H_2O_2 Regulation of Vascular Function Through sGC mRNA Stabilization by HuR

Abel Martín-Garrido, Marta González-Ramos, Mercedes Griera, Brenda Guijarro, Jorge Cannata-Andia, Diego Rodriguez-Puyol, Manuel Rodriguez-Puyol, Marta Saura

Objective—Hydrogen peroxide (H_2O_2) 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 H_2O_2 on sGC expression and function and to explore the molecular mechanism involved.

Methods and Results—H_2O_2 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 H_2O_2, and it was prevented by ODQ (sGC inhibitor). cGMP production in both freshly isolated vessels and RASMCs exposed to H_2O_2 was greatly increased after sodium nitroprusside treatment. The H_2O_2-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 H_2O_2 on sGCβ1 levels and cGMP synthesis.

Conclusion—Our results identify H_2O_2 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 ■ vasodilation

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 (H_2O_2), formed from superoxide (O_2^-) as a result of the activity of various superoxide dismutases, is an important regulator of the function of the vascular wall.[6,7] H_2O_2 has been detected in people under both physiological and pathophysiological conditions.[8–10] In humans, H_2O_2 can be detected in normal controls, as well as in patients with cardiovascular or pulmonary disease. H_2O_2 may modulate the contraction, proliferation, and apoptosis rate and the synthesis of ECM proteins in endothelial or smooth muscle cells.[11]

H_2O_2 has been proposed to be an endothelium-derived hyperpolarizing factor in peripheral arteries.[12,13] H_2O_2 may also dilate arteries by alternative mechanisms, such as the stimulation of the production of prostanoids[14] or the activation of soluble guanylate cyclase (sGC).[15] Moreover, under some circumstances, H_2O_2 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 al[26] 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 H_2O_2, to modulate the cellular content of

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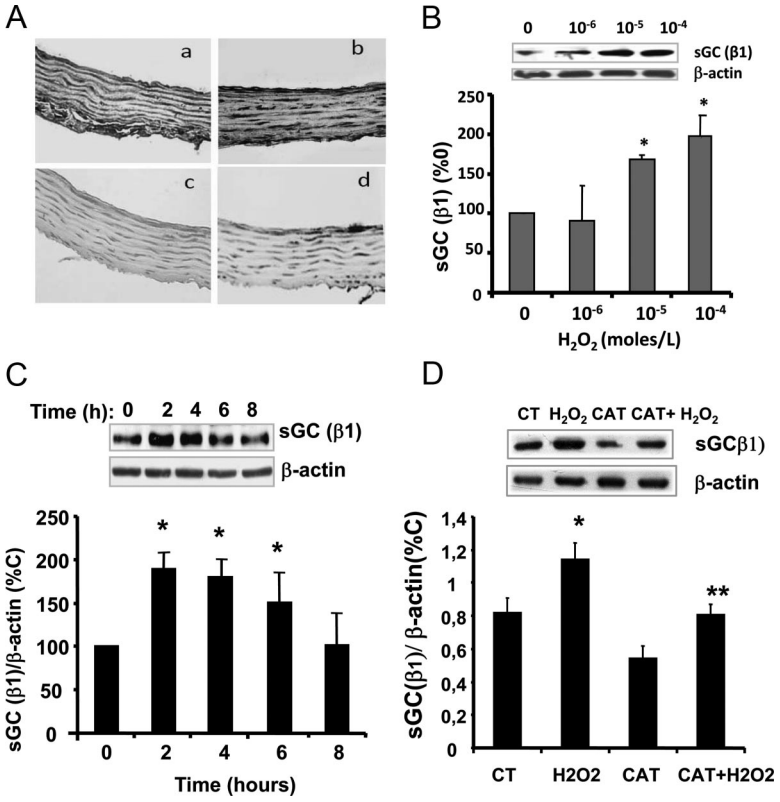


