Aldosterone Increases Oxidant Stress to Impair Guanylyl Cyclase Activity by Cysteinyl Thiol Oxidation in Vascular Smooth Muscle Cells

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Hyperaldosteronism is associated with impaired endothelium-dependent vascular reactivity owing to increased reactive oxygen species and decreased bioavailable nitric oxide (NO); however, the effects of aldosterone on vasodilatory signaling pathways in vascular smooth muscle cells (VSMC) remain unknown. Soluble guanylyl cyclase (GC) is a heterodimer that is activated by NO to convert cysytolic GTP to cGMP, a second messenger required for normal VSMC relaxation. Here, we show that aldosterone (10⁻⁹–10⁻⁷ mol/liter) diminishes GC activity by activating NADPH oxidase in bovine aortic VSMC to increase reactive oxygen species levels and induce oxidative posttranslational modification(s) of Cys-122, a cysteinylic thiol residue demonstrated previously to modulate NO sensing by GC. In VSMC treated with aldosterone, Western immunoblotting detected evidence of GC β₁-subunit disulfide bonding, whereas mass spectrometry analysis of a homologous peptide containing the Cys-122-bearing sequence revealed NO oxidation-induced disulfide formation of cysteinylic sulfinic acid (m/z 435), sulfonic acid (m/z 443), and disulfide (m/z 836) bond formation. The functional effect of these modifications was examined by transfecting COS-7 cells with wild-type GC or mutant GC containing an alanine substitution at Cys-122 (C122A). Exposure to aldosterone or hydrogen peroxide (H₂O₂) significantly decreased cGMP levels in cells expressing wild-type GC. In contrast, aldosterone or H₂O₂ did not influence cGMP levels in cells expressing the mutant C122A GC, confirming that oxidative modification of Cys-122 specifically impairs GC activity. These findings demonstrate that pathophysiologically relevant consequences of aldosterone increase oxidative stress to convert GC to an NO-insensitive state, resulting in disruption of normal vasodilatory signaling pathways in VSMC.

Elevated levels of the mineralocorticoid hormone aldosterone are associated with impaired vascular reactivity in patients with aldosterone-producing adenoma, hypertension, and congestive heart failure that is remediable following surgical resection of the tumor or treatment with a mineralocorticoid receptor antagonist (1–5). It has been suggested that aldosterone-induced vascular dysfunction is a consequence of a vasculopathy that results from the propensity for aldosterone to generate ROS² and decrease bioavailable NO (6). In the vascular endothelium, aldosterone has been shown to promote endothelial dysfunction by inducing an acquired antioxidant-deficient state that disrupts cellular redox homeostasis to increase ROS accumulation and diminish NO levels. In vivo, this results in diminished endothelium-dependent vascular reactivity (7). The relationship between aldosterone-induced oxidant stress and NO-stimulated vasodilatory signaling pathways in VSMC, however, remains unknown.

The influence of oxidant stress on NO-activated GC signaling in VSMC has been the subject of investigation for over three decades (8–12). Guanylyl cyclase is a heterodimeric enzyme that requires coexpression of the α₁-, or α₂-, and β₁-subunits to achieve catalytic activity and convert cytosolic GTP to cGMP, which, in turn, induces VSMC relaxation (13). When GC is functionally deficient, vasodilatory signaling is disrupted and vascular compliance is decreased, an effect that is associated with myocardial infarction, stroke, and progression of coronary artery disease (3, 6, 14).

