H₂O₂ Regulation of Vascular Function Through sGC mRNA Stabilization by HuR

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Objective—Hydrogen peroxide (H₂O₂) is an important mediator in the vasculature, but its role in the regulation of soluble guanylate cyclase (sGC) activity and expression is not completely understood. The aim of this study was to test the effect of H₂O₂ on sGC expression and function and to explore the molecular mechanism involved.

Methods and Results—H₂O₂ increased sGCβ1 protein steady-state levels in rat aorta and aortic smooth muscle cells (RASMCs) in a time- and dose-dependent manner, and this effect was blocked by catalase. sGCα2 expression increased along with β1 subunit, whereas α1 subunit remained unchanged. Vascular relaxation to an NO donor (sodium nitroprusside) was enhanced by H₂O₂, and it was prevented by ODQ (sGC inhibitor). cGMP production in both freshly isolated vessels and RASMCs exposed to H₂O₂ was greatly increased after sodium nitroprusside treatment. The H₂O₂-dependent sGCβ1 upregulation was attributable to sGCβ1 mRNA stabilization, conditioned by the translocation of the mRNA-binding protein HuR from the nucleus to the cytosol, and the increased mRNA binding of HuR to the sGCβ1 3' untranslated region. HuR silencing reversed the effects of H₂O₂ on sGCβ1 levels and cGMP synthesis.

Conclusion—Our results identify H₂O₂ as an endogenous mediator contributing to the regulation of vascular tone and point to a key role of HuR in sGCβ1 mRNA stabilization. (Arterioscler Thromb Vasc Biol. 2011;31:567-573.)

Key Words: antioxidants ■ cell physiology ■ endothelial function ■ reactive oxygen species ■ vascular biology ■ vascular muscle ■ vasodilatation

Reactive oxygen species (ROS) play a role in the development of vascular damage in different pathophysiological conditions. It has been demonstrated that vascular production of ROS can increase substantially in diseases such as diabetes, hypertension or hypercholesterolemia, and these ROS may modulate the phenotype of vascular cells or even the deposit or composition of extracellular matrix (ECM) in the vessel wall.¹⁻⁵ Hydrogen peroxide (H₂O₂), formed from superoxide (O₂⁻) as a result of the activity of various superoxide dismutases, is an important regulator of the function of the vascular wall.⁶⁻⁷ H₂O₂ has been detected in people under both physiological and pathophysiological conditions.⁸⁻¹⁰ In humans, H₂O₂ can be detected in normal controls, as well as in patients with cardiovascular or pulmonary disease. H₂O₂ may modulate the contraction, proliferation, and apoptosis rate and the synthesis of ECM proteins in endothelial or smooth muscle cells.¹¹ H₂O₂ has been proposed to be an endothelium-derived hyperpolarizing factor in peripheral arteries.¹²⁻¹³ H₂O₂ may also dilate arteries by alternative mechanisms, such as the stimulation of the production of prostanoids¹⁴ or the activation of soluble guanylate cyclase (sGC).¹⁵ Moreover, under some circumstances, H₂O₂ may increase the vascular content of endothelial nitric oxide synthase and to stimulate its activity, with subsequently increased synthesis of nitric oxide (NO).¹⁶⁻¹⁸

Most of cellular effects of NO depend on the interaction with its main receptor, sGC, and ROS could interact with this receptor, thus modifying the cellular response to NO. In fact, several studies have shown that cGMP levels increase during ischemia, a pathological condition characterized by increased ROS synthesis.¹⁹⁻²⁰ In addition, sGC can be modulated by ROS, modifying its activity after brief exposition to oxidants,²¹⁻²³ and recent work suggests that sGC expression and activity may be attenuated by ROS in rat aortic smooth muscle cells (RASMCs)²⁴ and ovine pulmonary artery smooth muscle cells.²⁵ Moreover, Courtois et al²⁶ proposed that a superoxide anion–dependent sGC downregulation may be involved in the genesis of the hypertension induced by chronic lead administration.

The present study was devoted to analyzing the ability of ROS, particularly H₂O₂, to modulate the cellular content of...
sGC, as well as identifying the molecular mechanism involved and its biological consequences.

