Rivero-Vilches, F.J. et al. (2003) 'Differential relaxing responses to particulate or soluble guanylyl cyclase activation on endothelial cells: A mechanism dependent on PKG-Iα activation by NO/cGMP', American Journal of Physiology: Cell Physiology, 285(4), pp. C891–C898.

Available at https://doi.org/10.1152/ajpcell.00590.2002

(Article begins on next page)

Articles in PresS. Am J Physiol Cell Physiol (June 18, 2003). 10.1152/ajpcell.00590.2002

Title Page

Title:

Differential relaxing responses to particulate or soluble guanylyl cyclase activation on endothelial cells: a mechanism dependent on PKG-Iα activation by NO/cGMP.

Running title:

Differential actions of cGMP on endothelial cell relaxation.

Authors:

F. J. Rivero-Vilches [a,b,#], S. De Frutos [a,b,#], M. Saura [a,b] *, D. Rodriguez-Puyol [b,c], M. Rodriguez-Puyol [a,b,].

Institutional Affiliations:

[a] Departmento de Fisiología, Universidad de Alcalá, Alcalá de Henares, Madrid, Spain.

[b] Instituto Reina Sofia de Investigación Nefrológicas, Madrid, Spain.

[c] Sección de Nefrología, Hospital Príncipe de Asturias, Alcalá de Henares, Madrid, Spain.

#: F. J. Rivero-Vilches and S. De Frutos have contributed equally to this work.

*Correspondence to: Dr. Marta Saura.

Dpto. Fisiología, Facultad de Medicina, Universidad de Alcalá, Crta. Madrid-Barcelona Km 33.600, Alcalá de Henares, 28871 Madrid, SPAIN. Phone: 34-91-885-4551; fax: 34-91-885-4590.

E-mail address: marta.saura@uah.es

Abstract

cGMP is generated in endothelial cells after stimulation of soluble guanylyl cyclase (sGC) by nitric oxide (NO) or of particulate guanylyl cyclase (pGC) by natriuretic peptides (NP). We examined whether localized increases in cytosolic cGMP have distinct regulatory roles on the contraction induced by H_2O_2 treatment in human umbilical vein endothelial cells (HuVEC). cGMP concentrations and temporal dynamics were different upon NO stimulation of sGC or C-type NP (CNP) activation of pGC, and did not correlate with their relaxing effects measured as planar cell surface area (PCSA) after H_2O_2 challenge. cGMP production due to sGC stimulation was always smaller and brief than that induced by pGC stimulation with CNP, which was greater and remained elevated longer. The NO effects on cell relaxation were cGMP dependent since they were blocked by sGC inhibition with ODQ and mimicked by 8-Br-cGMP. An antagonist of the cGMP-dependent protein kinase type-I (PKG-I) also inhibited the NO-induced effects. The cell contraction induced by H_2O_2 produces myosin light chain (MLC) phosphorylation and NO prevented it completely whereas CNP only produced a partial inhibition. Transfection with a dominant negative form of PKG type-Iα completely reversed the NO-induced effects on MLC phosphorylation while it only partially inhibited the effects due to CNP. Taken together these results demonstrate that the NO/sGC/cGMP pathway induces endothelial cell relaxation in a more efficient manner than CNP/pGC/cGMP pathway, an effect that might be related to a selective stimulation of PKG-1α by NO-derived cGMP. Consequently, stimulated PKG-Iα may phosphorylate important protein targets that are necessary to inhibit the endothelial contractile machinery activated by oxidative stress.

Keywords:

Nitric Oxide, C-type Natriuretic Peptide, Myosin light chain, cGMP-dependent Protein Kinase type Iα, Endothelial Cell Barrier Dysfunction.

Introduction

Vascular homeostasis depends on the ability of the endothelium to maintain its integrity, serving as a non-adherent non-thrombogenic surface and as a barrier that regulates the exchange of fluid and macromolecules between the blood and the extracellular tissue (9, 15). During vascular disease, endothelial cells are exposed to excess reactive oxygen species that can alter the endothelial cell phenotype by inducing several signaling pathways to generate second messengers that modulate the structure and organization of cytoskeletal proteins (20). These cytoskeletal alterations lead to changes in cell shape and the formation of paracellular gaps that impair the endothelial cell barrier function (2, 10, 36, 39).

Several laboratories, including ours, have demonstrated that reactive oxygen species can induce endothelial cell contraction, which could be responsible for the increased endothelial permeability that often accompanies ischemia/reperfusion injuries (5, 17, 18, 20). Agents that activate guanylyl cyclases (GC) have been shown to relax smooth muscle cells; similarly cGMP-elevating agents seem to attenuate oxidant-induced endothelial cell barrier dysfunction in some vascular beds (6, 10, 21, 24).

cGMP is a second messenger involved in many physiological processes such as smooth muscle tone, neural excitability, epithelial electrolyte transport, phototransduction in the retina and cell proliferation (26). Despite the enormous importance of cGMP in cell physiology, little attention is given to the fact that its formation is not uniformly distributed within the cell. cGMP can be formed from

GTP by the action of two distinct guanylyl cyclases: a soluble form (sGC) and a particulate membrane–bound form (pGC) (19), each of which form is activated by different agonists. Nitric oxide (NO) and NO donors activate sGC whereas pGC is a plasma membrane receptor for natriuretic peptides and related hormones. The pathways that control cGMP levels are complex due to the existence of several ubiquitously expressed phosphodiesterases (PDE), which hydrolyse cGMP (4). Some PDE are soluble while others are plasma membrane bound. The intracellular actions of cGMP are primarily mediated by cGMP-dependent protein kinases (PKG) but several types of cyclic nucleotide-activated ion channels also appear to be involved (7, 16)

Given the separate sources of cGMP within the cell, it is possible to conceive a functional compartmentalization of cGMP due to localized elevation of these second messengers within the cell i.e.: membrane and cytosol. The purpose of this study was to examine the endothelial cell relaxing activities of the cytosolic and particulated pools of cGMP in human endothelial cells exposed to hydrogen peroxide (H_2O_2) and to determine the mechanism by which cGMP inhibits contractility. We previously demonstrated that NO and natriuretic peptides are able to reverse the contraction induced by H_2O_2 in bovine endothelial cells, an effect that was mimicked by a cGMP analogue and mediated in part by PKG (17). The present study extends those results and addresses the hypothesis that cGMP generated via activation of soluble and particulate guanylyl cyclases may have differential activities in promoting endothelial cell relaxation. Our study will help to clarify how stimulation of different receptors that act via the same second messenger can elicit the appropriate functional response.