The mechanism by which oxidant stress adversely affects GC function remains controversial. NO is the primary, biologically active stimulator of GC. In the presence of excess ROS, it has been suggested that GC enzymatic function may be decreased by: (i) a peroxynitrite (ONOO⁻)-mediated decrease in GC specific activity (15); (ii) oxidation of the β₁-subunit-associated prosthetic heme group that converts GC to an NO-insensitive state (14); (iii) oxidation-induced disulfide formation of β₁-subunit-thiol groups (16, 17); or (iv) NO-dependent posttranslational modification (e.g. S-nitrosation) of a β₁-subunit-thiol...
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Based on these observations, we hypothesized that aldosterone-mediated ROS formation promotes oxidative post-translational modification of GC and conversion of the enzyme to an NO\textsuperscript{-} insensitive state, thereby disrupting a key pathway essential for normal VSMC relaxation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatments**—Bovine aortic VSMC (G-1) were grown to confluence using phenol-free Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, at 37 °C, 5% CO\textsubscript{2}. Cells were passaged twice weekly using 0.5% trypsin/EDTA, and experiments were performed on cells from passages 4–10. Aldosterone (Steraloids) and apocynin (Cayman) were dissolved in dimethyl sulfoxide (10 nmol/liter), which also served as the vehicle control. Cells were treated with aldosterone (10\textsuperscript{−3}-10\textsuperscript{−7} mol/liter) for 24 h, and in selected experiments, coincubated with apocynin (3 × 10\textsuperscript{−5} mol/liter) or 2-amino-5,6-dihydro-6-methyl-4H-1,2-thiazin (AMT) (100 μmol/liter)(Cayman) for 24 h. NOS\textsubscript{2} was induced using the combination of irradiated lipopolysaccharide (30 μg/ml)(Endogen), and interferon-γ (50 ng/ml)(Endogen) as described previously (19).

**Dichlorodihydrofluorescein Fluorescence**—ROS generation was assessed using 6-carboxy-2’,7’ dichlorodihydrofluorescein diacetate ester fluorescence (5 μmol/liter) (Molecular Probes) as described previously (7).

**Amplex Red Activity Assay**—Hydrogen peroxide was measured using the horseradish peroxidase-linked Amplex Red assay per kit instructions (Invitrogen). Phenol-red free tissue culture medium from experimental samples was incubated in 100 μM Amplex Red reagent, and absorbance was measured at an excitation wavelength of 560 nm and an emission wavelength of 590 nm in a microplate fluorometer (Spectramax Gemini XPS, Molecular Devices). Data are standardized to sample protein concentration.

**NADPH Oxidase Activity**—NADPH oxidase activity was measured using lucigenin (5 μmol/liter) chemiluminescence. Photon emission was measured every 10 s for 5 min in a luminometer (Turner Biosystems 20/20\textsuperscript{a}), and the rate of enzyme activity was calculated as described previously (20).

**NO\textsuperscript{−} Metabolites**—Nitrite (NO\textsubscript{2}\textsuperscript{−}) and nitrate (NO\textsubscript{3}\textsuperscript{−}) were measured from cell culture medium containing 2% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, at 37 °C, 5% CO\textsubscript{2}. Cells were passaged twice weekly using 0.5% trypsin/EDTA, and experiments were performed on cells from passages 4–10. Aldosterone (Steraloids) and apocynin (Cayman) were dissolved in dimethyl sulfoxide (10 nmol/liter), which also served as the vehicle control. Cells were treated with aldosterone (10\textsuperscript{−3}-10\textsuperscript{−7} mol/liter) for 24 h, and in selected experiments, coincubated with apocynin (3 × 10\textsuperscript{−5} mol/liter) or 2-amino-5,6-dihydro-6-methyl-4H-1,2-thiazin (AMT) (100 μmol/liter)(Cayman) for 24 h. NOS\textsubscript{2} was induced using the combination of irradiated lipopolysaccharide (30 μg/ml)(Endogen), and interferon-γ (50 ng/ml)(Endogen) as described previously (19).

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**3-Nitrotyrosine Immunochemistry**—Cells were grown to confluence on glass chamber slides, exposed to anti-3-nitrotyrosine antibody (Santa Cruz Biotechnology), and stained using the 3,3’-diaminobenzidine substrate method (Vector laboratories) as described previously (21). Images were viewed under 200× magnification with the Olympus BX51\textsuperscript{TM} microscope and acquired by Picture Taker\textsuperscript{TM}.

**cGMP Measurement**—Confluent cells were washed twice with ice-cold phosphate-buffered saline and then exposed to 6% trichloroacetic acid. Cells were collected and centrifuged at 1,500 × g for 10 min at 4 °C, cGMP formation was measured by immunosay according to the manufacturer’s instructions (Cayman), and bicinchoninic acid (Bio-Rad) was used for protein determination.