Methods
Details regarding materials, animal studies, RASMC isolation, and immunohistochemical studies; measurement of cGMP, RNA isolation, and RNA interference; RNA–electrophoretic mobility shift assay (RNA-EMSA); and statistics are provided in an expanded Methods section in the online Data Supplement, available at http://atvb.ahajournals.org.

Vascular Reactivity
The preparation of the aortic rings was essentially similar to that described. Aortas were cut into 3 mm segments. The aortic rings were suspended between 2 stirrups in a 5 mL of organ bath filled with prewarmed (37°C) oxygenated (95% O2/5% CO2) modified Krebs–Ringer bicarbonate solution at pH 7.4. The rings were set at an initial resting tension of 2 g, and changes in force were recorded with an isometric force transducer (AD Instruments, Panlab, BCN, Spain) connected to a computer equipped with a suitable software program. The tissues were allowed to equilibrate for 60 minutes before the experiments began. In that period, the resting tension was readjusted to 2 g, if required. The vessels were exposed to 75 mmol/L KCl to verify their functional integrity. After washing, the segments were first incubated for 2 hours with H2O2 and then contracted with noradrenaline (100 μmol/L). After that aortic rings were treated with different doses of sodium nitroprusside (SNP), and tension was recorded. Some experiments were performed in the presence of 1H-(1,2,4)-oxadiazolo-(4,3-a)-quinoxalin-1-one (ODQ) (1 μmol/L).

Results

H2O2 Increases sGCβ1 Subunit Protein Expression in Rat Aorta and in Cultured RASMCs
To assess the effect of H2O2, freshly isolated rat aortic rings were incubated with increasing concentrations of H2O2 (10^-6 to 10^-4 mol/L) for 4 hours, and the expression of the β1 subunit of sGC was analyzed by immunohistochemistry and immunoblot. Figure 1A shows an increased immunostaining of sGC in the media of the aorta in response to H2O2 treatment. Figure 1B shows that H2O2 increased sGCβ1 levels in a dose-dependent manner. As observed in Figure 1C, H2O2 treatment of cultured RASMCs induced an increase in sGCβ1 expression over time, reaching a peak after 4 hours of treatment, and returning to basal levels after 8 hours. This effect was dose-dependent because the β1 subunit increased in response to increasing concentrations of H2O2 (supplemental Figure I). In contrast, α1 subunit protein levels were not affected by H2O2 (supplemental Figure II). Because sGC functions as an heterodimer, we also tested α2 subunit expression observing an increase in response to H2O2 treatment, which was time- and concentration-dependent (supplemental Figure III). The effects of H2O2 on sGCβ1 expression were specific because catalase abolished the increase in sGCβ1 levels in response to H2O2 treatment of cultured RASMCs (Figure 1D).

H2O2 Enhances sGC Function in Rat Aorta and RASMCs
To test the biological consequences of the enhanced sGC levels in the presence of H2O2, the vascular reactivity to an
H2O2 abrogated the vasorelaxant responses to both SNP and consistent with an increase in sGC function because ODQ the aortic rings to SNP (Figure 2A). This response was plotted control; # 0.05 vs SNP alone (n = 6).

For 30 minutes, and cGMP was measured. * P<0.05 vs unstimulated control; #P<0.05 vs SNP alone (n = 6).

Figure 2. H2O2 enhances sGC function in rat aorta and RASMCs. A, Arterial relaxant responses to increasing doses of sodium nitroprusside (SNP) in rat aortic rings pretreated with saline (white circles), 10−4 mol/L H2O2 (black circles), saline+ODQ (white squares), or H2O2+ODQ (black squares) for 4 hours and contracted with noradrenaline. Each point represents measurements from 10 individual rings. Results are expressed as percentages of contraction against time 0.

NO donor (SNP; 10−9 to 10−4 mol/L) was studied. Endothelium-denuded rat aortas were pretreated with saline or H2O2 (10−4 mol/L) for 2 hours followed by incubation with a sGC inhibitor, ODQ, or saline for 30 minutes and then contracted with noradrenaline. Contraction to noradrenaline was not affected by ODQ or H2O2 treatment (supplemental Figure IV). H2O2 enhanced the relaxation observed after exposure of the aortic rings to SNP (Figure 2A). This response was consistent with an increase in sGC function because ODQ abrogated the vasorelaxant responses to both SNP and H2O2+SNP. To confirm this result, cGMP accumulation in rat aortic rings exposed to NO or H2O2+NO was measured (Figure 2B). After SNP treatment, cGMP levels increased 7-fold in aortic tissue. Treatment with H2O2 produced a further increase, up to 16-fold, in the amount of cGMP synthesized by aortic tissue compared to NO treated samples.