Materials and Methods

Materials

E-199 medium, fetal calf serum, L-glutamine, penicillin, streptomycin sulphate, and Hanks balanced salt solution were purchased by Biomedia (Boussens, France). Collagenase type IA from *Clostridium histolyticum*, hydrogen peroxide (H_2O_2), natriuretic peptide type C (CNP), sodium nitroprusside (SNP, NO donor), 8-Br-cGMP, 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, sGC inhibitor), Rp-8-[(4-Clorophenil)tyo]-cGMPs triethylamine (Rp-cGMPs, PKG type-I inhibitor), 3-isobutyl-1-methyl-xanthine (IBMX, PDE inhibitor), Zaprinast (PDE type-5 inhibitor) and monoclonal anti-myosin (light chains) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Spermidine-NONOate (sp-NO, NO donor) was purchased from Alexis Biochemicals (San Diego, CA, USA). EMEM medium was from BioWhittaker (Walkersville, MD, USA). Pansorbin was obtained from Calbiochem (La Jolla, CA, USA). cGMP RIA kit and [^{32}P]-orthophosphate were purchased from Amersham Pharmacia (Bukinghanshire, UK). VASP and P-VASP antibodies were purchased by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Plasmids

Flag-tagged PKG type-Iα regulatory region (fcGK-IαR) which acts as a dominant negative mutant when overexpressed in cells was a kind gift from Dr. D. Browning (3). pcDNA 3.1 plasmid obtained from Invitrogen Co. (Carlsbad, CA, USA) was used as a control plasmid in the transfection experiments.

Cell Culture

Human endothelial cells from umbilical vein (HuVEC) were obtained and cultured as described previously (8). Cells were seeded on dishes coated with gelatine 0.2% at 37ºC in a humidified atmosphere of 95% O_2 and 5% CO_2. Individual clones were established and subcloned to obtain pure cell populations. Clones were characterised by their typical cobblestone morphology, by the presence of factor VIII-related antigen and by the uniform uptake of fluorescent acetylated low-density lipoprotein, as described (13).Cells were fed every two days with E-199 medium supplemented with 20% fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 20 mM HEPES and 300 µg/mL endothelial cell growth factor. Cells were passaged when confluence was reached with trypsin-EDTA. Toxicity was evaluated in every experimental condition by the trypan blue dye exclusion method.

Measurement of planar cell surface area (PCSA)

Cells were grown at low density in 20-mm plates, and were studied before confluence. In every experiment, cells were washed twice, discarding the culture media and placed in buffer A (Tris 20 mM, NaCl 130 mM, KCl 5 mM, sodium acetate 10 mM, glucose 5 mM, pH 7.45) containing 2.5 mM Ca^{2+} and

maintained at room temperature. After 15 minutes of temperature equilibration, the experiments were started.

In the first group of experiments, the cells were preincubated with buffer, 1µM sp-NONOate or 0.1µM CNP for five minutes. H_2O_2 (100 µM) was subsequently added to each treatment. Microphotographs were taken before H_2O_2 addition (time 0) and 30 minutes after this addition (time 30).

In the second group, cells were preincubated with buffer, 10µM 8-Br cGMP (cGMP analogue), sp-NONOate or CNP. Five minutes later, H_2O_2 was added and the experiment was carried out as described previously.

In the third group of experiments, cells were preincubated with buffer, 1µM ODQ (sGC inhibitor) or 1µM Rp-cGMPs (PKG type I inhibitor) for 5 minutes. Then, sp-NONOate was added and cells were incubated for five additional minutes after which H_2O_2 was added. Microphotographs were taken as described previously, just before H_2O_2 addition and 30 min afterwards.

During each experiment, cells were observed under phase contrast with an inverted photomicroscope (Olympus IMT 2, Tokyo, Japan) with a 150 magnification. Photographs of the same cells were taken under the experimental conditions cited above. Every cell with a sharp margin suitable for the planimetric analysis was considered, and six to twelve cells were analysed per photograph, PCSA was determined by computer aid planimetric techniques (17, 35). Measurements were performed by two different researchers in a blind fashion. The intraobserver and interobserver variations were 2 % and 5%, respectively.

Measurement of cGMP synthesis by HuVEC

Cells were washed twice with buffer A. Cells were then preincubated in the same buffer containing 2.5 mM Ca^{2+} at room temperature. Reactions were started after addition of the reagents as indicated previously. At different intervals from 30 seconds to 30 minutes, the medium was aspirated and 1 mL of ice-cold ethanol was added to the plates, which were maintained at 4ºC for 30 minutes. Cell extracts were centrifuged for 20 minutes at 2000 x g, the supernatant fraction was evaporated to dryness and cGMP levels were determined with the use of a commercial [^{125}I]-cGMP radioimmunoassay kit as described (28). Protein concentration in the pellets was determined according to the Bradford method.

Measurement of myosin light chain phosphorylation

Phosphorylation of the myosin light chain (MLC) was determined after immunoprecipitation and protein separation by SDS-polyacrylamide gel electrophoresis, as reported previously (34). Briefly, after labelling the cells with 50 µCi/mL of neutralized, carrier-free sodium [^{32}P]-orthophosphate (3 h, 37ºC), incubations were performed under the conditions detailed elsewhere (see figure legends). Thereafter, the incubation media was removed, and cells were precipitated with ice-cold ethanol. After solubilizing the proteins with a pyrophosphate buffer (100 mM NaF, 8 mM sodium pyrophosphate, 250 mM NaCl, 5 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulphonyl fluoride, 50 µg/mL leupeptin and 1% Nonidet P-40), the samples were centrifuged. The supernatants were collected and incubated with human anti-platelet myosin

antibody at 4ºC for 90 min, and Pansorbin was used to precipitate the immuno-linked MLC. This fraction was separated by 12% SDS-polyacrylamide gel electrophoresis, the gel was frozen and exposed to X-OMAT films. The phosphorylated MLC was identified on the autoradiographs, and the absorbance of the 20-kDa band was measured by densitometry. Results were calculated in arbitrary density units and corrected for the protein concentration in the sample.

Transient Transfection Experiments.

HuVEC were plated at 65% confluency on either 100 mm dishes or 6 well plates. The cells were transfected to express exogenous DNA using LipofectAMINE (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's instructions. Subconfluent cell cultures were transfected with 2 µg of the fcGK-IαR plasmid, which expresses a dominant negative form of PKG type α. The pcDNA3.1 plasmid DNA was used as a control. Transfection was performed during 6 hours after which regular media was added. Cells were treated with the corresponding reagents twenty-four hours after transfection.

Western blotting

HuVEC were washed briefly in PBS and solubilized in lysis buffer (10mM tris-HCl pH 7.4, 1 mM EDTA, 1% triton-X-100, 0.1% sodium deoxycholate, 500 nM sodium orthovanadate, 50 nM sodium fluoride, 1 µg/ml pepstatin/ leupeptin/ aprotinin, 1 mM phenylmethylsulfonyl fluoride) for 40 minutes at 4ºC. Lysates were spun down for 5 minutes and the supernatants were collected. Protein concentration was determined by the Bradford method. Proteins (100 µg) were

separated in a15% SDS-polyacrilamide gel overnight and transferred to a PDVF membrane (Polyscreen, Dupont, Wilmington, USA). For protein detection, the membranes were incubated with a phospho-myosin light chain specific antibody, generously supplied by Dr. J. Staddon (25), at a dilution of 1:500 at 4ºC overnight. Following washing, the blots were incubated with secondary antibody and ECL detection was performed using the manufacturer's instructions. The membranes were reprobed with anti-VASP and anti-P-VASP antibodies and developed as described above.