**Soluble Guanylyl Cyclase Activity Assay**—Intracellular GC activity was measured using the methodology of Waldman et al. (22) with minor modifications. All samples were incubated with 0.5 mM 3-isobutyl-1-methylxanthine and DETA NONOate (10 mM, Cayman) at 37° for 10 min, and the reaction was initiated by the addition of 4 mM MgCl\textsubscript{2} and 1 mM GTP (Sigma). After 10 min, 6% trichloroacetic acid was added, supernatant fractions were extracted using water-saturated ethyl ether (Fisher), and samples were dried under nitrogen gas. cGMP levels were measured by immunosay according to the manufacturer’s instructions (Cayman).

**Immunoblotting**—Proteins were size-fractionated electrophoretically using SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were incubated with anti-NOS2 (Santa Cruz Biotechnology) and anti-GC α1-subunit and anti-GC β1-subunit (Cayman) antibodies overnight at 4 °C and visualized using the ECL detection system (Amersham Biosciences).

**Western Blot to Detect GC Disulfide Bond Formation**—VSMC were lysed in an alkalizing buffer (0.1 M Tris–HCl, pH 6.8, 1% SDS, 100 mM iodoacetamide, 100 mM N-ethylmaleimide) and sonicated on ice. Following a 30-min incubation at 25 °C, alkylated proteins were precipitated with acetone. Proteins were resuspended in 50 μl of 0.1 M Tris–HCl, pH 7.4, 1% SDS, and disulfides were reduced by adding a final concentration of 5 mM Tris(2-carboxyethyl)phosphine hydrochloride. Following a 20-min incubation at 25 °C, Tris(2-carboxyethyl)phosphine hydrochloride was removed with a Micro Bio-Spin column 6 (Bio-Rad), and 1% SDS was added to the eluant. The previously oxidized (now reduced) cysteines were labeled with 1 mM polyethylene glycol-conjugated maleimide (molecular mass 10 kDa, Fluka). After a 1-h incubation at 25 °C, proteins were precipitated with acetone, resuspended in 50 μl of non-reducing SDS electrophoresis buffer, size-fractionated electrophoretically using SDS-PAGE, and transferred to a polyvinylidene fluoride membrane (23). To detect disulfides specific to the sGC β1-subunit, the membrane was immunoblotted with an sGC β1-subunit antibody (Santa Cruz Biotechnology).

**Site-directed Mutagenesis and Transfection**—Expression constructs in pCMV5 were used to overexpress α1-, β1-, and β1-C122A subunits of rat GC as described previously (18). COS-7 cells, which do not express endogenous GC, were plated in 6-well tissue culture dishes and transfected with 5 μg of DNA for 4 h with Lipofectamine 2000\textsuperscript{TM} (Invitrogen) (24). After this time, medium was replaced with full growth media, and experiments were performed after 48 h.

**Mass Spectrometry**—In-gel trypsin digestion of immunoprecipitated GC from bovine VSMC whole cell extracts was performed according to the method by Shevchenko et al. (25). Extracted peptides were subjected to liquid chromatography-mass spectrometry (LC-MS) analysis, and modified cysteinyl thiols in digested peptides were monitored using MS1 analysis. A doubly charged ion of m/z 435 corresponding to a Cys-122-containing peptide (CTDADKGK) with the addition of two oxygen was identified. A model peptide containing CTDADKGG was synthesized in the Biopolymers Laboratory at Har-
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Aldosterone increases oxidant stress in VSMC. A, vascular smooth muscle cells were exposed to aldosterone (ALDO) (10^{-8}-10^{-7} mol/liter) or vehicle control (V) for 24 h, and ROS accumulation was measured by 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate ester (DCF) fluorescence (n = 3). B, hydrogen peroxide (H2O2) generation was evaluated by Amplex Red assay fluorescence (n = 3), p < 0.01 versus vehicle control. Data are presented as mean ± S.E.