We also measured cGMP accumulation in RASMCs exposed to NO or H2O2+NO (Figure 2C). NO treatment induced a 6-fold elevation in cGMP levels in control cells. Treatment with H2O2 produced an additional increase in cGMP levels compared to NO-treated cells.

H2O2 Induces sGCβ1 mRNA Expression by Increasing Its mRNA Stability

Northern blot analysis for specific sGCβ1 mRNA was next performed to determine whether the stimulatory effect of H2O2 was attributable to differences in mRNA expression. As shown in Figure 3A, sGCβ1 mRNA steady-state levels increased after 4 hours of exposure to increasing concentrations of H2O2 in a dose-dependent manner.

The effect of H2O2 on the activity of the sGC promoter was studied by transfecting RASMCs with the full-length sGCβ1 promoter luciferase construct (p3.0-Luc) followed by treatment with H2O2 (10−4 mol/L) for 24 hours. Human sGCβ1 promoter transactivation was measured as firefly luciferase activity normalized to Renilla luciferase activity. H2O2 did not produce any significant change in promoter activity, thus suggesting that a transcriptional induction was not involved in the observed effects (Figure 3B).

To determine whether changes in mRNA stability contributed to the H2O2-dependent increase in sGCβ1 mRNA levels, RASMCs were exposed to H2O2 (10−4 mol/L) for 4 hours, transcription was stopped by addition of DRB (5,6-dichloro-1-β-D-ribofuranosyl benzimidazole) (10−5 mol/L), and mRNA expression was followed for 6 hours. As shown in Figure 3C, DRB-treated cells show a progressive decline in sGCβ1 mRNA levels. In contrast, H2O2 prolonged the half-life of sGC mRNA for more than 4 hours.

H2O2 Induces HuR Cytoplasmic Translocation and HuR Binding to the 3′ Untranslated Region of sGCβ1 mRNA

The adenylate- and uridylate-rich elements (AREs) located in the 3′ untranslated regions (UTRs) are among the most frequent cis-regulatory elements targeting mRNAs for rapid decay.28 It has been reported that sGCβ1 mRNA contains several conserved AREs that are targeted for a rapid mRNA degradation by specific endonucleases.29 Because human (Hu) antigen R (HuR), a RNA-binding protein, has been described to regulate the mRNA stability of many genes by binding to ARE sequences in their 3′UTR including sGC, we decided to explore the effect of H2O2 on the levels of the mRNA-stabilizing protein, HuR. As shown in Figure 4A, H2O2 induced an increase in HuR levels in the cytosol of RASMCs 4 hours after treatment and HuR decreased expression in the nuclear fraction.

To test whether HuR could bind and stabilize sGCβ1 mRNA, a biotin-labeled ARE-containing oligoribonucleotide probe from the 3′ UTR of sGCβ1 mRNA was incubated for 30 minutes with nuclear extracts from control or H2O2-treated RASMCs. The protein–riboprobe complex formation was detected by RNA-EMSA. As observed in Figure 4B, a protein–riboprobe complex consisting of 4 distinct bands was formed in the H2O2-treated cells (lane 2) compared to controls (lane 1). The specificity of these bands was con-
firmed by a competition assay with a 100-fold excess of unlabeled oligonucleotide riboprobe (lane 4). Preincubation of the nuclear extracts from H2O2-treated cells with an antibody against HuR prevented the formation of the protein–riboprobe complex, whereas incubation with a nonspecific IgG had no effect (lanes 5 and 3).

To further study the involvement of HuR in H2O2 stabilization of sGC, HuR was silenced by means of small interfering (si)RNA nucleofection of RASMCs. As shown in Figure 5A, siHuR prevents the H2O2-induced upregulation of sGCβ1 in RASMCs compared to cells nucleotransfected with siNeg (as a control). In addition, we performed studies using SiHuR-nucleofected RASMCs to measure cGMP release in response to H2O2 treatment. Results shown in Figure 5B show that the increase in cGMP release in response to H2O2 is completely prevented by HuR downregulation compared to siNeg-transfected cells.

