orange in an acidic solution with sorbitol and ammonium iron sulphate together with H₂O₂ to produce a purple colour that is proportional to the H₂O₂ concentration in the sample.

Blood Glutathione Peroxidase

Glutathione Peroxidase (GPx) was quantified in whole blood using a commercially available kit from Randox Chemicals (RANSEL). This method is based on that of Paglia and Valentine 1967.

Statistical Analysis

Statistical analysis was performed using Graphpad InStat (Version 5.0) and are represented as Mean ± SEM. All means of the control and the experimental groups

were compared with a Students t-test and a $p < 0.05$ was considered statistically significant.

Results

Blood Pressure

Both SBP and DBP showed slow increases over the successive weeks in the experimental group, whereas the control group showed no significant change in SBP and DBP. Both the SBP and DBP was significantly higher (20%) in the experimental group as compared to the control group at the end of the study at 28 days. Results are shown in table 2.

Plasma Angiotensin II

There was no significant change in the circulating plasma Ang II levels, results are shown in table 3.

Aorta AT-1a mRNA Expression

The experimental group had a significant 126% net increase in AT-1a mRNA expression as compared to the control group as shown in table 3.

Aorta p22phox mRNA Expression

The experimental group had a significant 154% net increase in p22phox mRNA expression as compared to the control group as shown in table 3.

Aorta Cu/Zn SOD mRNA Expression

The experimental group had a significant 81% net decrease in Cu/Zn SOD mRNA expression as compared to the control group as shown in table 3.

Glutathione Peroxidase

There was no significant change to the GPx levels in the blood as shown in table 3.

Hydrogen Peroxide

There was no significant change to the plasma H₂O₂ levels as shown in table 3.

Discussion

In this study we report that a subpressor dose of Ang II administered over a 28 day period in a normotensive rat model induces a significant elevation in blood pressure, from the start of the study to the end of the study at day 28. The elevation in blood pressure appears to be linked with the concomitant increase in oxidative stress in the vascular compartment that the Ang II infusion produces.

This study has shown that a subpressor dose of Ang II significantly increases p22phox mRNA expression in the vasculature. A previous study by Fukui *et al* 1997, showed that an increase in p22phox mRNA expression translates to an increase in NADPH oxidase activity. The increase in NADPH oxidase activity would result in a concomitant increase in O_2^- production as shown in a well-controlled study by Zalba *et al* 2000. We thus propose that the consequent increase in O_2^- is responsible for the following blood pressure elevating mechanisms. Firstly O_2^- is known to scavenge nitric oxide, thereby reducing its bioavailability, and thus decreasing its vasodilatory capacity and secondly O_2^- is known to be a direct vasoconstrictor (de Champlain *et al* 2004, Bryan *et al* 2006). Thus these two oxidative stress related mechanisms would act independently to increase total peripheral resistance and ultimately blood pressure as shown in the proposed scheme in Figure 1.

The decrease in Cu/Zn SOD levels during Ang II infusion would blunt the neutralization of the superoxide radical in the vasculature. This action coupled with the increased NADPH oxidase activity would result in oxidative stress due to O_2^- accumulation and its associated blood pressure elevating consequences as outlined in Figure 1.

The subpressor dose of Ang II resulted in no change to the circulating plasma Ang II levels, however it did increase the expression of the AT-1a receptor in the vasculature. It is suspected that the infusion of the subpressor dose of Ang II acts on renin release due to the elevation in blood pressure, in a classic negative feedback, to decrease circulating Ang II levels. This appears to be an attempt to thwart an increase in blood pressure by the pressor effects of Ang II. However the Ang II infusion appears to stimulate AT-1a and NADPH oxidase activity and thus potentiate only this specific modality of Ang II. The AT-1a receptor is widely distributed in various tissues including the vasculature, adrenal glands, kidneys, heart, lung, liver, testis, pituitary gland and brain and is known to mediate the blood pressure elevating properties of Ang II. In the vasculature it modulates vasoconstriction and the activity of NADPH oxidase (de Champlain *et al* 2004). Thus the subpressor dose appears to stimulate NADPH oxidase activity via the AT-1a receptor and this result is fortified by a previous study that has shown that the administration of Ang II receptor blockers decreased NADPH oxidase activity in the vasculature and thus decreased O_2^- production by the vasculature (de Champlain *et al* 2004). Our results show that the activity of NADPH oxidase is directly proportional to the activity of the AT-1a receptor where the infusion increased AT-1a by 126% and NADPH oxidase by 154%. This is of clinical significance as Ang II receptor blockers would thus also be able to attenuate the deleterious effects of oxidative stress due to O_2^- accumulation and its associated blood pressure elevating actions, as well as its blockage of the normal pressor effects of Ang II.