Statistical Analysis

Every experimental condition was duplicated within each experiment and each experiment was repeated at least three times. The data are expressed as means $\pm$ SE. Comparisons were made with analysis of variance followed by Dunnett's modification of the t-test, whenever comparisons were made with a common control, whereas the unpaired two-tailed Student's test was used for other comparisons. The level of statistically significant difference was defined as $p< 0.05$.

Results

CNP and NO display different relaxing activities on H_2O_2 induced HuVEC contraction

Preliminary experiments were performed to determine the amount of cGMP released by different concentrations of C-type natriuretic peptide (CNP) and two NO donors, sodium nitroprusside (SNP) and Spermidine-NONOate (sp-NO) in human endothelial cells in the absence of the phosphodiesterase inhibitor IBMX. CNP (10^{-5} M to 10^{-9} M) was used to activate pGC and the NO donors, SNP and sp-NO (10^{-5} M to 10^{-9} M) were used to activate sGC. After a 15 min incubation, cGMP levels were determined by RIA and the results represented in Fig. 1A. Both SNP and sp-NO generated a concentration-dependent elevation of cGMP levels. sp-NO (10^{-6} M) increased cGMP levels from a control value of 2.5 $\pm$ 0.1 to 7 $\pm$ 0.06 fmol/ g protein while incubation of the endothelial cells with CNP led to an increase of intracellular cGMP from a control value of 2.5 $\pm$ 0.1 to 33 $\pm$ 0.045 fmol/ g protein at the highest concentration used (half-maximal effective concentration EC_{50}= 1.8 x 10^{-7} M). The cGMP production in response to CNP was much more pronounced than that produced due to NO even when the highest doses of the NO donors were employed. In view of these results, endothelial cells were incubated with sp-NO 10^{-6} M and CNP 10^{-7} M in all subsequent experiments.

To examine the differential effects of cGMP on H_2O_2 induced endothelial cell contraction, HuVEC were preincubated during 5 min with exogenous NO from sp-NO (NO, 10^{-6} M), CNP (10^{-7} M) or vehicle in buffer A without IBMX after

which H_2O_2 (10^{-4} M) was added (time 0) and the incubation continued for another 30 min. Microphotographs were taken at times 0 and 30 minutes and planar cell surface area (PCSA) was measured. As shown in figure 1B, H_2O_2 (10^{-4} M, 30 min) induced a significant contraction of cultured HuVEC expressed as a reduction of the PCSA, which was abolished by preincubation with the NO donor. CNP preincubation also diminished the H_2O_2 contractile effect but to a lesser extent than NO. Similar results were obtained when another NO donor (SNP,10^{-6} M) was used instead of sp-NO (PCSA after SNP plus H_2O_2: 100,2 $\pm$ 2 % of control value).

In order to determine if a correlation exists between intracellular cGMP levels and the relaxing potencies of the GC agonists, we measured cGMP production under the same experimental conditions on which microphotograph experiments were carried out. To this aim, HUVECs were preincubated with NO and CNP with or without H_2O_2 as described above, in the absence of the phosphodiesterase inhibitor IBMX. cGMP levels after NO or CNP stimulation varied both in magnitude and temporal pattern. While the NO donor induced low cGMP levels that peaked at 2 minutes after the start of the experiment (Fig. 2A) and faded at 5 minutes, CNP mediated cGMP production resulted 10 times higher and remained elevated for at least 10 minutes after the start of the experiment, declining to control levels at 30 minutes probably due to phosphodiesterase activity (figure 2B). H_2O_2 addition did not affect cGMP production induced by either NO or CNP, since cGMP produced in CNP/H_2O_2- and NO/H_2O_2-treated cells were comparable to those obtained in CNP- or NO-treated cells. cGMP determination was conducted after 30 min of NO or CNP treatment in the presence of IBMX (Fig. 2C). Phosphodiesterase inhibition

caused a more pronounced difference between cGMP levels obtained after particulate GC stimulation and the levels obtained via sGC, thus confirming that the decline in cGMP observed in figures 2A and 2B is due to phosphodiesterase activity. Similar results were obtained using Zaprinast (10^{-6} M), a selective PDE type-5 (PDE-5) inhibitor (data not shown). In both cases, H_2O_2 did not have any effect on cGMP production when used in combination with CNP or NO.

Taken together, these results show that the cGMP levels produced by CNP stimulation of pGC, which were one order of magnitude higher and remained elevated longer than the levels obtained by NO activation of sGC, did not correlate with the corresponding relaxing response elicited by this second messenger .

Recent findings indicate that H_2O_2 treatment of endothelial cells increases myosin light chain (MLC) phosphorylation suggesting that endothelial contraction plays an important role in the oxidative stress-induced endothelial barrier dysfunction (18, 39). cGMP dependent relaxation mechanisms involve MLC dephosphorylation via PKG activation (33). PKG-I is expressed in HuVEC in our experimental conditions (data not shown). To analyse if this is the mechanism involved in our study, we labelled HuVEC with [^{32}P] orthophosphate, as described in materials and methods. H_2O_2 increased phosphate incorporation into MLC, an effect which was completely prevented by preincubation with NO. By contrast, preincubation with CNP was unable to significantly prevent MLC phosphorylation in response to H_2O_2 (Figure 3).

Effects of NO on endothelial cell relaxation depend on cGMP production

Because of the differences observed between NO and CNP relaxing potencies and cGMP production levels, we decided to test if NO could produce its effects by means of an additional mechanism such as direct protein modification. As shown in figure 4, the effects of NO on PCSA were mimicked by the addition of a soluble cGMP analogue (8-Br cGMP, 10^{-5}M) and were inhibited by treatment with a sGC inhibitor (ODQ, 10^{-6} M). In addition, a PKG-I inhibitor (Rp-cGMPs, 2.5 10^{-6} M) blocked the NO inhibitory effect on PCSA reduction obtained after HuVEC treatment with H_2O_2. This result suggests the involvement of the cGMP/PKG signaling pathway in the observed effects.

In order to probe if the NO effect on MLC phosphorylation was also dependent on the cGMP/PKG pathway, HuVEC were preincubated with the sGC antagonist (ODQ) or the PKG-I antagonist (Rp-cGMPs). MLC phosphorylation levels were analyzed by [^{32}P] orthophosphate cell labelling. As shown in figure 5, MLC phosphorylation induced by H_2O_2 was inhibited by NO and ODQ whereas Rp-cGMPs treatment reversed the NO-induced effects. These results indicate that NO mediates HuVEC relaxation mainly through the activation of sGC followed by cGMP production and the activation of PKG.

