NADH/NADPH Oxidase and Enhanced Superoxide Production in the Mineralocorticoid Hypertensive Rat

Richard A. Beswick, Anne M. Dorrance, Romulo Leite, R. Clinton Webb

Abstract—We previously reported increased aortic reactive oxygen species (ROS) production in mineralocorticoid (deoxycorticosterone acetate [DOCA]-salt) hypertensive rats. In the present study, we tested the hypothesis that NADH/NADPH oxidase is responsible for increased ROS production, namely superoxide (O$_2^-$), in aorta of the DOCA-salt rat. Treatment of aortic rings from DOCA-salt rats with the NO synthase inhibitor N-nitro-L-arginine and the xanthine oxidase inhibitor allopurinol did not significantly change O$_2^-$ production. Furthermore, deendothelialization of aorta from DOCA-salt rats did not affect O$_2^-$ production compared with that of sham-operated rats. Thus, xanthine oxidase and uncoupled endothelial NO synthase were not responsible for increased O$_2^-$ production in the DOCA-salt rats. In contrast, treatment with the NADPH oxidase inhibitor apocynin significantly decreased O$_2^-$ production in aortic rings from DOCA-salt rats compared with sham-operated rats. Moreover, long-term administration of apocynin (in drinking water, 1.5 mmol/L, 28 days) to DOCA-salt rats significantly decreased systolic blood pressure compared with that of rats treated with DOCA-salt alone. Furthermore, O$_2^-$ production in aortic rings from DOCA-salt rats treated with apocynin for 28 days was reduced compared with that of untreated DOCA-salt rats. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis demonstrated that DOCA-salt rats have significantly greater mRNA levels of the NADPH oxidase subunit p22phox than do sham-operated rats. These findings suggest that NADPH oxidase is increased and is responsible for increased O$_2^-$ production and possibly contributes to increased blood pressure in the DOCA-salt hypertensive rat. (Hypertension. 2001;38:1107-1111.)

Key Words: deoxycorticosterone acetate • NADH/NADPH • mineralocorticoids • hypertension, mineralocorticoid

Hypertension, atherosclerosis, and mechanical injury exert common pathological effects on the vessel wall, such as vascular smooth muscle cell (VSMC) proliferation, monocyte/macrophage infiltration, endothelial dysfunction, and increased connective tissue deposition. In addition, these vascular diseases have been shown to be associated with oxidative stress, and there is good evidence implicating angiotensin II in the oxidative stress associated with hypertension. However, we and others have reported increased oxidative stress in mineralocorticoid (deoxycorticosterone acetate [DOCA]) hypertension, which is characterized by low angiotensin II levels. We also found that oxidative stress causes increased transcription of genes responsible for the early inflammatory response in the DOCA-salt hypertensive rat. Moreover, a potentially significant consequence of oxidative stress is vascular smooth muscle cell proliferation and NO inactivation, which could play an important role in the vascular changes and causes of hypertension. However, the source of the oxidative (reactive oxygen species [ROS]) stress is not known. Early studies have identified endothelial xanthine oxidase and uncoupled endothelial NO synthase (eNOS) as possible sources of endothelium-dependent O$_2^-$ production. It has since been established that smooth muscle cells and fibroblasts account for the majority of O$_2^-$ produced by the vessel wall. It also has been known for years that cardiac tissues are rich in ROS, such as O$_2^-$, H$_2$O$_2$, and NO, and that virtually every cell in the vascular wall produces and is regulated by ROS.