Endogenously Produced H2O2 Increases sGCβ1 Protein Expression, mRNA Stability, and HuR Translocation to Cytosol

We had previously demonstrated that angiotensin II (Ang II) increases intracellular H2O2 levels.30 To study whether endogenous H2O2 can regulate sGC expression, RASMCs were treated with Ang II or catalase. As observed in Figure 6A, Ang II mimicked the effects of exogenously added H2O2 on sGCβ1 protein expression, and this increase was abolished by catalase. Ang II also increased sGCβ1 mRNA stability, and catalase treatment blocked this effect (Figure 6B). Additionally, Ang II modified HuR localization, inducing an increased cytosolic and a decreased nuclear HuR content, an effect that was also reversed by catalase (Figure 6C). These experiments support that H2O2 could act as an endogenous regulator of sGCβ1 expression, under certain pathophysiological conditions.
Discussion

The main finding of our study is that H$_2$O$_2$ increases sGC$_1$ gene expression and protein content, as well as sGC activity, by a posttranscriptional mechanism that involves HuR binding to the sGC$_1$ mRNA 3’UTR, increasing its stability. The increase in sGC$_1$ protein levels was associated with an increase in vascular relaxant responses to NO in rat aortic rings.

There is evidence supporting the role of ROS in the regulation of sGC expression. There is evidence supporting the role of ROS in the regulation of sGC expression. Our results show that H$_2$O$_2$ can upregulate the expression of the $\beta_1$ subunit of sGC in a time- and dose-dependent manner in both cells and aortic tissue. sGC is a heterodimer made up of 2 subunits, $\alpha$ and $\beta$. Two isoforms are known to exist (a1$b_1$ and a2$b_1$) in which the $\beta_1$ subunit acts as the dimerizing partner for either $\alpha$ subunit. Our results show that although a1 expression does not change, a2 subunit is also upregulated by H$_2$O$_2$ in a time-a dose-dependent manner; therefore, a2$b_1$ sGC may be the isoform responsible for increased cGMP and vascular relaxation to H$_2$O$_2$. The increase in sGC expression was associated with an increase in NO-dependent cGMP release, indicating that sGC was functional. Moreover, the increase in sGC levels had a biological effect because vascular relaxation responses to NO in rat aortic rings was enhanced in presence of H$_2$O$_2$. Recently, an opposite role for ROS in regulating sGC expression has been described. In their study, Gerassimou et al. describe a downregulation in sGC$_1$ mRNA caused by a transcriptional effect. However, the H$_2$O$_2$ concentration used in their study was significantly higher than the one used in our study. In addition, when these investigators exposed cells to a lower concentration of H$_2$O$_2$ (10$^{-5}$ mol/L), they observed no change in a1 subunit protein expression and an increase in $\beta_1$ subunit expression, which is consistent with our results. Indeed, by using Ang II to increase endogenous H$_2$O$_2$ production we also observed enhanced sGC$_1$ expression which is blocked by catalase. This increase in was attributable to mRNA stabilization, and it was specific because it was avoided by catalase.
Ang II upregulation of sGCβ1 is mediated by H2O2 stabilization of sGC mRNA and increased cytoplasmic HuR translocation. A, Western blot for sGCβ1 in protein extracts from RASMCs treated with saline (CT), 10−6 mol/L All, catalase (80 U/mL) (CAT), or CAT+All for 6 hours. Bottom, Densitometric analysis of 5 independent experiments expressed as percentage increase of control. *P<0.05 vs other groups. B, Cells were treated with All (10−6 mol/L) or CAT+All for 4 hours. DRB 10−5 mol/L was added to stop transcription, and mRNA expression was analyzed at 0 and 6 hours after DRB addition by Northern blot. sGCβ1 mRNA levels are expressed as percentage control (time 0). *P<0.05 vs DRB alone (n=6). C, HuR detection in cytosolic and nuclear extracts of RASMCs treated with vehicle alone (CT), All, CAT, or CAT+All as above. For control purposes, poly(ADP-ribose) polymerase (PARP), and β-actin were also detected. A representative experiment of 3 independent is shown.