The Iα isoform of PKG is involved in the differential effects of NO/cGMP and CNP/cGMP on H_2O_2-induced HuVEC contraction

It has been recently shown that PKG type-Iα (PKG-Iα) activates the MLC phosphatase by phosphorylating its myosin binding subunit, thereby inhibiting MLC phosphorylation and contraction (33). In order to clarify the mechanism involved in the differential relaxing effect of NO-derived cGMP compared to

CNP-derived cGMP, we transfected HuVEC with a dominant negative form of PKG type-Iα (fcGK-IαR). The overexpression of this form of PKG, which lacks the PKG catalytic subunit, is able to block cGMP stimulated activity of the endogenous kinase, having no basal kinase activity itself (3). Figure 6 shows MLC phosphorylation of transfected cells using either fcGK-IαR or an empty vector (pcDNA 3.1). In the pcDNA 3.1-transfected cells, H_2O_2-induced MLC phosphorylation was completely prevented by NO while CNP produced only a moderate inhibition. By contrast, fcGK-IαR transfection resulted in a complete reversal of the NO inhibitory effect on MLC phosphorylation. However, the CNP effects were not completely reversed by fcGK-IαR. The endogenous PKG activity of transfected HuVECs was assessed by examinating the phosphorylation status of its vascular substrate, the vasodilator-stimulated phosphoprotein (VASP), at serine (239) (31). Figure 6 shows an increase of phosphorylated VASP (P-VASP) levels in NO or CNP-treated cells transfected with an empty plasmid. By contrast, P-VASP levels are lower in cells transfected with a PKG negative-dominant construct, even in the presence of NO or CNP, which confirms the biological activity of the transfected constructs

Discussion

The main finding of this study is that different pools of cGMP produced in human endothelial cells via pGC activation by CNP or NO stimulation of sGC have different functional activities. CNP has a moderate relaxing effect compared with NO on endothelial cells of human origin exposed to H_2O_2. However, cGMP production by the CNP/pGC system is higher (in all the concentration ranges studied) than that due to sGC stimulation by NO. We

demonstrate that this discrepancy is due to a more efficient stimulation of PKG-Iα by the NO/sGC-derived cGMP. This result strongly suggests a role for the Iα isoform of PKG in transducing the signals of certain pools of cGMP to produce differential effects.

The concept of functional compartmentalization of second messengers is not new. One well established example is the spatial control of [Ca^{2+}] and cAMP signals (14, 38). However, parallelisms between those systems and cGMP have not been properly established. Zolle and co-workers recently demonstrated that the activation of pGC inhibit Ca^{2+} extrusion via a plasma membrane Ca^{2+} ATP-ase, while the activation of sGC leads to an increase of Ca^{2+} uptake into the intracellular stores (40). The specific source of cGMP seems to be important for this effect. In our study NO-derived cGMP produced a more intense relaxing effect than CNP-derived cGMP on endothelial cell contraction induced by H_2O_2. cGMP levels evoked by stimulation of sGC with NO or of pGC with CNP differ both in amplitude and duration. While NO produced a typical pulse of cGMP during the first 5 minutes of incubation which faded 15 minutes after the start of the experiment, CNP produced cGMP levels which was ten times greater and which remained elevated throughout the experiment. Despite the differences in cGMP production, there was no correlation with the observed cellular effect.

The first question that arises from this observation is whether the NO-induced effects are dependent on cGMP production. NO can act through cGMP independent pathways to directly modify amino acid residues in several proteins and thus, alter their function (32). In support of this hypothesis, Hart and co-workers showed that NO effects on endothelial barrier dysfunction in porcine coronary artery endothelial cells stimulated by H_2O_2 were cGMP-independent

(11). In our study, incubation of HuVEC with inhibitors of either sGC or PKG and incubation with a cGMP analogue demonstrated that the NO-induced effects were mainly due to cGMP production rather than to a protein modification.

cGMP regulates cell responsiveness through PKG stimulation, which comprises a major mechanism for cGMP action (12). There are two reported forms of PKG: a soluble type I PKG which is expressed and present in endothelial cells, smooth muscle cells and neurons, and a membrane bound type II PKG (16). Two isoforms of PKG type I (Iα and Iβ) are produced by alternate splicing of the same gene and differ only in their amino terminus. Both PKG type-Iα and -Iβ isoforms are involved in the control of smooth muscle cell relaxation. PKG type-Iβ–dependent phosphorylation of inositol 1,4,5-triphosphate receptor-associated G-kinase (IRAG) decreases Ca^{2+} release from the sarcoplasmic reticulum (1) whereas PKG-Iα phosphorylates the myosin binding subunit of myosin light chain phosphatase, activating it and therefore inhibiting MLC phosphorylation and contraction (33). The similarities between the contractile apparatus in smooth muscle and endothelial cells prompted us to investigate the role of the two PKG isoforms in the differential effects of cGMP originated by the activation of the two sets of guanylate cyclases. Transfections with the dominant negative form of PKG-Iα completely abrogated the responses elicited by NO on MLC phosphorylation induced by H_2O_2. In this case, the responses to CNP showed only partial inhibition. Therefore, these results suggest that PKG-Iα is involved in the transduction of NO/cGMP signalling rather than the CNP/cGMP system. This could be simply due to a different spatial confinement of guanylate cyclases and PKG-Iα. PKG-Iα was originally described as a cytosolic enzyme. However, under certain situations it is partially

associated with the cytoskeleton (31, 37). During oxidant injury there are several changes that promote endothelial cell contraction and rearrangement of the actin cytoskeleton that compromises the endothelial barrier function, producing tissular edema (20). PKG-Iα is able to phosphorylate VASP, a protein member of the ENA/VASP family of proteins involved in the regulation of the actin cytoskeleton, causing its detachment from sites of focal adhesions. This could explain some of the effects of NO on H_2O_2-induced endothelial cell contraction since focal adhesions provide additional adhesive forces in the endothelial barrier regulation (22).

In addition to MLC kinase, MLC phosphorylation in endothelial cells can be induced by the Rho/Rho kinase pathway. PKG-Iα can phosphorylate Rho *in vitro* and *in vivo* causing its inhibition (29). The role of a selective inhibition of Rho kinase in our results awaits further investigation.

Besides the possible phosphorylation by PKG-Iα of different substrates involved in cell relaxation, PKG-Iα can control the level and subcellular distribution of cGMP directly by regulating phosphodiesterase activity. PDE-5 is the major PDE that degrades cGMP and PKG-Iα can phosphorylate PDE-5 both *in vivo* and *in vitro*, activating it (23, 27). Although the physiological function for the phosphorylation and activation of PDE-5 has not been analysed in endothelial cells, a similar mechanism has been described for platelets on NO sensitization (30). Activation of PDE-5 may then provide a negative feedback regulation of cGMP and PKG-Iα, because PDE-5 activation by PKG can also control the ability of PKG to phosphorylate other substrates when the intracellular concentration of cGMP reaches a high level, such as after CNP stimulation.

In summary, our results demonstrate that cGMP originated from separate sources within the endothelial cell plays a different role in the control of cell relaxation. Additional studies will be necessary to address whether these differences are due to a different subcellular location of the cGMP effectors such as PKG and its substrates and cGMP degrading systems. This has potential implications for understanding the role of natriuretic peptides versus NO in endothelial dependent vascular relaxation and endothelial barrier function upon oxidant injury.

Acknowledgments

This work was supported in part by grants from the “Ministerio de Ciencia y Tecnología” (PM 97-0067, BFI 2001-1036), “Comunidad de Madrid” (08.4/0012/2001-2) and “Fondo de Investigación Sanitaria” (FISS 01-0434).