Aldosterone increases oxidant stress in VSMC. To study the effect of oxidant stress on Cys-122, the peptide (0.1 mM) was incubated with 200 μmol/liter H2O2 and 0.2 mmol/liter ferrous sulfate (Sigma) in 50 mM ammonium acetate, pH 5.9, for 30 min at 22 °C. Reaction products were mixed with 10 volumes of 30% acetonitrile and 0.1% formic acid in water and analyzed with an HPLC system (Thermo Electron Co., San Jose, CA) equipped with a static nanospray ion source probe. The spray voltage was set to 1.4 kV, and the heated capillary was set to 200 °C. MS1 scanning was performed using an enhanced scan mode to monitor the size and charge state of the ions. MS-MS scanning was acquired for definitive identification of the oxidation products of the peptide.

Statistical Analysis—Continuous data were expressed as means ± S.E. Comparison between groups was performed by Student’s paired two-tailed t test, and p < 0.05 is considered significant.

RESULTS

Aldosterone Induces Oxidant Stress in VSMC—To evaluate the effect of aldosterone on ROS accumulation, bovine aortic VSMC were exposed to aldosterone (10^{-8}-10^{-7} mol/liter) for 24 h. When compared with vehicle-treated cells, there was a concentration-dependent increase in ROS levels in aldosterone-treated cells (117.9 ± 5.7 versus 124.5 ± 6.1 versus 144.9 ± 4.6% control, p < 0.01, n = 3) (Fig. 1A). As no further increase in ROS formation was observed after 24 h, experiments were performed following this incubation period in VSMC exposed to 10^{-7} mol/liter aldosterone, a pathophysiologically relevant concentration comparable with levels achieved in patients with primary or secondary hyperaldosteronism (3).

Aldosterone-mediated ROS generation resulted from activation of NAPDH oxidase, a predominant source of ROS in VSMC. Aldosterone increased NAPDH oxidase activity nearly 2-fold when compared with vehicle-treated cells (61.7 ± 1.5 versus 117.9 ± 5.7 nmol O2·/min/mg of protein, p < 0.001, n = 3). Coincubation of aldosterone-treated cells with apocynin (30 μmol/liter), an NAPDH oxidase inhibitor, decreased ROS levels significantly (145.1 ± 1.8 versus 115.6 ± 8.6% control, p < 0.04, n = 3), confirming that the majority of aldosterone-mediated ROS formation resulted from NAPDH oxidase activation.

Next, the effect of aldosterone on H2O2 levels was assessed as H2O2 has been shown to modify protein function by reacting with free thiol moieties and GC contains several cysteinylish thioles. When compared with vehicle, aldosterone increased H2O2 formation significantly as determined by Amplex Red fluorescence (5.24 ± 0.02 versus 9.31 ± 1.06 μM/μg of protein, p < 0.03, n = 3) (Fig. 1B). Coincubation with apocynin decreased aldosterone-induced H2O2 production by 66.1% (p < 0.01, n = 3). Together, these studies demonstrate that aldosterone activates NADPH oxidase to increase oxidant stress in VSMC.

Aldosterone and NO Metabolism—Under basal conditions in VSMC, bioavailable NO has one of two metabolic fates. It may either remain unoxidized to bind the Fe^{2+} center of the prosthetic heme group to activate GC and increase cGMP levels or undergo oxidation to nitrite (NO_{2}^-) and nitrate (NO_{3}^-). Therefore, to assess the influence of aldosterone on endogenously produced NO' bioactivity, we induced iNOS expression to increase NO_{2}^- production (0.0 ± 0.0 versus 0.08 ± 0.01 μM/μg of protein, p < 0.01, n = 4) (Fig. 2, A and B). AMT (100 μmol/liter), a specific iNOS inhibitor, decreased NO_{2}^- formation to non-detectable levels in iNOS-expressing cells, indicating that iNOS was the primary source of NO_{2}^-.