Figure 1. H_2O_2 increases sGCβ1 protein levels in rat aorta and in cultured RASMCs. A, a and b, Detection of sGCβ1 in rat aortic rings treated with saline (a) or 10^{-4} mol/L H_2O_2 (b) for 4 hours. c and d, Negative controls. Magnification=×200. Microphotographs are representative of 8 independent experiments. B, Western blot for sGCβ1 in protein extracts from rat aortic rings treated with and without H_2O_2 at different concentrations (10^{-6} to 10^{-4} mol/L) for 4 hours. Bottom, Densitometric analysis of 10 independent experiments expressed as percentage increase of control. *$P<0.05$ vs control (0). C and D, Western blot for sGCβ1 in protein extracts from RASMCs treated with and without H_2O_2 (10^{-4} mol/L) at different time points (C) or saline (CT), H_2O_2 (10^{-4} mol/L) , catalase (80 U/mL) (CAT), or H_2O_2+CAT for 4 hours (D). Bottom, Densitometric analysis of 5 independent experiments expressed as percentage increase of control. *$P<0.05$ vs the other groups; **$P<0.05$ vs H_2O_2.

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.[27] 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% O_2/5% CO_2) 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 K^+ to verify their functional integrity. After washing, the segments were first incubated for 2 hours with H_2O_2 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 1-*H*-(1,2,4)-oxidiazolo-(4,3-a)-quinoxalin-1-one (ODQ) (1 μmol/L).

Results

H_2O_2 Increases sGCβ1 Subunit Protein Expression in Rat Aorta and in Cultured RASMCs

To assess the effect of H_2O_2, freshly isolated rat aortic rings were incubated with increasing concentrations of H_2O_2 (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 H_2O_2 treatment. Figure 1B shows that H_2O_2 increased sGCβ1 levels in a dose-dependent manner.

As observed in Figure 1C, H_2O_2 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 H_2O_2 (supplemental Figure I). In contrast, α1 subunit protein levels were not affected by H_2O_2 (supplemental Figure II). Because sGC functions as an heterodimer, we also tested α2 subunit expression observing an increase in response to H_2O_2 treatment, which was time- and concentration-dependent (supplemental Figure III). The effects of H_2O_2 effects on sGCβ1 expression were specific because catalase abolished the increase in sGCβ1 levels in response to H_2O_2 treatment of cultured RASMCs (Figure 1D).

H_2O_2 Enhances sGC Function in Rat Aorta and RASMCs

To test the biological consequences of the enhanced sGC levels in the presence of H_2O_2, the vascular reactivity to an

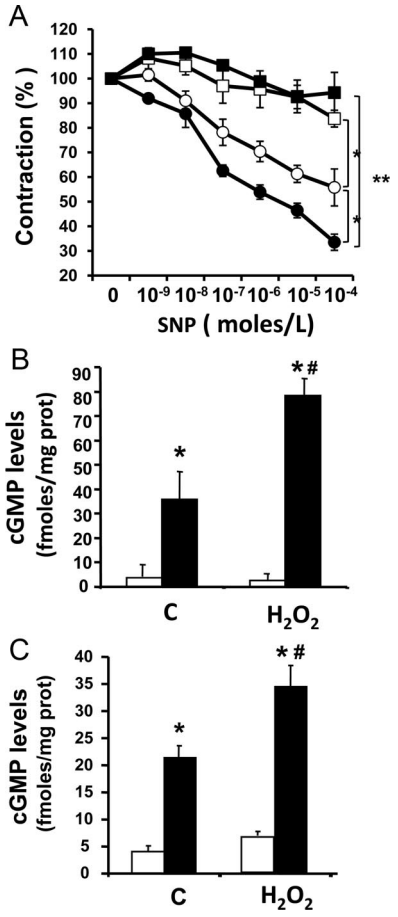


Figure 2. H_2O_2 enhances sGC function in rat aorta and RASMCs. A, Arterial relaxant responses to increasing doses of sodium nitroprusside (SNP) in rat aortic rings pretreated with saline (white circles), 10^{-4} mol/L H_2O_2 (black circles), saline+ODQ (white squares), or H_2O_2+ODQ (black squares) 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. *$P<0.05$ vs saline; **$P<0.05$ vs H_2O_2. Rat aortic rings (B) or RASMCs (C) were isolated in IBMX buffer, treated with H_2O_2 for 4 hours, and stimulated (gray bars) or not with SNP (white bars) for 30 minutes, and cGMP was measured. *$P<0.05$ vs unstimulated control; #$P<0.05$ vs SNP alone (n=6).