More recently, it has been shown that aldosterone decreases sGC activity because of disulfide bond formation, and ROS seem to be involved in the genesis of this effect.35 No changes in protein levels were reported. We failed to observe a reduction in sGC activity in the presence of ROS, but the manner in which aldosterone induces oxidative stress may explain the effects observed. It is believed that aldosterone promotes a defect in antioxidant defenses that increase ROS accumulation, decreases NO levels, and diminishes endothelium-dependent vascular reactivity. In our study, we directly tested the effects of H2O2 without perturbing oxidant or antioxidant enzymatic cellular systems. In addition, our functional experiments confirm increased sGC activity in vivo and in vitro, and this increase was specific of H2O2 because the increase in sGCβ1 could be blocked by catalase. In addition, H2O2 can exert its vasorelaxant effects by different mechanisms that may be vascular bed–specific. In our study, a sGC inhibitor, ODQ, reverses the NO-dependent vasodilator effect enhanced by H2O2, suggesting that that sGC upregulation may be responsible for the effects observed, although the participation of other pathways cannot be excluded. Thus, H2O2 can stimulate endothelial nitric oxide synthase expression and also increase its activity, thereby increasing NO release and cGMP synthesis and producing a vasorelaxant effect.16–18 Alternatively, H2O2 may stimulate the production of prostanoids, which also produce vasodilatation. It is generally accepted that vasodilation produced by prostacyclin is mediated by cAMP, which may relax vascular smooth muscle by several different mechanisms.14

The expression of sGC mRNA can be regulated by different agents. It has been described that NO and cAMP can downregulate sGC by modifying its mRNA stability. Furthermore, hypoxia and ECM can also modulate sGC mRNA expression.36–40 The 3′UTR region of the sGC gene contains several AUUUA-rich sequences that intervene in mRNA stability. HuR binds to these sequences, thereby stabilizing mRNA in the cytoplasm.41 Our results show that after H2O2 treatment sGCβ1 mRNA half-life increases and HuR binds to the ARE sequences present in the 3′UTR region of sGCβ1 mRNA. The binding is specific because it could be competed by a nonlabeled RNA probe and prevented by incubation of extracts with a HuR antibody. The increased binding of HuR could be explained by an increased expression of HuR in response to H2O2. The mechanisms that regulate the localization or function of HuR are not fully understood, but recent studies have provided some important clues. In the majority of studies in which an upregulation of mRNA stability has been described, cytosolic HuR does increase.42 Indeed, our results show a diminished presence of HuR in the nuclei of H2O2-treated cells, favoring a cytosolic localization that could account for the increase in sGC mRNA stability. Endogenous H2O2 induced by Ang II also increased HuR presence on the cytosol, and this effect was reversed by catalase. HuR silencing reversed only not the effects of H2O2 in sGCβ1 protein levels but also sGC function because cGMP did not increase in response to NO in HuR-silenced cells exposed to H2O2.

It appears that H2O2 is a vasoactive agent and may play an important role as an endogenous mediator contributing to the regulation of vascular tone and possibly local blood flow in the microcirculation under physiological and/or pathophysiological conditions. In view of our results, it is possible that endogenous H2O2 can contribute to vasodilation by stabilizing sGC mRNA through HuR, increasing the expression of sGC and hence cGMP formation.

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Disclosures

None.

References

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Acrylamide and bisacrylamide were from Merck (Darmstadt, Germany). Hydrogen peroxide, phenylmethylsulfonylfluoride (PMSF), leupeptin, ammonium persulfate, sodium nitroprusside, salmon sperm DNA, formaldehyde, guanidinium thiocyanate, formamide, 1H-(1,2,4) Oxadiazolo (4,3-a)quinoxalin-1-one (ODQ), collagenase IV, anti-sGC-β1 and α1 antibodies, and α-actin antibody were purchased from Sigma Chemical (St. Louis, MO, USA). Tissue culture media and growth supplements were from BioWhittaker (Walkersville, MD, USA). The random prime labeling system (Rediprime II), nylon filters (Hybond N), and deoxy-[32P] cytidine triphosphate and RIA kit were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). The bicinecinonic acid (BCA) assay kit and LightShift Chemiluminescent EMSA kit were from Pierce (Rockford, IL, USA). The polyvinylidene difluoride membrane was from Perkin Elmer (Boston, MA, USA). All the reagents employed were of the highest commercially available grade.