Exposure of iNOS-expressing cells to aldosterone had no further influence on either iNOS protein expression or NO_{2}^- levels. To demonstrate that these findings were not unique to iNOS-expressing cells, we treated VSMC with the exogenous NO' donor, DETA NONOate (1 mmol/liter). DETA NONOate-treated cells exhibited a significant increase in NO_{2}^- formation (0.0 ± 0.0 versus 1.34 ± 0.02 μM/μg of protein, p < 0.03, n = 3), and aldosterone had no additional effect on these levels (1.35 ± 0.02 μM/μg of protein, p = not significant, n = 3).

In addition to the observed lack of effect of aldosterone on NO_{2}^- levels, aldosterone had no influence on total NO' metabolites (NO\_2^\text{-}\text{NO}_3^\text{-}\text{NO}_3^\text{N}_{\text{O}}) in iNOS-expressing cells (Fig. 2C). It has been shown previously that following iNOS induction in vitro, the NO\_3^- to NO\_2^- ratio is ~2:1; however, in the presence of increased ROS generation, enhanced peroxynitrite (ONOO^-) formation may shift this ratio in favor of NO\_3^- (26). Here, in iNOS-expressing cells, the NO\_2^-/NO\_3^- ratio was found to be 1.86:1, and aldosterone decreased this ratio by 31.7% (p < 0.04, n = 6). To explain the observed shift in the NO\_2^-/NO\_3^- ratio, we examined ONOO^- formation (Fig. 2D). In iNOS-expressing cells, aldosterone induced a 54% rise in ONOO^- formation when compared with untreated cells as assessed by 3-nitrotyrosine immunostaining (p < 0.02, n = 3).

Inhibition of NAPDH oxidase activity with apocynin in aldosterone-treated cells fully restored the NO\_2^-/NO\_3^- ratio, increasing it modestly to ~3:1 (Fig. 2C). Taken together, these studies reveal that aldosterone increases ONOO^- formation in iNOS-expressing cells and offer an explanation for the observed decrease in the NO\_2^-/NO\_3^- ratio.

Aldosterone Decreases GC Activity and cGMP Production—Next, the influence of aldosterone on NO'-GC signaling was examined. Although there was no effect of aldosterone on GC \alpha_\text{51}^- or \beta_\text{55}-subunit protein expression (Fig. 3A), we observed a 46% decrease in basal GC activity in aldosterone-treated when compared with vehicle-treated cells (0.016 ± 0.001 versus 0.008 ± 0.002 pmol cGMP/10 min/μg of protein, p < 0.02, n =
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FIGURE 2. Aldosterone and NO\textsuperscript{2+} byproduct formation. A, vascular smooth muscle cells were exposed to ALDO (10\textsuperscript{-7} mol/liter) or vehicle control (V) in the presence or absence of cytokines (lipopolysaccharide (LPS) = 30 μg/ml, interleukin-1β (IL-1β) = 50 ng/ml, interferon-γ (IFN-γ) = 50 ng/ml) for 24 h to induce iNOS expression. The effect of ALDO on iNOS protein expression was determined by Western blotting (∗, p < 0.001 versus vehicle control). B, iNOS expression was determined by immunofluorescence (∗, p < 0.001 versus vehicle control). C, the influence of ALDO on NO\textsuperscript{2+} formation was determined by Western blotting (∗, p < 0.001 versus vehicle control). D, the influence of ALDO on NADPH oxidase activity with apocynin restored iNOS-stimulated GC formation (∗, p < 0.001 versus vehicle control). E, the influence of ALDO on NO\textsuperscript{2+} byproduct formation was determined by Western blotting (∗, p < 0.001 versus vehicle control).