NO donor (SNP; 10^{-9} to 10^{-4} mol/L) was studied. Endothelium-denuded rat aortas were pretreated with saline or H_2O_2 (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 H_2O_2 treatment (supplemental Figure IV). H_2O_2 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 H_2O_2+SNP. To confirm this result, cGMP accumulation in rat aortic rings exposed to NO or H_2O_2+NO was measured (Figure 2B). After SNP treatment, cGMP levels increased 7-fold in aortic tissue. Treatment with H_2O_2 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 H_2O_2+NO (Figure 2C). NO treatment induced a 6-fold elevation in cGMP levels in control cells. Treatment with H_2O_2 produced an additional increase in cGMP levels compared to NO-treated cells.

H_2O_2 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 H_2O_2 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 H_2O_2 in a dose-dependent manner.

The effect of H_2O_2 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 H_2O_2 (10^{-4} mol/L) for 24 hours. Human sGCβ1 promoter transactivation was measured as firefly luciferase activity normalized to Renilla luciferase activity. H_2O_2 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 H_2O_2-dependent increase in sGCβ1 mRNA levels, RASMCs were exposed to H_2O_2 (10^{-4} mol/L) for 4 hours, transcription was stopped by addition of DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole) (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, H_2O_2 prolonged the half-life of sGC mRNA for more than 4 hours.

H_2O_2 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 H_2O_2 on the levels of the mRNA-stabilizing protein, HuR. As shown in Figure 4A, H_2O_2 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 H_2O_2-treated RASMCs. The protein–riboprobe complex formation was detected by RNA-EMSA. As observed in Figure 4B, a protein–riboprobe complex consisting on 4 distinct bands was formed in the H_2O_2-treated cells (lane 2) compared to controls (lane 1). The specificity of these bands was con-