Recent studies have focused on NADPH oxidase, which is found within the membrane of smooth muscle cells, as a critical source of ROS production within the vascular wall. NADPH oxidase is one of the primary enzyme complexes involved in the antipathogenic actions of neutrophils and other phagocytic white cells; however, the vascular NADPH oxidase appears to be structurally and functionally different from the neutrophil NADPH oxidase. Currently, all components of the NADPH oxidase have been found in endothelial cells, whereas only the p22phox subunit has been identified in VSMCs, and several studies have examined the mechanism of activation of this enzyme in the pathophysiology of hypertension. From these studies, there have been 2 emerging common themes: (1) hypertension increases oxidative stress by increasing O$_2^-$ production, and (2) NADPH oxidase in vascular cells and myocytes may play an important role in this response.
We and others have shown that DOCA-salt rats have increased aortic $\text{O}_2^-$ production and that treatment with antioxidants normalizes $\text{O}_2^-$ production and attenuates hypertension. In the present study, we tested whether NADPH oxidase, xanthine oxidase, or uncoupled eNOS is responsible for the increased $\text{O}_2^-$ production observed in the DOCA-salt rat. We used apocynin, allopurinol, and N-nitro-L-arginine (L-NNA) to inhibit NADPH oxidase, xanthine oxidase, and eNOS, respectively. Apocynin, a methoxy-substituted catechol from the medicinal herb *Picroria kurroa*, has been used in primitive cultures for the treatment of inflammatory diseases. It has also been shown to impede the assembly of the membrane NADPH oxidase complex. Allopurinol is an analog of hypoxanthine and has been shown to be a potent inhibitor of xanthine oxidase in human and animal models. The third potential source of vascular ROS production is eNOS, which requires tetrahydrobiopterin to transfer electrons to L-arginine and has been shown to be a potent inhibitor of nitric oxide production in these ring segments. Details of this assay experimentation. The cDNA was resolved on an 8% polyacrylamide gel, and the amount of DNA present was identified by PhosphorImager analysis (Bio-Rad) and quantified by Multi-analyst software.

**Statistical Analysis**

Data are presented as mean±SEM. Statistically significant differences among groups were tested by ANOVA, Tukey’s multiple range test, or $t$ test as appropriate. A value of $P<0.05$ was considered statistically significant.

An expanded Methods section can be found in an online data supplement available at [http://www.hypertensionaha.org](http://www.hypertensionaha.org).

**Results**

Systolic blood pressure increased significantly in DOCA-salt rats compared with sham-operated rats after 28 days of treatment (196±4 versus 111±2 mm Hg, respectively; $n=5$ in all groups) (Figure 1A). In addition, $\text{O}_2^-$ counts were significantly higher in aortic segments from DOCA-salt rats compared with those from sham-operated rats (6591±353 in DOCA-salt rats versus 2513±158 counts $\cdot$ mg$^{-1}$ $\cdot$ min$^{-1}$ in sham-operated rats; $n=5$ in all groups) (Figure 1B).

**Role of NADPH Oxidase in $\text{O}_2^-$ Production**

$\text{O}_2^-$ counts in aortic rings from DOCA-salt rats averaged 6591±353 counts $\cdot$ mg$^{-1}$ $\cdot$ min$^{-1}$, and treatment for 1 hour

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**Methods**

Experiments were conducted in male Sprague-Dawley rats (Harlan, Indianapolis, Ind), and all procedures were approved by the institutional animal care and use committee. Rats were anesthetized with an intramuscular injection of ketamine (100 mg/kg)/xylazine (20 mg/kg) (The Butler Company), and a Silastic (Dow Corning) sheet containing DOCA-salt (Sigma; 200 mg/kg body weight) was inserted intramuscular injection of ketamine (100 mg/kg)/xylazine (20 mg/kg) (The Butler Company), and a Silastic (Dow Corning) sheet containing DOCA-salt (Sigma; 200 mg/kg body weight) was inserted into their water to block NADPH oxidase. Sham-operated rats underwent uninephrectomy without implantation of the Silastic/DOCA sheet and were given tap water. Twenty-eight days after implantation, systolic blood pressure was measured according to the tail-cuff method, and rats were anesthetized with an intramuscular injection of ketamine (100 mg/kg)/xylazine (20 mg/kg) (The Butler Company), and a Silastic (Dow Corning) sheet containing DOCA-salt (Sigma; 200 mg/kg body weight) was inserted into their water to block NADPH oxidase. Sham-operated rats underwent uninephrectomy without implantation of the Silastic/DOCA sheet and were given tap water. Twenty-eight days after implantation, systolic blood pressure was measured according to the tail-cuff method, and rats were anesthetized with ketamine/xylazine.