Posttranslational Oxidative Modification of GC Decreases NO\textsuperscript{2+} Sensing—To support our hypothesis that aldosterone impairs GC activity by inducing oxidative modification of a key cysteine residue located within the NO\textsuperscript{2+}-sensing region of GC, we used liquid chromatography-mass spectrometry (LC-MS) to target three key β\textsubscript{1}-subunit-associated cysteine residues (Cys-78, Cys-122, Cys-214). These cysteines were selected for analysis based on prior studies that implicated them as integral to normal NO\textsuperscript{2+} sensing by GC (10, 18). Immunoprecipitation of GC using a β\textsubscript{1}-subunit antibody was performed on VSMC exposed to aldosterone for 24 h, and after separation of peptides by capillary column liquid chromatography, MS1 scanning suggested potential oxidative modification of Cys-122 (peptide sequence: CTDADKGK). To examine the effect of oxidant stress on Cys-122, a model peptide containing the above amino acid sequence was synthesized. After exposure of this peptide to H\textsubscript{2}O\textsubscript{2} (200 μmol/liter) for 30 min, MS1 scanning identified three doubly charged ions at m/z 435, m/z 443, and m/z 836 that corresponded to the CTDADKGK oxidation products cysteinyl sulfonic acid, cysteinyl sulfonic acid, and cysteinyl disulfide, respectively. MS-MS scanning confirmed the identity of these products (Fig. 4, B–D). Collision-induced dissociation of ion m/z 435 resulted in a spectrum with a characteristically predominant [M-81] ion (m/z = 394 at doubly charged state), representing the loss of NH\textsubscript{3} and SO\textsubscript{2} in the parent ion after a rearrangement of the N-terminal cysteinyl sulfonic acid in the peptide (for details of this reaction, see Ref. 27) (Fig. 4B). In addition, fragmentation of ion m/z 836 resulted in a spectrum with the most abundant daughter ion being derived from the homolytic cleavage of the disulfide bond present in the parent ion (Fig. 4D). These mass spectrometry analyses reveal that oxid-
dation of CTDADKGK results in oxidation products by either the addition of oxygen or disulfide bond formation.

To determine whether oxidative modification of Cys-122 has functional implications for normal NO sensing by GC, we transiently transfected COS-7 cells with rat GC DNAs coding for the wild-type (WT) α₁- and β₁-subunit of GC or the wild-type α₁-subunit and a mutant β₁-subunit containing a substitution of alanine for cysteine at position 122 (α₁/β₁-C122A). Although a single amino acid dissimilarity exists at position 126 between rat (glutamate) and bovine (aspartate) GC DNA, this difference is conservative and unlikely to affect the microenvironment of the catalytically active subunit protein expression in VSMC exposed to either ALDO (10⁻⁷ mol/liter) or vehicle control (V) for 24 h was assessed by Western blotting (n = 3). B and C, the effect of aldosterone (10⁻⁷ mol/liter) on GC activity under basal conditions (B) and iNOS-expressing cells in the presence or absence of NADPH oxidase inhibition with apocynin (Apo) (3 × 10⁻⁵ mol/liter) (n = 4). C. D, cGMP levels were determined in iNOS-expressing cells (n = 4). *p < 0.02 versus vehicle control, **p < 0.04 versus INOS, #p < 0.03 versus ALDO. Representative blots are shown. Data are presented as mean ± S.E.

FIGURE 3. Aldosterone and GC subunit expression and activity. A. GC α₁- and β₁-subunit protein expression in VSMC exposed to either ALDO (10⁻⁷ mol/liter) or vehicle control (V) for 24 h was assessed by Western blotting (n = 3). B and C, the effect of aldosterone (10⁻⁷ mol/liter) on GC activity under basal conditions (B) and iNOS-expressing cells in the presence or absence of NADPH oxidase inhibition with apocynin (Apo) (3 × 10⁻⁵ mol/liter) (n = 4). C. D, cGMP levels were determined in iNOS-expressing cells (n = 4). *p < 0.02 versus vehicle control, **p < 0.04 versus INOS, #p < 0.03 versus ALDO. Representative blots are shown. Data are presented as mean ± S.E.

Next, we investigated the functional effect of these findings on GC activity. Although WT-transfected COS-7 cells treated with aldosterone demonstrated decreased NO-stimulated cGMP formation when compared with vehicle-treated cells (100 ± 8.1 versus 67.6 ± 8.8% control, p < 0.05, n = 3), aldosterone failed to decrease NO-stimulated cGMP levels in α₁/β₁-C122A mutant-transfected cells (100 ± 13.4 versus 120.1 ± 5.1% control, p = not significant, n = 3) (supplemental Fig. 1D). These findings confirm that aldosterone-mediated oxidative modification of Cys-122 limits GC NO sensing.