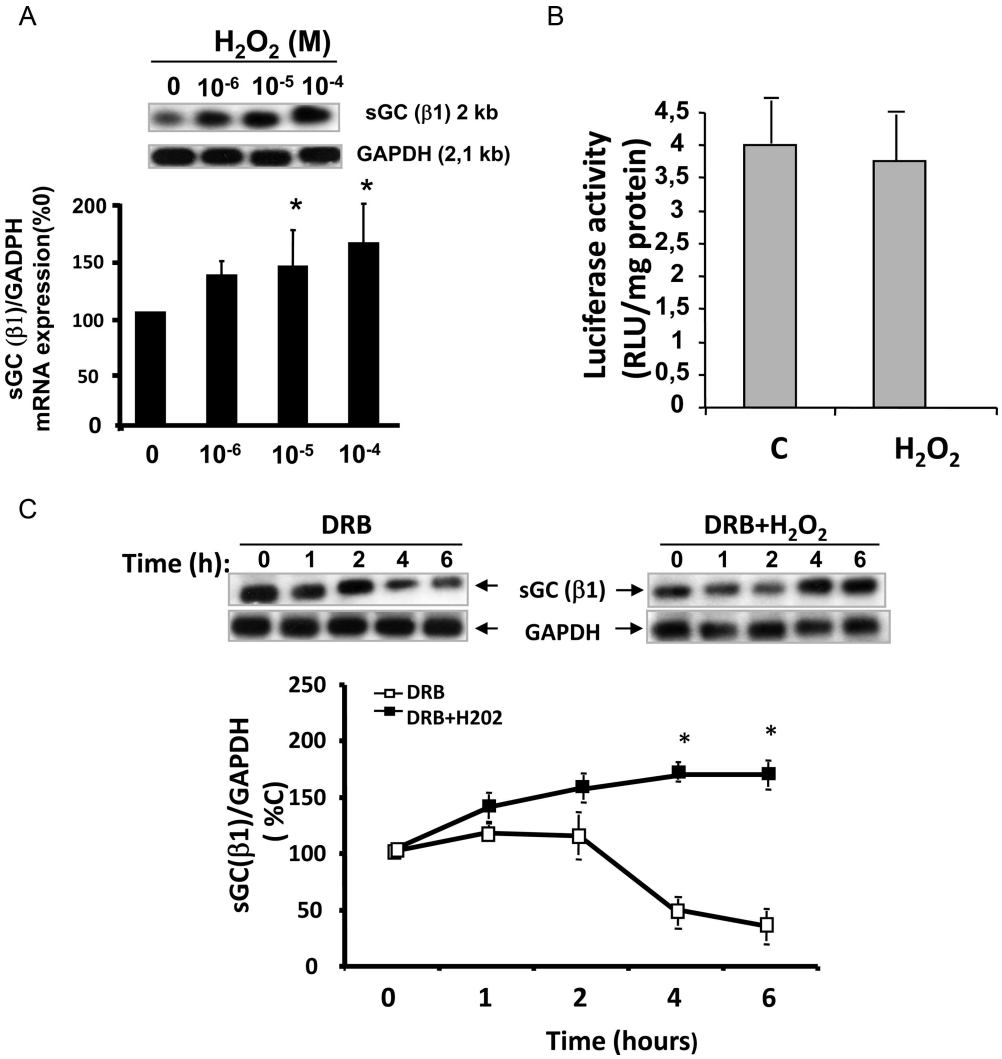


Figure 3. H_2O_2 increases sGCβ1 subunit mRNA stability. A, mRNA expression of sGCβ1 subunit from RASMCs treated or not with H_2O_2 for 4 hours at different concentrations (10^{-6} to 10^{-4} mol/L). Bottom, Densitometric analysis expressed as percentage increase of control (0). *$P<0.05$ vs C (n=3). B, RASMCs were transfected with the reporter construct of the sGCβ1 promoter, treated with or without H_2O_2, and luciferase activity was determined (n=4). C, Cells were treated with H_2O_2 (10^{-4} mol/L) for 4 hours, DRB 10^{-5} mol/L was added to stop transcription, and mRNA expression was analyzed at different time points by Northern blot. sGCβ1 mRNA levels are expressed as percentage control (time 0). *$P<0.05$ vs DRB alone (n=6).

firmed by a competition assay with a 100-fold excess of unlabeled oligonucleotide riboprobe (lane 4). Preincubation of the nuclear extracts from H_2O_2-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 H_2O_2 stabilization of sGC, HuR was silenced by means of small interfering (si)RNA nucleofection of RASMCs. As shown in Figure 5A, siHuR prevents the H_2O_2-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 H_2O_2 treatment. Results shown in Figure 5B show that the increased cGMP release in response to H_2O_2 is completely prevented by HuR downregulation compared to siNeg-transfected cells.

Endogenously Produced H_2O_2 Increases sGCβ1 Protein Expression, mRNA Stability, and HuR Translocation to Cytosol

We had previously demonstrated that angiotensin II (Ang II) increases intracellular H_2O_2 levels.[30] To study whether endogenous H_2O_2 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 H_2O_2 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 H_2O_2 could act as an endogenous regulator of sGCβ1 expression, under certain pathophysiological conditions.