Rivero-Vilches F.J. is a recipient of a postgraduate research scholarship from the "Ministerio de Educación, Cultura y Deporte". De Frutos S. is a recipient of a postgraduate research scholarship from the “Comunidad de Madrid”. Saura M. is a research investigator from the "Ramón y Cajal" program (MCYT).

We must thank Dr. D. Browning for his kind donation of PKG-Iα regulatory region plasmid (fcGK-IαR).

The authors also thank Dr. Federico Gago, Dr. Carlos Zaragoza and Dr. Lillian Puebla for their review of this manuscript.

References

1. Ammendola A, Geiselhoringer A, Hofmann F, and Schlossmann J. Molecular determinants of the interaction between the inositol 1,4,5-trisphosphate receptor-associated cGMP kinase substrate (IRAG) and cGMP kinase Ibeta. *J Biol Chem* 276: 24153-9., 2001.

2. Bradley JR, Thiru S, and Pober JS. Hydrogen peroxide-induced endothelial retraction is accompanied by a loss of the normal spatial organization of endothelial cell adhesion molecules. *Am J Pathol* 147: 627-41., 1995.

3. Browning DD, Mc Shane M, Marty C, and Ye RD. Functional analysis of type 1alpha cGMP-dependent protein kinase using green fluorescent fusion proteins. *J Biol Chem* 276: 13039-48., 2001.

4. Conti M, and Jin SL. The molecular biology of cyclic nucleotide phosphodiesterases. *ProgNucleic Acid Res Mol Biol* 63: 1-38, 1999.

5. Dhalla NS, Temsah RM, and Netticadan T. Role of oxidative stress in cardiovascular diseases. *J Hypertens* 18: 655-73., 2000.

6. Draijer R, Atsma DE, van der Laarse A, and van Hinsbergh VW. cGMP and nitric oxide modulate thrombin-induced endothelial permeability. Regulation via different pathways in human aortic and umbilical vein endothelial cells. *Circ Res* 76: 199-208., 1995.

7. Finn JT, Grunwald ME, and Yau KW. Cyclic nucleotide-gated ion channels: an extended family with diverse functions. *Annu Rev Physiol* 58: 395-426, 1996.

8. Gonzalez-Santiago L, Lopez-Ongil S, Rodriguez-Puyol M, and Rodriguez-Puyol D. Decreased nitric oxide synthesis in human endothelial cells cultured on type I collagen. *Circ Res* 90: 539-45., 2002.

9. Griffith TM. Endothelial control of vascular tone by nitric oxide and gap junctions: a haemodynamic perspective. *Biorheology* 39: 307 -18, 2002.

10. Gupta MP, Evanoff V, and Hart CM. Nitric oxide attenuates hydrogen peroxide-mediated injury to porcine pulmonary artery endothelial cells. *Am J Physiol* 272: L1133-41., 1997.

11. Gupta MP, Ober MD, Patterson C, Al-Hassani M, Natarajan V, and Hart CM. Nitric oxide attenuates H(2)O(2)-induced endothelial barrier dysfunction: mechanisms of protection. *Am J Physiol Lung Cell Mol Physiol* 280: L116-26., 2001.

12. Hood J, and Granger HJ. Protein kinase G mediates vascular endothelial growth factor-induced Raf-1 activation and proliferation in human endothelial cells. *J Biol Chem* 273: 23504-8., 1998.

13. Jaffe EA, Grulich J, Weksler BB, Hampel G, and Watanabe K. Correlation between thrombin-induced prostacyclin production and inositol trisphosphate and cytosolic free calcium levels in cultured human endothelial cells. *J Biol Chem* 262: 8557-65., 1987.

14. Johnson JD, and Chang JP. Function- and agonist-specific Ca2+ signalling: the requirement for and mechanism of spatial and temporal complexity in Ca2+ signals. *Biochem Cell Biol* 78: 217-40, 2000.

15. Levi M, ten Cate H, and van der Poll T. Endothelium: interface between coagulation and inflammation. *Crit Care Med* 30: S220-4., 2002.

16. Lincoln TM, Dey N, and Sellak H. Invited review: cGMP-dependent protein kinase signaling mechanisms in smooth muscle: from the regulation of tone to gene expression. *J Appl Physiol*9 1: 1421-30., 2001.

17. Lopez-Ongil S, Gonzalez-Santiago L, Griera M, Molpeceres J, Rodriguez-Puyol M, and Rodriguez-Puyol D. Mechanisms involved in the relaxation of bovine aortic endothelial cells. *Life Sci* 70: 699-714., 2001.

18. Lopez-Ongil S, Torrecillas G, Perez-Sala D, Gonzalez-Santiago L, Rodriguez-Puyol M, and Rodriguez-Puyol D. Mechanisms involved in the contraction of endothelial cells by hydrogen peroxide. *Free Radic Biol Med* 26: 501-10., 1999.

19. Lucas KA, Pitari GM, Kazerounian S, Ruiz-StewartI, Park J, Schulz S, Chepenik KP, and Waldman SA. Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol Rev* 52: 375-414., 2000.

20. Lum H, and Roebuck KA. Oxidant stress and endothelial cell dysfunction. *Am J Physiol Cell Physiol* 280: C719-41., 2001.

21. McQuaid KE, and Keenan KA. Endothelial barrier dysfunction and oxidative stress: roles for nitric oxide? *Exp Physiol* 82: 369-76., 1997.

22. Mehta D, Tiruppathi C, Sandoval R, Minshall RD, Holinstat M, and Malik AB. Modulatory role of focal adhesion kinase in regulating human pulmonary arterial endothelial barrier function. *J Physiol* 539: 779 89., 2002.

23. Murthy KS. Activation of phosphodiesterase 5 and inhibition of guanylate cyclase by cGMP-dependent protein kinase in smooth muscle. *Biochem J* 360: 199-208., 2001.

24. Okayama N, Kevil CG, Correia L, Jourd'heuil D, Itoh M, Grisham MB, and Alexander JS. Nitric oxide enhances hydrogen peroxide-mediated endothelial permeability in vitro. *Am J Physiol* 273: C1581-7., 1997.

25. Ratcliffe MJ, Smales C, and Staddon JM. Dephosphorylation of the catenins p120 and p100 in endothelial cells in response to inflammatory stimuli. *Biochem J* 338: 471-8., 1999.

26. Rivero-Vilches F, de Frutos S, Rodriguez-Puyol M, Rodriguez-Puyol D, and Saura M. [Guanylate cyclases: physiological processes mediated by cyclic GMP]. *Nefrologia* 21: 233-9., 2001.

27. Rybalkin SD, Rybalkina IG, Feil R, Hofmann F, and Beavo JA. Regulation of cGMP-specific phosphodiesterase (PDE5) phosphorylation in smooth muscle cells. *J Biol Chem* 277: 3310-7., 2002.

28. Saura M, Lopez S, Rodriguez Puyol M, Rodriguez Puyol D, and Lamas S. Regulation of inducible nitric oxide synthase expression in rat mesangial cells and isolated glomeruli. *Kidney Int* 47: 500-9., 1995.

29. Sawada N, Itoh H, Yamashita J, Doi K, Inoue M, Masatsugu K, Fukunaga Y, Sakaguchi S, Sone M, Yamahara K, Yurugi T, and Nakao K. cGMP-dependent protein kinase phosphorylates and inactivates RhoA. *Biochem Biophys Res Commun* 280: 798-805., 2001.