The aorta was carefully removed, cleaned of excess fat and adventitia, and placed in PSS composed of (in mmol/L) NaCl 130, KCl 4.7, KH$_2$PO$_4$ 1.18, MgSO$_4$ 7H$_2$O 1.17, NaHCO$_3$ 14.9, dextrose 5.5, EDTA 0.26, and CaCl$_2$ 1.6. Aorta was cut into 2- to 3-mm rings and treated for 1 hour with either 10$^{-6}$ mol/L L-NNA, 10$^{-6}$ mol/L apocynin, or 10$^{-6}$ mol/L apocynin. These concentrations were selected based on previously reported inhibitory activity. Some rings were also de-endothelialized and treated as previously stated. Luciferin chemiluminescence with 5$^{-15}$ was used to measure $\text{O}_2^-$ production in these ring segments. Details of this assay have been published previously.

**Reverse Transcriptase–Polymerase Chain Reaction**

RNA was extracted from aorta using a preparative kit (Qiagen). RNA (1 $\mu$g) was used for first-strand cDNA synthesis with AMV reverse transcriptase (RT) and oligo(dT) as primer. Polymerase chain reaction (PCR) amplifications were carried out on a portion of the cDNA produced using specific oligonucleotide primers for p22phox (5$'$-GGAGCAACACCTTTGGA-3$'$ and 3$'$-GAGCAACACCTTTGGA-5$'$) and cyclophilin. Reactions were performed using a PE Applied Biosystems GeneAmp thermal cycler and *Thermus aquaticus* DNA polymerase (Taq). The reaction contained 5 pmol/L concentration of each oligonucleotide primer, 200 pmol/L dNTP, 0.2 U Taq, 1.5 pmol/L MgCl$_2$, and 1 pmol $^{[35]}$P$dCTP$ in the manufacturer’s buffer. Optimum annealing temperature, cycle number, and template dilution factor were determined for each amplicon before experimentation. The cDNA was resolved on an 8% polyacrylamide gel, and the amount of DNA present was identified by PhosphorImager analysis (Bio-Rad) and quantified by Multi-analyst software.

**Figure 1.** A, Systolic blood pressure in DOCA-salt rats was significantly higher than that of sham-operated rats. B, $\text{O}_2^-$ production was significantly increased in DOCA-salt rats compared with sham-operated rats. Results are expressed as mean±SEM (n=5 rats per group). *$P<0.05$ vs sham.
with either allopurinol or L-NNA had no significant effect (6739 ± 343 versus 6350 ± 420 counts · mg⁻¹ · min⁻¹, respectively; n=5 in all groups, P<0.05). However, treatment with the NADPH oxidase inhibitor apocynin decreased O₂⁻ counts significantly (from 6591 ± 353 counts · mg⁻¹ · min⁻¹ in DOCA-salt rats to 4409 ± 347 counts · mg⁻¹ · min⁻¹; n=5 in all groups, P<0.05) (Figure 2). In agreement with the effect of apocynin to inhibit NADPH oxidase O₂⁻ production in vitro, long-term treatment of DOCA-salt rats with 1.5 mmol/L apocynin significantly decreased systolic blood pressure compared with that of rats treated with DOCA-salt alone (149 ± 4 versus 193 ± 4 mm Hg, respectively; n=5 in all groups, P<0.001). Treatment of DOCA-salt rats with apocynin for 28 days also decreased O₂⁻ production significantly in aortic rings compared with that of rats treated with DOCA-salt alone (5433 ± 472 versus 6591 ± 352 counts · mg⁻¹ · min⁻¹, respectively; n=5 in all groups, P<0.05) (Figure 4B).

**p22phox mRNA Expression**

RT-PCR analyses of aortic p22phox mRNA in sham-operated, DOCA-salt, and DOCA-salt plus apocynin–treated rats are shown in Figure 5. The aorta from the DOCA-salt rats had significantly greater mRNA for p22phox compared with that from the sham-operated animals (0.628 ± 0.085 versus 0.391 ± 0.035 arbitrary PhosphorImager units normalized to cyclophilin, respectively; n=5 in all groups, P<0.05), and treatment of the DOCA-salt rats with apocynin did not reduce the expression of p22phox mRNA (0.628 ± 0.085 versus 0.493 ± 0.095 arbitrary PhosphorImager units normalized to cyclophilin for DOCA-salt versus DOCA-salt plus apocynin; P<0.05) (Figure 5).

**Discussion**

These results provide the first evidence that NADPH oxidase is involved in aortic O₂⁻ production in the DOCA-salt rat. Furthermore, we found that inhibition of NADPH oxidase with apocynin attenuates systolic blood pressure, which may be due in part to increased NO bioavailability. DOCA-salt rats also exhibited an increase in mRNA levels of the NADPH oxidase subunit p22phox, and interestingly, treat-
may be responsible for enhancing NADPH oxidase activity. Recent studies in vitro raise the possibility that stretch of the vasculature could enhance $O_2^-$ and $H_2O_2$ production by NADPH oxidase during a relatively short period of time. Therefore, it is conceivable that direct stretch of vasculature may have these same effects.

Previously, workers at our laboratory and others found that DOCA-salt hypertension markedly increased $O_2^-$ production, which in turn diminished NO-dependent relaxation and hypertension. Furthermore, treatment of DOCA-salt rats with the antioxidants pyrrolinedithiocarbamate and Tempol decreased systolic blood pressure. The present study adds to these findings by showing that NADPH oxidase plays an important role in vascular $O_2^-$ production in the DOCA-salt rat. On the other hand, we did not find that inhibition of NOS with L-NNa significantly affected $O_2^-$ production in aortic rings from DOCA-salt rats, suggesting, therefore, that uncoupled eNOS plays little, if any, role in $O_2^-$ production in the DOCA-salt rat model. This is further supported by our observation that de-endothelialization of aortic rings from DOCA-salt rats did not affect $O_2^-$ production. The reason for these different findings is not known, but other studies have also reported that oxypurinol had no significant effect on $O_2^-$ production in mechanically stretched human cultured aortic endothelial cells. In the present study, we show that $O_2^-$ production in aortic segments from hypertensive and normotensive rats was not blocked by the xanthine oxidase inhibitor allopurinol. This does not support a major role for xanthine oxidase in $O_2^-$ production in aortic segments from hypertensive and normotensive rats, which was reported by others.

Figure 5. Quantification of aortic $p22phox$ mRNA by RT-PCR analysis. DOCA-salt rats had significantly increased amounts of $p22phox$ mRNA levels compared with those of sham-operated rats. Treatment of DOCA-salt rats with the NADPH oxidase inhibitor apocynin (APO) resulted in a decrease in $p22phox$ mRNA levels compared with DOCA-salt, but the difference was not statistically significant. Results are expressed as mean±SEM (n=5 rats per group). *P<0.05 vs sham.

NADPH oxidase is expressed in endothelial cells, adventitial fibroblasts, VSMCs, and infiltrating monocytes and macrophages. Although the exact location of NADPH oxidase was not localized in the present study, several previous studies strongly suggest NADPH oxidase is found mainly in VSMCs and is responsible for the formation of ROS within the vasculature. For example, Zalba et al. reported that removal of the adventitia from the aorta of spontaneously hypertensive rats (SHR) does not significantly change ROS production. Furthermore, the same authors showed that de-endothelialization of SHR aorta did not affect NADPH oxidase activity. In addition, it has been reported that hypertension causes aortic monocyte/macrophage infiltration, thus implicating a role for leukocyte infiltration in ROS production within the aorta. In contrast to this observation, we found through immunofluorescence analysis that aorta from DOCA-salt rats exhibited no monocyte/macrophage infiltration (R.A.B. and R.C.W., unpublished observations, 2000). Furthermore, de-endothelialization did not affect ROS production, suggesting that vascular smooth muscle cells or fibroblasts were the source.

It is not presently clear which factors are responsible for NADPH oxidase activity. However, it is interesting that SHR exhibit no significant difference in NADPH oxidase activity when young but develop a difference by adulthood, suggesting that long-term hypertension may be necessary for the activation of NADPH oxidase. Alternatively, nonhemodynamic factors, such as tissue hormones, stretch, or cytokines,
more, long-term inhibition of NADPH oxidase in DOCA-salt rats significantly decreased O$_2^-$ production and systolic blood pressure.

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