DISCUSSION

These studies demonstrate that pathophysiologically relevant concentrations of aldosterone activate NADPH oxidase in bovine VSMC to increase ROS levels, which, in turn, create a redox milieu that favors oxidative posttranslational modification(s) of the catalytically active β₁-subunit of GC. Furthermore, these studies implicate oxidative modification of Cys-122, a key cysteine moiety resides in the NO-sensing domain of the GC β₁-subunit (28), as a mechanism by which oxidant stress impairs GC enzyme activity. Our functional studies of GC support this observation. NO sensing by WT (α₁/β₁) GC was inhibited significantly in cells exposed to either H₂O₂ or aldosterone; however, a mutant form of GC, with a substitution of alanine for cysteine at position 122 (α₁/β₁-C122A), was resistant to oxidative modification under the same conditions and exhibited normal NO sensing. Furthermore, our studies reveal that aldosterone had no effect on total NO metabolite levels, and, therefore, confirm the hypothesis that aldosterone-mediated ROS influences GC directly to limit GC activation rather than by decreasing levels of bioavailable NO.
Aldosterone has been shown to increase both H$_2$O$_2$ levels and ONOO$^-$ formation in hearts and peripheral blood mononuclear cells isolated from rats supplemented with dietary salt (29, 30). Our data are in concert with these observations; VSMC exposed to aldosterone demonstrated elevated levels of H$_2$O$_2$ and ONOO$^-$ formation. This finding has consequence for our studies of GC activity in a redox-active milieu as both species have been suggested to modulate GC function. Elevated levels of H$_2$O$_2$ may induce posttranslational oxidative modification(s) of regulatory cysteinyl thiols to generate protein sulfenic acid, which, in an oxygen-rich environment, is rapidly oxidized to yield sulfinic or sulfonic acid or can form disulfide bonds (31). Peroxynitrite has also been shown to decrease GC activity in cytosolic extracts of rat aorta and animal models of vascular disease (14, 15). Although ONOO$^-$ generation is associated with reduced levels of bioavailable NO$, we found that aldosterone-induced increases in ONOO$^-$ formation did not offset total NO$^+$ oxidative product levels but merely altered the NO$_2^-$/NO$_3^-$ ratio. This result is not surprising as in the presence of ONOO$^-$ and CO$_2$, preferential formation of NO$_3^-$ over NO$_2^-$ has been described owing to the scavenging of NO$_2^-$ by O$_2$ to form peroxynitrate (O$_2$NOO$^-$) (32). Our data are in agreement with those others who have shown that by limiting the source of NO$_2^-$ scavenging, accomplished here with apocynin, an NADPH oxidase inhibitor and free radical antioxidant, the NO$_2^-$/NO$_3^-$ ratio is restored (26, 33, 34).

Prior work has suggested that, in the presence of increased ROS formation, decreased GC activity may be attributed to down-regulation of GC $\alpha_1$- and/or $\beta_1$-subunit protein expression (35–37). In contrast, we found that aldosterone-induced
oxidant stress had no influence on α1- or β1-subunit expression. These earlier investigations examined GC expression in the vasculature of aged hypertensive rat models, whereas our studies were performed in vitro in bovine vascular smooth muscle cells. Interestingly, other non-hypertensive animal models of impaired vascular reactivity, including hypercholesterolemic rabbits and nitrate-tolerant rats, demonstrated an increase in GC subunit expression (38). More recently, it was shown that rat aortic smooth muscle cells exposed to elevated levels of H$_2$O$_2$ (500 μmol/liter) for 24 h demonstrated decreased GC α1- and β1-subunit mRNA and protein expression; however, exposure to this concentration of H$_2$O$_2$ was associated with a 25% decrease in cell viability. In addition, when these investigators exposed cells to a lower concentration of H$_2$O$_2$ (150 μmol/liter), they saw no change in α1-subunit protein expression and an increase in β1-subunit expression (39). In our experiments, the levels of H$_2$O$_2$ generated by aldosterone were similar to the lower concentration of H$_2$O$_2$ studied by these investigators, and aldosterone treatment at these concentrations did not influence cell viability.