30. Sly MK, Eberhart RC, and Prager MD. Anti-platelet action of nitric oxide and selective phosphodiesterase inhibitors. *Shock* 8: 115-8., 1997.

31. Smolenski A, Poller W, Walter U, and Lohmann SM. Regulation of human endothelial cell focal adhesion sites and migration by cGMP-dependent protein kinase I. *J Biol Chem* 275: 25723-32., 2000.

32. Stamler JS, Lamas S, and Fang FC. Nitrosylation. the prototypic redox-based signaling mechanism. *Cell* 106: 675 83., 2001.

33. Surks HK, Mochizuki N, Kasai Y, Georgescu SP, Tang KM, Ito M, Lincoln TM, and Mendelsohn ME. Regulation of myosin phosphatase by a specific interaction with cGMP- dependent protein kinase Ialpha. *Science* 286: 1583-7., 1999.

34. Torrecillas G, Boyano-Adanez MC, Medina J, Parra T, Griera M, Lopez-Ongil S, Arilla E, Rodriguez-Puyol M, and Rodriguez-Puyol D. The role of hydrogen peroxide in the contractile response to angiotensin II. *Mol Pharmacol* 59: 104-12., 2001.

35. Torrecillas G, Diez-Marques ML, Garcia-Escribano C, Bosch RJ, Rodriguez-Puyol D, and Rodriguez-Puyol M. Mechanisms of cGMP-dependent mesangial-cell relaxation: a role for myosin light-chain phosphatase activation. *Biochem J* 346 Pt 1: 217-22., 2000.

36. Valen GSA., Vaage J, Malm E, Kjelltrom BT. Hydrogen peroxide induces endothelial cell atipia and cytoskeleton depolymerization. *Free Radic Biol med* 26: 1480-1488, 1999.

37. Vo NK, Gettemy JM, and Coghlan VM. Identification of cGMP-dependent protein kinase anchoring proteins (GKAPs). *Biochem Biophys Res Commun* 246: 831-5., 1998.

38. Zaccolo Z and Pozzan T. Discrete microdomains with high concentration of cAMP in stimulated rat cardiac myocites. *Science* 295: 1711-1715, 2002.

39. Zhao YDH. Hydrogen peroxide-induced cytoskeletal rearrangement in cultured pulmonary endothelial cells. *J Cell Physiol* 174: 370-379, 1998.

40. Zolle O, Lawrie AM, and Simpson AW. Activation of the particulate and not the soluble guanylate cyclase leads to the inhibition of Ca2+ extrusion through localized elevation of cGMP. *J Biol Chem* 275: 25892-9., 2000.

Figure legends

Figure 1. Concentration-response curve for cGMP production by NO/sGC or CNP/pGC stimulation and NO and CNP effects on the reduction of the PCSA induced by H_2O_2. A: HuVEC were incubated with spermidine-NONOate (sp-NO), sodium nitroprusside (SNP), C-type natriuretic peptide (CNP) or vehicle at different concentrations ranging from 10^{-9} to 10^{-5} M in the absence of IBMX for 15 min. cGMP content was determined from the ethanol fixed cells by RIA. Results are the mean ± SE of 6 different experiments and are expressed as fmoles of cGMP per µg of protein. *p<0.05 vs SNPand sp-NO. **B:** HuVEC were preincubated with sp-NO (NO, 10^{-6} M), CNP (10^{-7}M) or vehicle (C) for 5 minutes. Hydrogen peroxide (H_2O_2, 10^{-4}M) was subsequently added (time 0) and the incubation was prolonged 30

Figure 2. NO- and CNP-derived cGMP production differ in concentration and temporal course: HuVEC were preincubated with sp-NO (NO, 10^{-6} M, panel **A**), C-type natriuretic peptide (CNP, 10^{-7}M, panel **B**), or vehicle (C) in the absence of IBMX for 5 minutes. Hydrogen peroxide (H_2O_2, 10^{-4}M) or vehicle (C, CNP and NO) were then added and cGMP content was determined from the ethanol-fixed cells by RIA from time 0 to 30 minutes**.** Results are the mean ± SE of 6 different experiments and are expressed as fmoles of cGMP per µg of protein *p<0.05 vs. C**. C.** HuVEC were incubated as above but in the presence of IBMX (10^{-4} M) and cGMP determinations were carried out at 30 minutes. Results are the mean ± SE of 6 different experiments and are expressed as

fmoles of cGMP per µg of protein. *p<0.05 vs. C and H_2O_2, **p<0.05 vs. NO and NO/H_2O_2

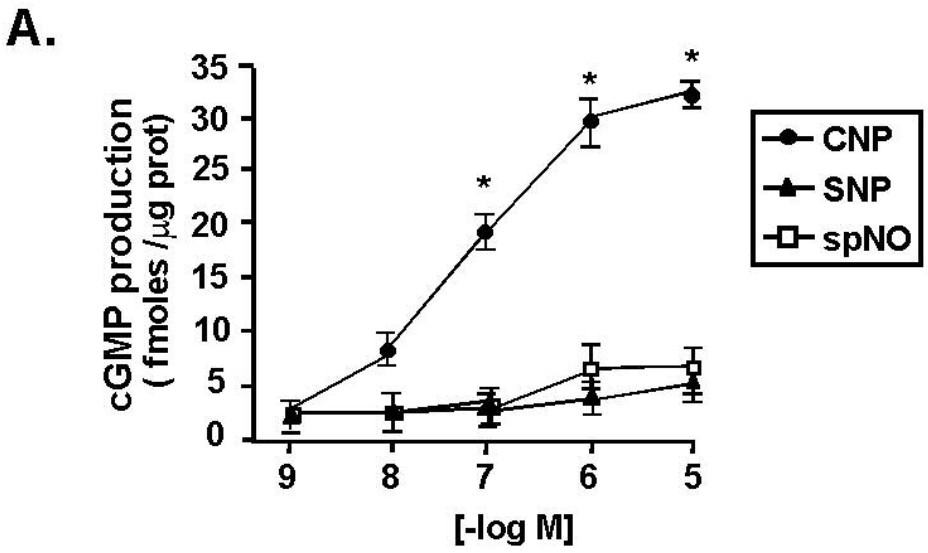
Figure 3. NO and CNP display different inhibitory effects on MLC phosphorylation (^{32}P-MLC) induced by H_2O_2. HuVEC were labelled with 50 µCi/mL sodium [^{32}P]orthophosphate and then preincubated for 5 minutes with sp-NO (NO, 10^{-6} M), C-type natriuretic peptide (CNP, 10^{-7}M) or vehicle (C). H_2O_2 was then added and the incubation proceeded for 30 additional minutes. A typical autoradiograph of an immunoprecipitation experiment is shown in the upper panel. The densitometric analysis of the immunoprecipitation experiments is shown below. Results are the mean ± SE of 3 different experiments. * p< 0.05 vs. C, **p< 0.05 vs H_2O_2.