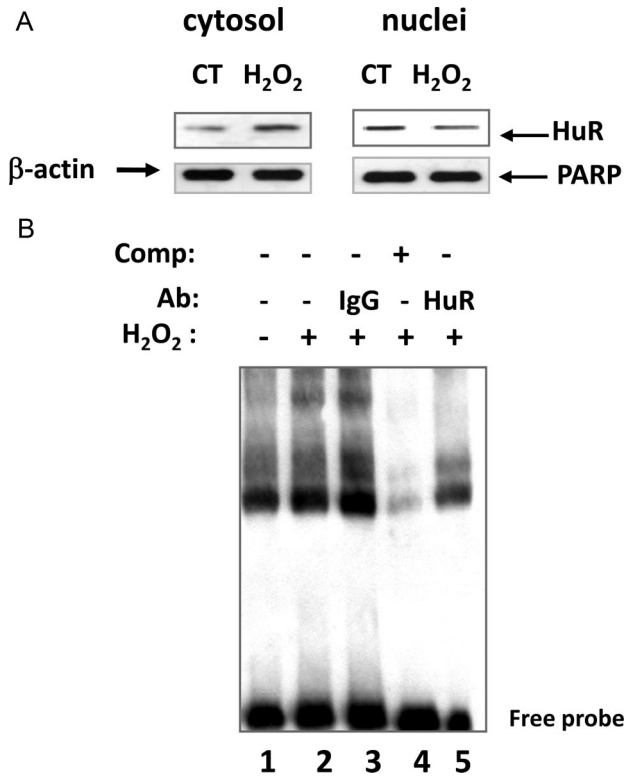


Figure 4. H_2O_2 promotes HuR cytoplasmic translocation and HuR binding to the 3′UTR of sGCβ1 subunit mRNA. A, HuR detection from cytosolic and nuclear extracts of RASMCs treated with vehicle alone or H_2O_2 10^{-4} mol/L. As a control, poly(ADP-ribose) polymerase (PARP), and α-actin were also detected. A representative experiment of 3 independent is shown. B, RNA-EMSA analysis of nuclear extracts from RASMCs treated with vehicle (lane 1) or 10^{-4} mol/L H_2O_2 (lanes 2 to 5) for 4 hours using a sGC(β1) mRNA 3′UTR biotin-labeled probe. In some cases an unlabeled excess of 3′UTR competitor (lane 4), a specific HuR Ab (lane 5), or a normal IgG (lane 3) was included in the assay (n=3).

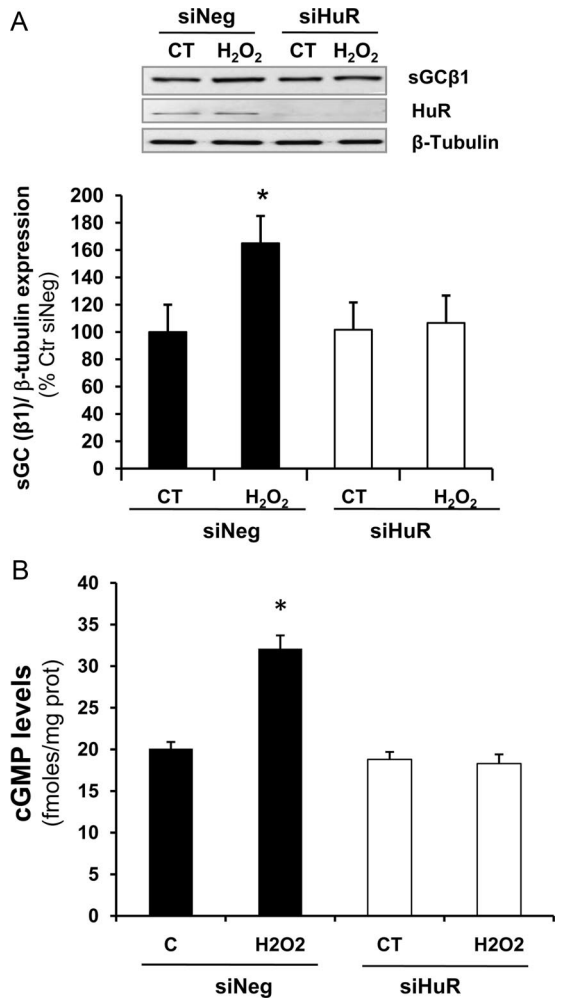


Figure 5. HuR silencing prevents H_2O_2-induced upregulation of sGCβ1 in RASMCs. A, Western blot for sGCβ1 and HuR in protein extracts from RASMCs nucleotransfected with siNeg as a control or siHuR and treated with and without H_2O_2 (10^{-5} mol/L) for 4 hours. Bottom, Densitometric analysis expressed as percentage increase of control (siNeg) (n=3). *$P<0.05$ vs the other groups. B, RASMCs nucleotransfected (white bars) or not (black bars) with siHuR were treated with H_2O_2 for 4 hours and then stimulated with SNP for 30 minutes. Cell were isolated in IBMX buffer, and cGMP was measured (n=5). *$P<0.05$ vs the other groups.

Discussion

The main finding of our study is that H_2O_2 increases sGCβ1 gene expression and protein content, as well as sGC activity, by a posttranscriptional mechanism that involves HuR binding to the sGCβ1 mRNA 3′UTR, increasing its stability. The increase in sGCβ1 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.[31–33] Our results show that H_2O_2 can upregulate the expression of the β1 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, α and β. Two isoforms are known to exist ($\alpha1\beta1$ and $\alpha2\beta1$) in which the β1 subunit acts as the dimerizing partner for either α subunit.[34] Our results show that although α1 expression does not change, α2 subunit is also upregulated by H_2O_2 in a time- a dose-dependent manner; therefore, $\alpha2\beta1$ sGC may be the isoform responsible for increased cGMP and vascular relaxation to H_2O_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 were enhanced in presence of H_2O_2. Recently, an opposite role for ROS in regulating sGC expression has been described.[24] In their study, Gerassimou et al[24] describe a downregulation in sGCα1 mRNA caused by a transcriptional effect. However, the H_2O_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_2O_2 (10^{-5} mol/L), they observed no change in α1 subunit protein expression and an increase in β1 subunit expression, which is consistent with our results. Indeed, by using Ang II to increase endogenous H2O2 production we also observed enhanced sGCβ1 expression which is blocked by catalase. This increase in was attributable to mRNA stabilization, and it was specific because it was avoided by catalase.

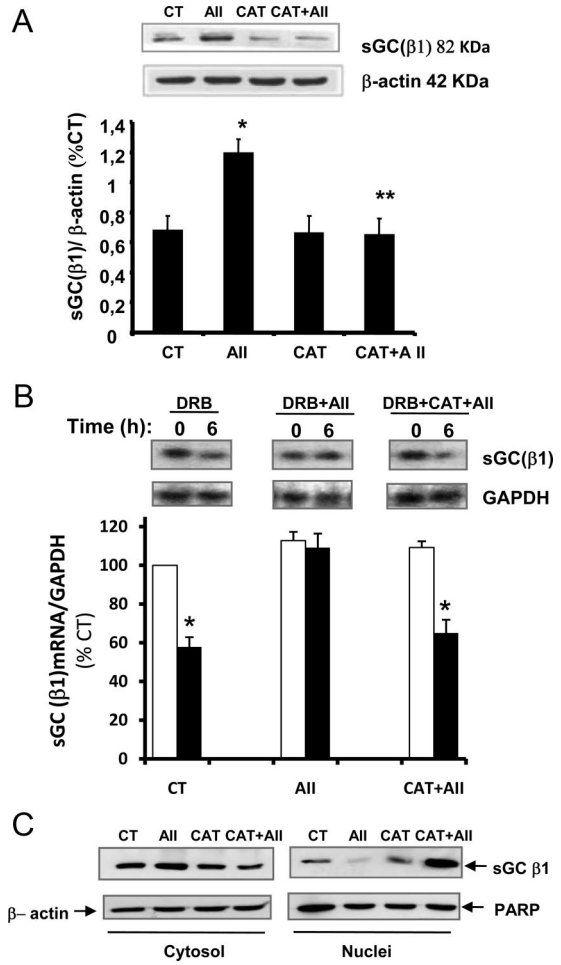


Figure 6. Ang II (AII) upregulation of sGCβ1 is mediated by H_2O_2 stabilization of sGC mRNA and increased cytoplasmic HuR translocation. A, Western blot for sGCβ1 in protein extracts from RASMCs treated with saline (CT), 10^{-6} mol/L AII, catalase (80 U/mL) (CAT), or CAT+AII for 6 hours. Bottom, Densitometric analysis of 5 independent experiments expressed as percentage increase of control. *$P<0.05$ vs the other groups. B, Cells were treated with AII (10^{-6} mol/L) or CAT+AII 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), AII, CAT, or CAT+AII 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 H_2O_2 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 H_2O_2 because the increase in sGCβ1 could be blocked by catalase.

In addition, H_2O_2 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 H_2O_2, suggesting that that sGC upregulation may be responsible for the effects observed, although the participation of other pathways cannot be excluded. Thus, H_2O_2 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, H_2O_2 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 H_2O_2 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 H_2O_2. 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 H_2O_2-treated cells, favoring a cytosolic localization that could account for the increase in sGC mRNA stability. Endogenous H_2O_2 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 H_2O_2 in sGCβ1 protein levels but also sGC function because cGMP did not increased in response to NO in HuR-silenced cells exposed to H_2O_2.

It appears that H_2O_2 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 H_2O_2 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.

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