In the presence of increased ROS levels, decreased GC activity has been observed in isolated enzyme preparations and vascular cells (40, 41). This effect has been attributed to a ROS-mediated decrease in bioavailable NO$^+$ to limit enzyme activation, as well as the direct influence of ROS or ONOO$^-$ on GC. In our studies, we found that aldosterone-mediated ROS adversely modulates GC activity directly, supported by the observation that GC activity and cGMP levels were restored to basal levels when NADPH oxidase was inhibited by apocynin. Furthermore, we observed an aldosterone-induced uncoupling of iNOS-generated NO$^+$ bioactivity; aldosterone selectively decreased cGMP formation without influencing total NO$^+$ metabolite levels. Our observed effects of aldosterone on GC activity, therefore, cannot be explained by decreased levels of NO$^+$ available to activate the enzyme. These results are supported by studies that examined the influence of ROS on GC activity using both purified enzyme and VSMC stimulated with YC-1, an NO$^+$-independent GC activator. In these studies, ROS generation via xanthine/xanthine oxidase inhibited YC-1-stimulated GC activity, an effect that could not be attributed to a ROS-mediated decrease in bioavailable NO$^+$ (40).

Our study is akin to work from others that implicates thiol redox status as a critical determinant of NO$^+$ sensing by GC (16–18). Early observations by Brandwein et al. (17) suggested disulfide bond formation as an explanation for decreased GC activity. Other investigators have shown that decreased GC activity associated with thiol oxidation was reversible by thiol reduction (16). Here, we demonstrate that cysteiny1 thiol oxidation occurs in the presence of increased levels of H$_2$O$_2$ generated by aldosterone-induced NADPH oxidase activation followed by dismutation of superoxide to H$_2$O$_2$ to yield disulfide bond(s) as well as sulfinic and sulfonic acid oxidative modifications. In the LC-MS experiments, the Cys-122-containing peptide was also exposed to ferrous sulfate, a constituent present in GC under physiological conditions. Cys-122 is located near the His-105-associated prosthetic heme group, and ferrous sulfate by-reacts with H$_2$O$_2$ to yield OH$^-$ radicals (Fenton reaction), which, in turn, have been shown previously to oxidize cysteiny1 thiols (42). Importantly, our studies do not exclude the possibility that aldosterone-mediated ROS oxidizes other key cysteiny1 thiols or the His-105-associated prosthetic heme moiety to influence GC activity.

We also confirmed prior studies that implicate Cys-122 as a key regulatory element involved in NO$^+$ sensing by GC. Specifically, substitution of an alanine for cysteine at position 122 resulted in preserved GC activity under conditions of oxidant stress. As the crystal structure of mammalian GC has not yet been resolved, the precise mechanism by which oxidative modification of GC impairs NO$^+$ sensing remains controversial (43). Analysis of non-mammalian proteins that are structurally similar to GC in which Cys-122 is highly conserved has revealed that subtle conformational changes at Cys-122 may limit NO$^+$ binding at the sixth coordinating position of reduced heme (at His-105). This, in turn, would prevent the nitrosyl-heme formation that is necessary for GC activation (18, 28).

Taken together, our findings suggest a novel mechanism by which aldosterone-mediated ROS formation adversely influences vasodilatory signaling pathways in VSMC. These studies demonstrate that increased ROS levels, as occur in aldosterone-treated VSMC, induce oxidative posttranslational modification(s) of GC and subsequent conversion of the enzyme to an NO$^+$-insensitive state, an effect that is abrogated by inhibition of NADPH oxidase. These data suggest further that impaired vascular reactivity associated with elevated levels of aldosterone may result, in part, from decreased GC activity and identify GC
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as a therapeutic target to ameliorate impaired vascular reactivity associated with hyperaldosteronism.

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