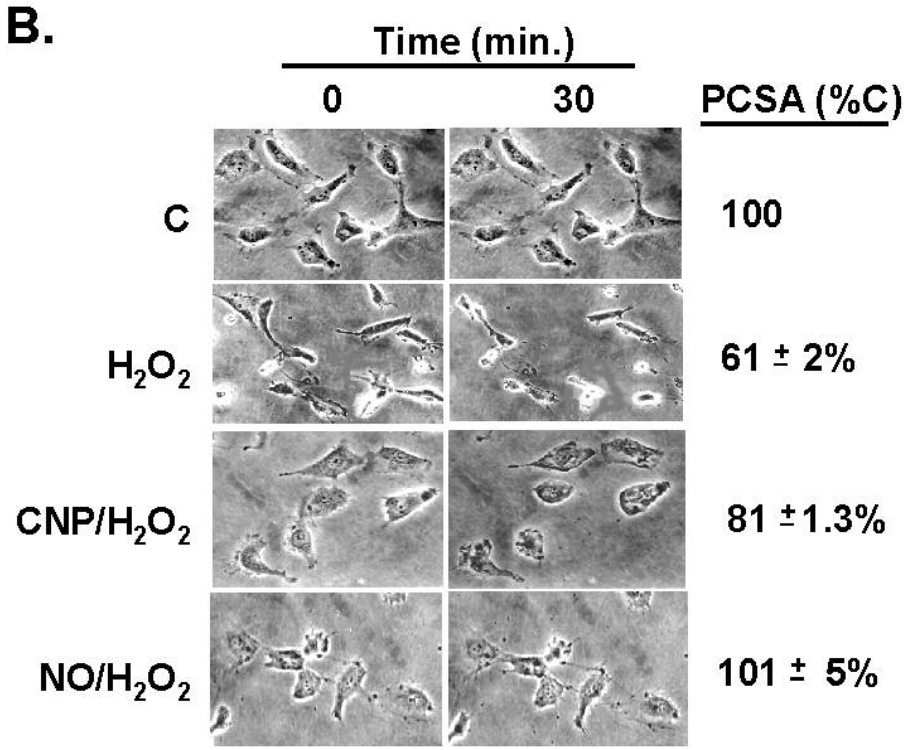
Figure 4. NO effects on PCSA are dependent on cGMP production and PKG stimulation. HuVEC were preincubated with sp-NO (NO, 10^{-6} M), 8-Br cGMP (10 µM) or vehicle (C) for 5 minutes. Hydrogen peroxide (H_2O_2, 10^{-4} M) was then added and the incubation was prolonged 30 minutes. In some cases an inhibitor of soluble guanylate cyclase (ODQ, 1 µM) or an inhibitor of PKG-Iα (Rp-cGMPs, 1 µM) were included in the incubation medium. Microphotographs were taken at time 0 and 30 and PCSA was analysed. Results are the mean ± SE of 6 different experiments and are expressed as % of control values. *p< 0.05 vs C, **p<0.05 vs H_2O_2.

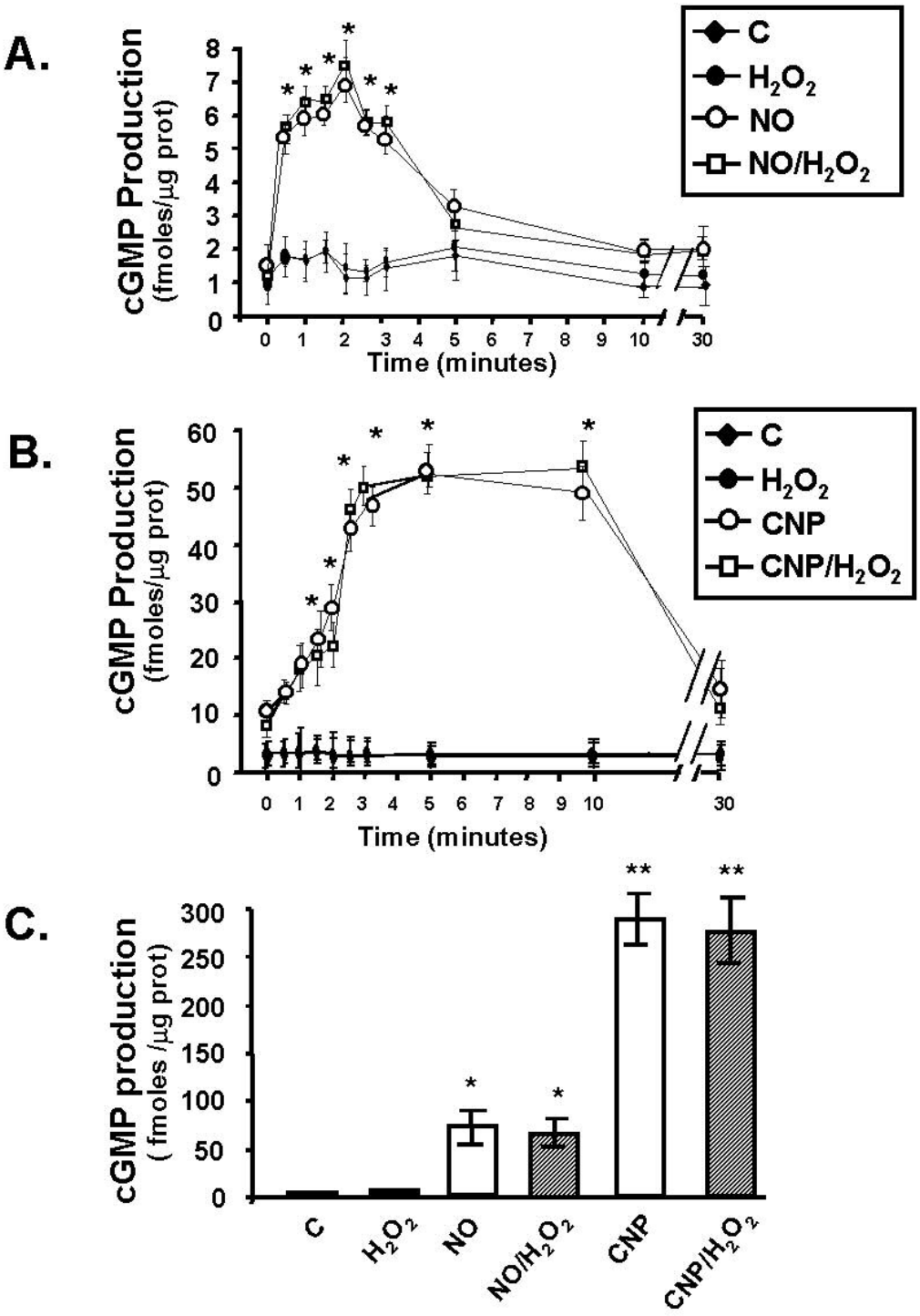
Figure 5. Inhibitory effect of NO on MLC phosphorylation (^{32}P-MLC) induced by H_2O_2 depends on the cGMP/PKG pathway. HuVEC were labelled