METHODS

Cell culture

Rats Aortic Smooth Muscle Cells (RASMC) were obtained from thoracic aortas of Wistar rats by methods described previously¹. Briefly, Wistar rats (125-200g) were sacrificed and exsanguinated. Thoracic aortas were removed, cleaned of surrounding tissues, dissected into small strips, and incubated in DMEM/Ham’s F-12 medium with collagenase type IV at 37°C for 45 min. Digested strips were seeded onto 100-mm diameter dishes and maintained DMEM/Ham’s F-12 with 10% fetal calf serum, at 37°C, in a humidified atmosphere of 5% CO₂. The cells were used between the third and fifth passages.
**Protein Extraction and Western Blot Analysis**

Following treatment, cells were washed in PBS and solubilized (10 mmol/L Tris-HCl pH 7.4, 1 mmol/L EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 50 nmol/L sodium orthovanadate, 50 nmol/L NaF, 1 mmol/L pepstatin/leupeptin/aprotinin, 1 mmol/L PMSF) for 30 min at 4ºC. Immunoblotting was performed as described. Immunoreactive bands were visualized with the SuperSignal detection system according to the manufacturer’s instructions (Pierce, Rockford, IL, USA).

**Immunohistochemistry**

Aortas were harvested from Wistar rats and treated with or without hydrogen peroxide for 2h, fixed with formalin and subjected to increasing concentrations of ethanol and xylene. Tissues were embedded in paraffin, and cut into serial sections 3-5 μm thick and incubated with anti sGC β1 (Sigma, dilution 1:100). The aortic sections were then incubated with biotin–labelled goat anti-mouse antibody (DAKO, Carpinteria, CA, USA) followed by exposure to avidin–peroxidase complex (DAKO). Finally, diaminobenzidine (DAB, DAKO) was added to serve as a substrate. Samples were counterstained with Mayer’s Haematoxylin. Negative controls were obtained by omitting primary antibodies (IgG).

**Measurement of cGMP Synthesized by sGC**

Control and hydrogen peroxide-treated cells were washed with buffer A (Tris 20 mmol/L, NaCl 130 mmol/L, KCl 5 mmol/L sodium acetate 10 mmol/L, glucose 5 mmol/L, pH 7.45). Cells were then preincubated with the same buffer containing 2.5 mmol/L Ca²⁺ and IBMX 10⁻⁴ mol/L. The reactions were started by the addition of a NO donor (SNP 10⁻⁶ mol/L) for 15 min. The medium was aspirated and 1 ml of ice-cold ethanol was added to the plates and maintained at 4ºC for 30 min. Concentrations of
cGMP were determined with a commercial $^{125}$IcGMP RIA kit. Protein concentration was
determined by the BCA assay.

**RNA Isolation and Northern Blot Analysis**

Total RNA from RASMC was isolated as described$^3$. For Northern analysis, a
set of primers for sGC-$\beta$1 (forward, base position 350, 5'-CGTGTCCTGGGCTCTAA-3'
and reverse, base position 774, 5'-ACCACTAGGTCCCGGTC-3') was used to amplify a
408 bp cDNA probe in a reverse transcription-polymerase chain reaction$^4$. The
fragment of human sGC $\beta$1 subunit and a GAPDH RNA probe were radiolabeled as
recommended by the manufacturer (Redi Prime, Amersham Pharmacia Biotech,
Buckinghamshire, UK). The membranes were hybridized at 60°C and autoradiography
was performed. Densitometric analysis of the exposed films was performed with a
scanner and analysed using NIH Image 1.55 software.

**Transient Transfection and Luciferase Assays**

RASMC were plated on six well plates for 24 h. RASMC were then incubated
with 1 $\mu$g of sGC b1 subunit promoter$^5$ and 4 $\mu$l of LipofectAMINE reagent according
to the manufacturer’s instructions (Invitrogen Co. Carlsbad, CA, USA). Luciferase activity
was determined using the Dual Luciferase Reporter Kit (Promega). Firefly luciferase
activity was evaluated (Monolight 2010 luminometer, Analytical Luminescence
Laboratory, Ann Arbor, MI, USA) and normalized against Renilla luciferase activity.
Results were normalized as relative luciferase light units/mg protein.

**RNA Interference.**

Cells were nucleotransfected by electroporation using a Nucleofector (Amaxa
Biosystems,) set to the U25 program with 2 $\mu$g of annealed small interfering (si)RNA
duplexes for HuR (Qiagen,) or nonsilencing control sequence (All-star Negative,
Qiagen) per 1$x$10$^6$ cells. siRNA duplexes were synthesized using a previously
published sequence ( sense: 5’-GAU GCC AAC UUG UAC AUC ATT -3’; antisense: 5’-
UGA UGU ACA AGU UGG CAU CTT -3'). After transfection, RAVSMC were allowed to attach overnight in complete medium.

**RNA-EMSA.**

RNA-electrophoretic mobility shift assays (EMSA) were carried out as described recently. The biotin-labeled oligoribonucleotides β1GC3UTR (5’AAACUGCUUUUCUGUAAAAUGUUGUCUUUCUUUAGUA-3’) comprising bases 2929 to 2968 of the 3'-UTR from the sGCβ1 mRNA were used as probes. The oligoribonucleotides (150 ng) were incubated with 20 μg of total protein extract from endothelium-denuded rat aorta and a reaction mixture [10x reaction buffer (100 mmol/L Tris, pH 7.5, 500 mmol/L KCl, 10 mmol/L DTT; LightShift chemiluminescent EMSA Kit, 1.5% glycerol, 5 mmol/L MgCl₂, 0.05% Nonidet P-40, 2 units/μl RNase inhibitors (40 units/μl, RNaseOUT), 200 ng/ml total tRNA] for 30 min at 4°C. Complexes were resolved by native 8% polyacrylamide gel electrophoresis for 2 to 3h at 4°C and electroblotted onto nitrocellulose filters. Blocking and detection of biotin-labeled bands was performed as described previously. For supershifts, 4 μg of the monoclonal HuR-antibody was incubated with the native protein extract for 1 h on ice before the specific riboprobe was added; all subsequent steps were performed as described for native gels.

**Statistical Analysis**

Data are presented as mean ± SEM of a variable number of experiments (see figure legends). For animal studies the n values refer to the number of individual animals in which the experiment was performed. The data are expressed as the mean ± S.E. Non-parametric statistics were used for comparisons (Friedman´s and Wilcoxon´s test). A p<0.05 was considered statistically significant.
REFERENCES


Supplemental figure I. Hydrogen peroxide (H$_2$O$_2$) increases soluble guanylate cyclase β1 subunit [sGC (β1)] protein levels in cultured rat aortic smooth muscle cells (RASMC) in a dose dependent manner. Western blot for sGC (β1) in protein extracts from RASMC treated with different H$_2$O$_2$ concentrations (10$^{-6}$-10$^{-4}$ mol/L) for 4 hours. Lower panel shows the densitometric analysis of 10 independent experiments expressed as % increase of control. *p< 0.05 vs. control (0).
Supplemental Figure II: Hydrogen peroxide does not change soluble guanylate cyclase α1 subunit [sGC (a1)] protein levels in cultured rat aortic smooth muscle cells (RASMC). Western blot for sGC (a1) in protein extracts from RASMC treated with and without H$_2$O$_2$ (10$^{-4}$ mol/L) at different time points (A) or H$_2$O$_2$ concentrations (10$^{-6}$-10$^{-4}$ mol/L) for 4 hours (B). Lower panel shows the densitometric analysis of 10 independent experiments expressed as % increase of control. *p< 0.05 vs. control (0).
Supplemental Figure III: Hydrogen peroxide increases guanylate cyclase α2 subunit [sGC (α2)] protein levels in cultured rat aortic smooth muscle cells (RASMC). Western blot for sGC (α2) in protein extracts from RASMC treated with and without H₂O₂ (10⁻⁴ mol/L) at different time points (A) or H₂O₂ concentrations (10⁻⁶-10⁻⁴ mol/L) for 4 hours (B). Lower panel shows the densitometric analysis of 10 independent experiments expressed as % increase of control. *p< 0.05 vs. control (0).
Supplemental Figure IV: Arterial responses to noradrenaline (NA) are not affected by \( \text{H}_2\text{O}_2 \) or ODQ treatment. Degree of arterial contraction to in rat aortic rings pretreated with saline (CT), \( 10^{-4} \text{ mol/L H}_2\text{O}_2 \) (\( \text{H}_2\text{O}_2 \)), ODQ (ODQ) or \( \text{H}_2\text{O}_2 + \text{ODQ} \) for 4h. Results are expressed as % increase of contraction to basal tension.