We speculate that the O_2^- radical appears to be the exclusively implicated free radical species with regards to the elevation in blood pressure, as there was no change in H_2O_2 levels. This therefore shows that the subpressor dose of Ang II does

not cause H₂O₂ accumulation. This is confirmed by the unchanged GPx levels which are usually elevated in response to elevated H₂O₂ levels (Jones 2002). The 'normal' H₂O₂ levels could also be due to the decreased SOD, which appears to result in a decreased dismutation of O₂⁻ to H₂O₂. This therefore eliminates H₂O₂ as the causative free radical species in the elevation of blood pressure. It should also be noted that the blood pressure elevating effects of H₂O₂ accumulation and dysregulation appear to be associated with progressive long term derangements viz. vascular remodelling and oxidative stress related tissue damage (Hyoudou *et al* 2006, Nishikawa *et al* 2009). Thus it appears that a short term elevation of blood pressure by the subpressor dose of Ang II is due exclusively to O₂⁻ accumulation in the vascular compartment.

Therefore our results indicate that the subpressor dose of Ang II induces oxidative stress in the vascular compartment due to O₂⁻ accumulation by specifically increasing O₂⁻ and suppressing SOD.

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Tables:

| mRNA | Oligonucleotides Sequence |
|----------------------------|----------------------------------|
| p22phox (forward) | 5'-GCTCATCTGTCTGCTGGAGTA-3' |
| p22phox (reverse) | 5'-ACGACCTCATCTGTCACTGGA-3' |
| Cu/Zn SOD (forward) | 5'-CGGATGAAGAGAGGCATGTTG-3' |
| Cu/Zn SOD (reverse) | 5'-TTGGCCACACCGTCCTTT-3' |
| AT1a (forward) | 5'-CGGATGAAGAGAGGCATGTTG-3' |
| AT1a (reverse) | 5'-TTGGCCACACCGTCCTTT-3' |
| GAPDH (forward) | 5'-TCCATGACAACCTTTGGCATC-3' |
| GAPDH (reverse) | 5'-CATGTCAGATCCACCACGGA-3' |

Table 1: Primer sequences used in real-time PCR assay. Information derived from Moritz *et al* 2003 for p22phox, Cu/Zn SOD and GAPDH, and Naito *et al* 2002 for AT1a.

| Group | Week 1 | Week 2 | Week 3 | Week 4 |
|--|---------------|---------------|---------------|---------------|
| Control (SBP) (mmHg) | 116 ± 2 | 126 ± 1 | 123 ± 1 | 121 ± 2 |
| Experiment (SBP) (mmHg) | 132 ± 3* | 134 ± 4* | 149 ± 7* | 152 ± 6* |
| Control (DBP) (mmHg) | 80 ± 1 | 82 ± 1 | 83 ± 1 | 79 ± 1 |
| Experiment (DBP) (mmHg) | 89 ± 2* | 89 ± 4* | 100 ± 4* | 99 ± 4* |

Table 2: Weekly SBP and DBP (* - Statistically significant to control – p < 0.05)

| Parameter | Control | Experiment |
|--|----------------|-------------------|
| Plasma Angiotensin II (pg/ml) | 1.13 ± 0.28 | 1.21 ± 0.25 |
| Glutathione Peroxidase (units/ml) | 87553 ± 3881 | 81922 ± 3163 |
| Hydrogen Peroxide (ng/ml) | 425.0 ± 58.6 | 499.0 ± 49.6 |
| AT-1a | 4.33 ± 2.89 | 9.77 ± 0.52* |
| p22phox | 5.75 ± 0.13 | 14.61 ± 1.25* |
| Cu/Zn SOD | 4.41 ± 1.49 | 0.83 ± 0.25* |

Table 3: Experimental parameters (* - Statistically significant to control – p < 0.05)

Figure 1 Legend:

Figure 1: Role of Angiotensin II derived oxidative stress in blood pressure elevation

Figure 1: