Glucocorticoids Inhibit Superoxide Anion Production and p22 Phox mRNA Expression in Human Aortic Smooth Muscle Cells

Takeshi Marumo, Valérie B. Schini-Kerth, Ralf P. Brandes, Rudi Busse

Abstract—Recent reports suggest that the increased production of reactive oxygen species (ROS) in the vascular wall may contribute to the functional and structural changes associated with hypertension and atherosclerosis. Although glucocorticoid therapy can promote atherosclerosis, protective effects of these compounds on vascular lesion formation have been reported. In the present study, we investigated whether ROS production in cultured human aortic smooth muscle cells (HSMCs) can be modulated by glucocorticoids. Pretreatment of HSMCs with dexamethasone for 24 hours attenuated the basal and platelet-derived growth factor (PDGF)-AB– and angiotensin II–induced superoxide anion (O$_2^-$) production. PDGF-AB–stimulated O$_2^-$ production was also inhibited by prednisolone and hydrocortisone but not by other steroids, such as testosterone and norgestrel. Incubation of HSMCs with glucocorticoids for 24 hours decreased 2',7'-dichlorodihydrofluorescein (DCHF) oxidation, an indicator of intracellular ROS levels. Dexamethasone decreased the mRNA expression of p22 phox, one of the components of NADPH oxidase, but had no effect on the activity of superoxide dismutase. The effects of dexamethasone on DCHF oxidation, and p22 phox mRNA expression and PDGF-AB–stimulated O$_2^-$ production were inhibited by the glucocorticoid receptor antagonist RU486. These results indicate that glucocorticoids decrease O$_2^-$ production by HSMCs via a receptor-dependent pathway. This effect is likely to be mediated by a decrease in the generating system, such as downregulation of p22 phox mRNA, rather than an increased inactivation of O$_2^-$.

Key Words: glucocorticoids ■ superoxide ■ free radicals ■ NADH ■ NADPH oxidase ■ muscle, smooth, vascular

Increased production of vascular reactive oxygen species (ROS) has been implicated in the development of hypertension and atherosclerosis. In particular, the excess production of superoxide anion (O$_2^-$) by vascular cells, including endothelial cells, has been reported to result in a decreased release of nitric oxide in stroke-prone spontaneously hypertensive rats. Increased vascular ROS production has also been observed in other animal models of hypertension and atherosclerosis. Endothelium and the smooth muscle layer are reportedly the predominant sources of vascular ROS production depending on the disease state. Vascular O$_2^-$ levels seem to have important functional consequences because impaired endothelium-dependent relaxation in angiotensin II (Ang II)–induced hypertension has been partly by elevated vascular superoxide dismutase (SOD) levels. In addition, recent reports have suggested that vascular smooth muscle cell (VSMC)–derived ROS may promote proatherogenic processes by affecting VSMC proliferation and chemotaxis, activation of nuclear factor-kB, oxidative modification of LDL, and the induction of the LDL scavenger receptor.

Although increasing evidence suggests an important role for ROS in cardiovascular diseases, the main O$_2^-$–generating system in VSMCs remains to be characterized. Recently, p22 phox, one of the components of NADPH oxidase in phagocytes, was shown to be expressed in VSMCs and involved in Ang II–induced ROS production. Moreover, the expression of p22 phox mRNA was upregulated in aortas from rats with Ang II–induced hypertension. However, little is known about the regulation of this oxidase component in VSMCs. Glucocorticoids are known to modify the course of vascular lesion formation, and long-term systemic glucocorticoid therapy induces risk factors for atherosclerosis, including hypertension, hypercholesterolemia, and impairment of glucose tolerance. However, protective effects of glucocorticoids on cholesterol-induced atherogenesis have also been reported. In particular, the local application of dexamethasone demonstrably inhibits neointimal proliferation in balloon-injured arteries. In the present study, we investigated whether ROS production and mRNA expression of p22 phox in human aortic smooth muscle cells (HSMCs) can be modulated by glucocorticoids.
Methods

Smooth Muscle Cell Culture
HSMCs, isolated from the thoracic aorta of a young healthy donor for cardiac transplantation using enzymatic disaggregation as described previously, were kindly provided by Dr T. Scott-Burden (Texas Heart Institute, Houston). Cells from passages 16 through 24 were deprived of serum for 24 hours by incubation with the medium containing 0.1% BSA after reaching confluence as described. These cells were then incubated with the same medium in the presence and absence of steroids as indicated. The steroids and RU486 were dissolved in DMSO (final concentration, 0.1% or 0.2%).

Human coronary artery smooth muscle cells obtained from Clonetics were maintained in smooth muscle cell basal medium (SmBM, Clonetics) with supplements (SingleQuots, Clonetics) containing 0.5 ng/mL epidermal growth factor, 5 μg/mL insulin, 2 ng/mL fibroblast growth factor, 50 μg/mL gentamicin, 50 ng/mL amphotericin-B, and 5% fetal bovine serum according to the manufacturer’s recommendations. After reaching confluence, the cells were incubated in SmBM supplemented with 10% SingleQuots in the presence of vehicle or dexamethasone for 24 hours. The cell numbers were not significantly different between groups treated with vehicle and dexamethasone at the end of the incubation.

O$_2^-$ Production
The release of O$_2^-$ was determined by measuring SOD-inhibitable reduction of ferricytochrome c as described previously. Briefly, after the pretreatment with 100 U/mL SOD or vehicle for 10 minutes, HSMCs were incubated with 81 μmol/L ferricytochrome c in the presence and absence of reagents for 1 hour. The absorbance of the medium at 550 nm was then measured, and SOD-sensitive O$_2^-$ production was calculated. The number of cells was counted using a Neubauer chamber (Fisher Scientific) after trypsinization.

Intracellular Oxidant Production
The determination of intracellular oxidant production was based on the oxidation of 2,7’-dichlorodihydrofluorescein (DCHF) by peroxide, resulting in the formation of the fluorescent compound 2,7’-dichlorofluorescein (DCF) using a Cytofluor 2300 multiplate fluorometer (Millipore) as described previously. HSMCs were incubated in Hanks’ balanced salt solution enriched with 20 μmol/L DCHF diacetate for 20 minutes. The cells were washed, and the fluorescence was measured with a multiplate fluorometer using excitation and emission wavelengths of 485 nm and 530 nm, respectively. For the experiments with hydrogen peroxide, the fluorescence was further measured at 5 and 15 minutes after the addition of 20 μmol/L hydrogen peroxide. The increase in fluorescence induced by exogenous hydrogen peroxide was calculated by subtracting the increase in fluorescence obtained without hydrogen peroxide, which represents the endogenous component of DCHF oxidation. To investigate the effects of dexamethasone on agonist-induced intracellular oxidant production, fluorescent signals were monitored for 1 hour after the DCHF loading. The cells were maintained at 37°C during this period.

SOD Activity
SOD activity was measured by monitoring the inhibition of the rate of xanthine oxidase–mediated reduction of cytochrome c, as previously described. The cells were collected and homogenized with sonication in a 20-mmol/L potassium phosphate buffer (pH 7.0) containing 1 mmol/L EGTA, 0.5 mmol/L PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 10 μg/mL trypsin inhibitor, 10 μg/mL chymostatin, and 10 μg/mL antipain. SOD activity was determined spectrophotometrically using homogenate (75 μg total protein) to inhibit reduction of ferricytochrome c by O$_2^-$ generated by the addition of xanthine and xanthine oxidase. Calibration was made using known amounts of SOD.

Northern Blot Analysis
Total RNA was extracted, size-fractionated, and transferred to nylon membranes (Hybond, Amersham-Buchler) as described previously. After hybridization with the labeled 780-bp p22 phox cDNA (kindly provided by Dr K.K. Griendling, Emory University, Atlanta, Ga) or the cDNA probe specific for mouse 18S rRNA, the blots were washed, exposed to x-ray film, and analyzed with a densitometer as described previously. The values obtained from blots hybridized with the p22 phox cDNA were corrected using the values obtained with the 18S rRNA probe and were expressed as a percentage of control.

Reagents
Recombinant human platelet-derived growth factor (PDGF)-AB was purchased from R&D Systems Inc. Ang II was from Bachem Biochemica GmbH. Ferricytochrome c, dexamethasone, prednisolone, norgestrel, hydrocortisone, and testosterone were from Sigma Chemical Co. SOD was from Boehringer Mannheim GmbH. Fetal bovine serum was from Biochrom KG. DCHF was from Molecular Probes Inc. RU486 was a gift from Roussel Uclaf. All other chemicals and reagents were obtained from commercial sources and were of reagent or molecular biology grade.

Statistics
Multiple comparisons were evaluated with ANOVA, followed by Fisher’s protected least-significant difference method. Data are presented as mean±SEM, and P<0.05 was considered to be statistically significant.

Results
PDGF-AB (30 ng/mL) increased O$_2^-$ production from HSMCs measured after a 1-hour incubation period (Figure 1A), which is consistent with our previous observation. Pretreatment of the cells with dexamethasone (1 μmol/L) for 24 hours significantly inhibited the generation of O$_2^-$ by PDGF-AB–treated HSMCs. However, dexamethasone did not modify PDGF-AB–induced O$_2^-$ production when applied at the same time as PDGF-AB. Therefore, the glucocorticoid was not added during O$_2^-$ measurement in subsequent experiments. As shown in Figure 1B, the treatment of HSMCs with 1 μmol/L dexamethasone for 24 hours also significantly decreased the basal and Ang II (1 μmol/L)–induced O$_2^-$ production. The inhibitory effect of dexamethasone was concentration-dependent (Figure 1C), and a significant attenuation of PDGF-AB–induced O$_2^-$ production was observed at concentrations >10 nmol/L.

Treatment of the cells with other glucocorticoids, including hydrocortisone (100 nmol/L) and prednisolone (100 nmol/L), for 24 hours also significantly decreased the PDGF-AB–induced O$_2^-$ production in HSMCs (Figure 2A). In contrast, neither testosterone (100 nmol/L) nor norgestrel (100 nmol/L) inhibited O$_2^-$ production in PDGF-AB–stimulated HSMCs (Figure 2B). In the presence of the glucocorticoid receptor antagonist RU486 (1 μmol/L), dexamethasone (100 nmol/L) failed to decrease PDGF-AB–induced O$_2^-$ production in HSMCs (Figure 2C), suggesting that the inhibitory effect of dexamethasone is dependent on the glucocorticoid receptor. None of the compounds used affected cell viability as determined by the exclusion of trypan blue.

The effect of glucocorticoids on the intracellular ROS production was determined by monitoring DCF fluorescence. In cells incubated with dexamethasone (100 nmol/L) and prednisolone (100 nmol/L) for 24 hours, DCF fluorescence
O$_2$ was significantly decreased compared with in control cells (Figure 3A). Testosterone (100 nmol/L) and norgestrel (100 nmol/L) were without effect, whereas RU486 (1 μmol/L) completely antagonized the inhibitory effect of dexamethasone (100 nmol/L) on the oxidation of DCFH (Figure 3B). In contrast to the oxidation of DCFH by endogenous hydrogen peroxide (20 μmol/L) was not significantly different between groups whether treated with dexamethasone (100 nmol/L) for 24 hours or not (vehicle 22.7±1.9 arbitrary units versus dexamethasone 24.0±1.5 arbitrary units at 5 minutes; vehicle 34.0±4.4 arbitrary units versus dexamethasone 38.0±3.5 arbitrary units at 15 minutes, n=4), suggesting that dexamethasone selectively decreases endogenous ROS production.

To investigate the effects of dexamethasone on agonist-induced intracellular oxidant production, increases in DCF fluorescence during a 1-hour incubation were measured in the presence and absence of PDGF-AB (30 ng/mL) or Ang II (1 μmol/L). PDGF-AB significantly enhanced the increase in fluorescence during the 1-hour incubation, whereas pretreatment of HSMCs with dexamethasone (1 μmol/L, 24 hours) significantly attenuated the increases in fluorescence: control 100.0±6.4%; PDGF-AB 159.9±17.1%, dexamethasone 52.0±3.4% (P<0.05 versus control values); dexamethasone+PDGF-AB 83.5±8.7% of control, n=7 to 9 (P<0.05 versus values with PDGF-AB). In addition, dexamethasone (100 nmol/L) significantly attenuated the increases in fluorescence signals obtained from Ang II–treated HSMCs: control 100.0±4.7%; Ang II 125.9±6.4%, dexamethasone 69.8±11.8% (P<0.05 versus control values); dexamethasone+Ang II 75.1±4.7% of control, n=4 (P<0.05 versus values with Ang II).

To clarify whether inhibition of O$_2$ production by dexamethasone is due to an increased inactivation of O$_2$ $^-$, SOD activity was measured. SOD activity was not significantly changed by the treatment of HSMCs with dexamethasone (100 nmol/L) for 24 hours (vehicle 6.99±0.23 U/mg versus dexamethasone 6.35±0.16 U/mg, n=3).

Because p22 phox has been suggested to be involved in ROS production in VSMCs, we determined whether the expression of p22 phox mRNA in HSMCs could be modified...
Glucocorticoids Inhibit ROS and p22 Phox in VSMCs

The present study demonstrates that dexamethasone attenuates the basal, Ang II–, and PDGF-AB–stimulated production of O$_2^-$ in HSMCs. These inhibitory effects appear to be a consequence of glucocorticoid receptor activation. This assumption is based on the observations that PDGF-AB–induced O$_2^-$ production was also decreased by hydrocortisone and prednisolone but not by other classes of steroids such as testosterone and norgestrel. Moreover, the inhibition of PDGF-AB–induced O$_2^-$ by dexamethasone was not observed in the presence of the glucocorticoid receptor antagonist RU486.

Glucocorticoids also decreased intracellular ROS levels measured by DCHF oxidation in HSMCs. PDGF-2 and cytomegalovirus-stimulated DCHF oxidation in VSMCs is reportedly inhibited by catalase, suggesting that hydrogen peroxide is the main substance that oxidizes DCHF under these conditions. When one considers that DCHF oxidation in HSMCs is paralleled by the production of O$_2^-$, a precursor of hydrogen peroxide, it is likely that the levels of DCHF oxidation reflect, at least to some extent, the intracellular production of hydrogen peroxide in HSMCs, although other hydroperoxides can also oxidize DCHF.

The effect of dexamethasone on O$_2^-$ production in HSMCs was unrelated to changes in SOD activity and can be at least partially attributed to a decrease in the expression of p22 phox. p22 phox is a component of the membrane-bound cytochrome b-558 complex, which transfers an electron from NADPH to oxygen in the phagocyte NADPH oxidase system.

Although there is some debate as to whether an identical enzyme is expressed in VSMCs, transfection of antisense p22 phox cDNA into VSMCs is reported to decrease Ang II–induced DCHF oxidation in these cells. Thus, an NADH/NADPH oxidase that is similar if not identical to that in leukocytes has been proposed to be expressed in VSMCs and to be involved in ROS production in response to Ang II. Indeed, we previously reported that the production of O$_2^-$ by PDGF-AB–stimulated HSMCs is sensitive to iodonyl diphenyl, an inhibitor of flavoenzymes including NADPH oxidase in phagocytes, further suggesting a role for NADH/NADPH oxidase in VSMCs. The glucocorticoid-induced decrease in p22 phox mRNA observed in the present investigation seems to be unique to VSMCs, because p22 phox mRNA levels are reported not to be influenced by dexamethasone in human polymorphonuclear leukocytes.

To understand the mechanism by which dexamethasone inhibits p22 phox mRNA expression in VSMCs, additional information such as analysis of the promoter region, determination of gene transcription rate, and evaluation of mRNA stability of p22 phox is required.

Expression of p22 phox mRNA has been reported to be increased in the aorta of Ang II–infused hypertensive rats. Impaired endothelium-dependent vascular relaxation observed in this animal model has been shown to be reversed in part by increasing vascular SOD levels, suggesting a crucial role for vascular O$_2^-$ in endothelial dysfunction. Additionally, a polymorphism of p22 phox, which alters an amino acid in the potential heme-binding sites, has been demonstrated to be more frequent in control subjects compared with patients with coronary artery disease. Collectively, these reports support the hypothesis that the alteration of p22 phox expression might modulate ROS levels in the vasculature and influence the progression of vascular diseases.

The effects of glucocorticoids on ROS production in VSMCs may, in addition to the downregulation of p22 phox, involve other intracellular mechanisms. For example, phospholipase A$_2$ (PLA$_2$), an enzyme that is essential for O$_2^-$ production in several cell types, is inhibited by glucocorticoids in VSMCs. In addition, activation of PLA$_2$ is suggested to be involved in cytomegalovirus-induced ROS production in VSMCs. Thus, it is conceivable that inhibition of PLA$_2$ is involved in the decreased ROS production observed after glucocorticoid treatment. However, an inhibitor of cytosolic 85-kDa PLA$_2$, AACOCF$_3$, which has been shown to inhibit PDGF-BB–induced arachidonic acid release from HSMCs, failed to inhibit PDGF-AB–stimulated O$_2^-$ production (T.M., V.B.S.-K., R.P.B., and R.B., unpublished data, 1998). Although we cannot rule out the possibility that some isoforms of PLA$_2$ are insensitive to AACOCF$_3$, in HSMCs, the inhibition of PLA$_2$ does not seem to have a major role in the glucocorticoid-induced inhibition of ROS production described here.

In addition to promoting risk factors of atherosclerosis, glucocorticoids have been shown to upregulate Ang II receptor type 1, to stimulate cholesteryl ester formation, and to induce angiotensin-converting enzyme mRNA and activity in VSMCs. Although these effects of glucocorticoids are potentially atherogenic, the hormone can also inhibit vascular...
lesion formation.5,6,17 Protective effects of glucocorticoids, including the inhibition of ROS production observed in the present study, may exceed their proatherogenic effects when locally applied.17

Interestingly, systemic administration of dexamethasone to adrenalec tomized spontaneous hypertensive rats, but not Wistar-Kyoto rats, has been demonstrated to increase oxidative stress in mesenteric arterioles.2 Because in these animals endothelial cells are proposed to be the source of ROS, it cannot be ruled out that ROS production is differentially regulated by glucocorticoids in endothelial cells and VSMCs. Alternatively, in this animal model increased ROS production in arterioles by dexamethasone might be achieved by systemic effects of the glucocorticoid, such as enhanced hemodynamic forces due to increased blood pressure and altered metabolism of glucose and lipid, all of which are potential stimuli of $O_2^-$ production.8–41

In summary, the present study shows that glucocorticoids decrease ROS production and p22 phox mRNA expression in HSMCs via a glucocorticoid receptor-dependent pathway. This inhibition of ROS production might contribute at least in part to the local beneficial effect of glucocorticoids on the progression of vascular injury.

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