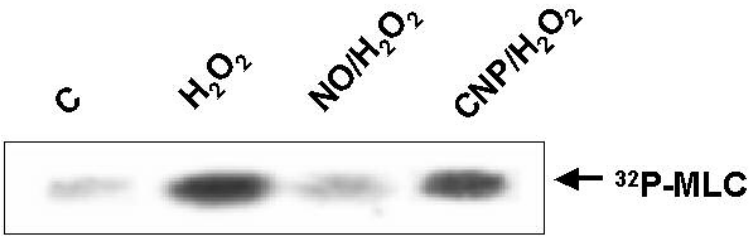
with 50 µCi/mL sodium [^{32}P] orthophosphate and then preincubated for 5 minutes with sp-NO (NO, 10^{-6} M), 8-Br cGMP (10 µM) or vehicle (C). H_2O_2 was then added and the incubation proceeded for 30 additional minutes. In some cases an inhibitor of sGC (ODQ, 1 µM) or an inhibitor of PKG-Iα (Rp-cGMPs, 1 µM) were included in the incubation medium. A typical autoradiograph of an immunoprecipitation experiment is shown in the upper panel. The densitometric analyses of the immunoprecipitation experiments are shown below. Results are the mean ± SE of 4 different experiments. * p< 0.05 vs. C, **p< 0.05 vs H_2O_2.

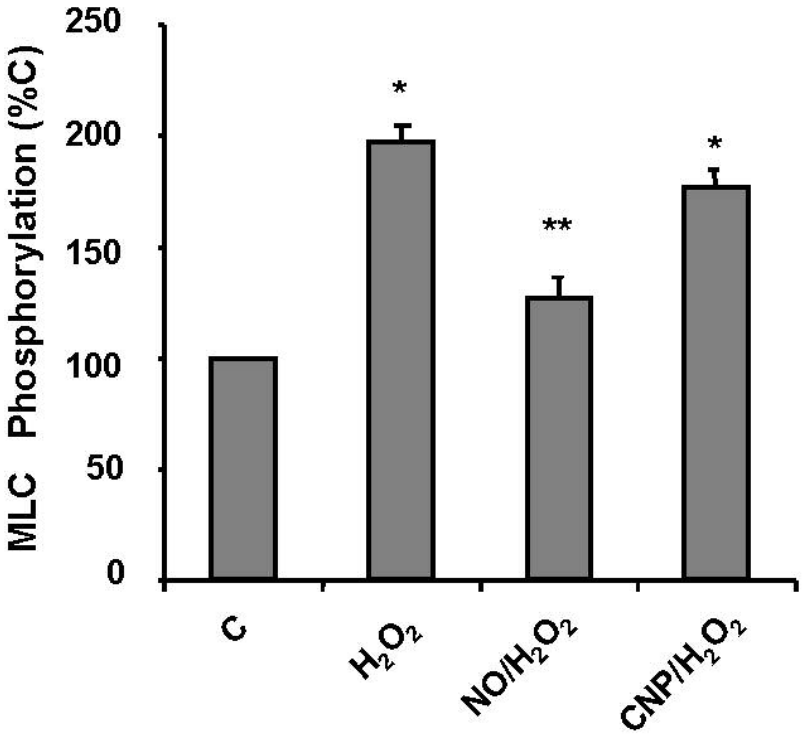
Figure 6. NO/cGMP and CNP/cGMP differentially activate PKG-Iα to inhibit MLC phosphorylation. HuVEC were transiently transfected either with a control plasmid (pcDNA 3.1) or with the dominant negative form of PKG-Iα (fcGK-IαR) and treated with the GC agonist and H_2O_2 as described (transfection efficiency 30% aprox.). A representative western blot against the di-phosphorylated form of MLC (PP-MLC) is shown. The same Western blot reprobed against phospho-VASP (P-VASP) and VASP (VASP) is shown below, as a way to test the biological activity of the transfected constructs. The densitometric analyses of the immunoprecipitation experiments are shown in the lower panel. Results are the mean ± SE of 4 different experiments. * p< 0.05 vs. C, **p< 0.05 vs H_2O_2.

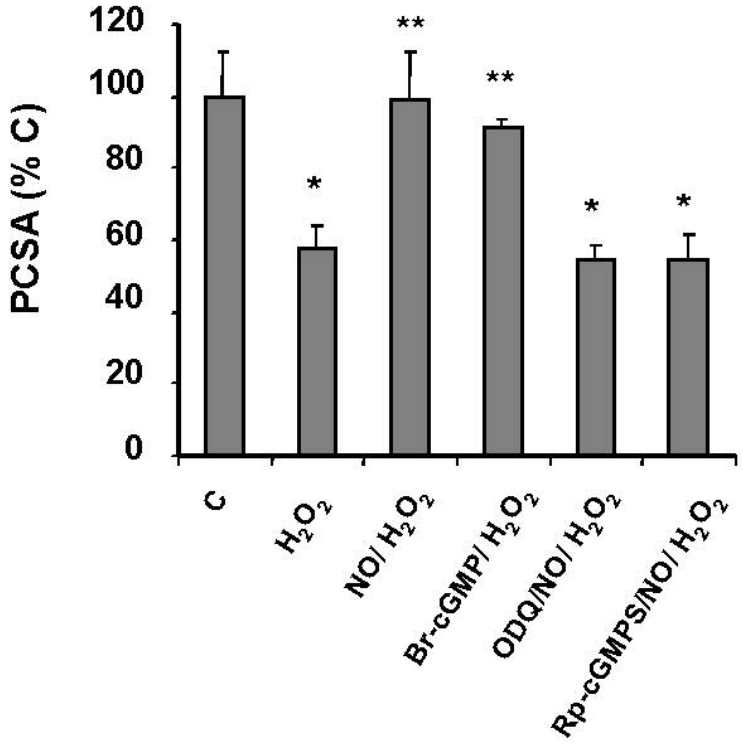

A.
cGMP production
(fmoles /μg prot)
35
30
25
20
15
10
5
0
9
8
7
6
5
[-log M]
*
*
*
CNP
SNP
spNO

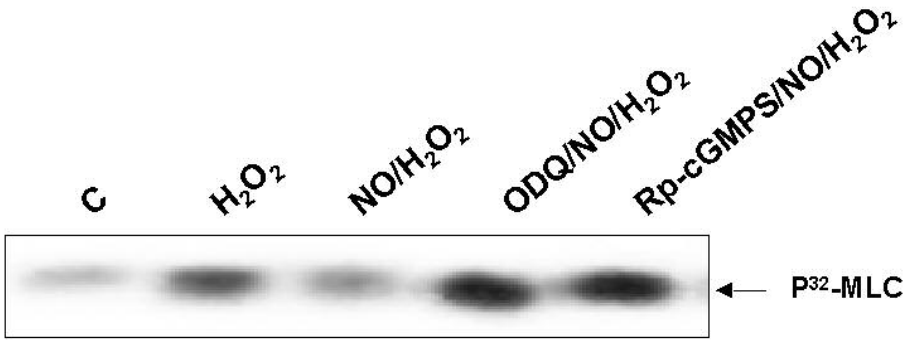

B.
Time (min.)
0
30
PCSA (%C)
C
100
H2O2
61 ± 2%
CNP/H2O2
81 ±1.3%
NO/H2O2
101 ± 5%

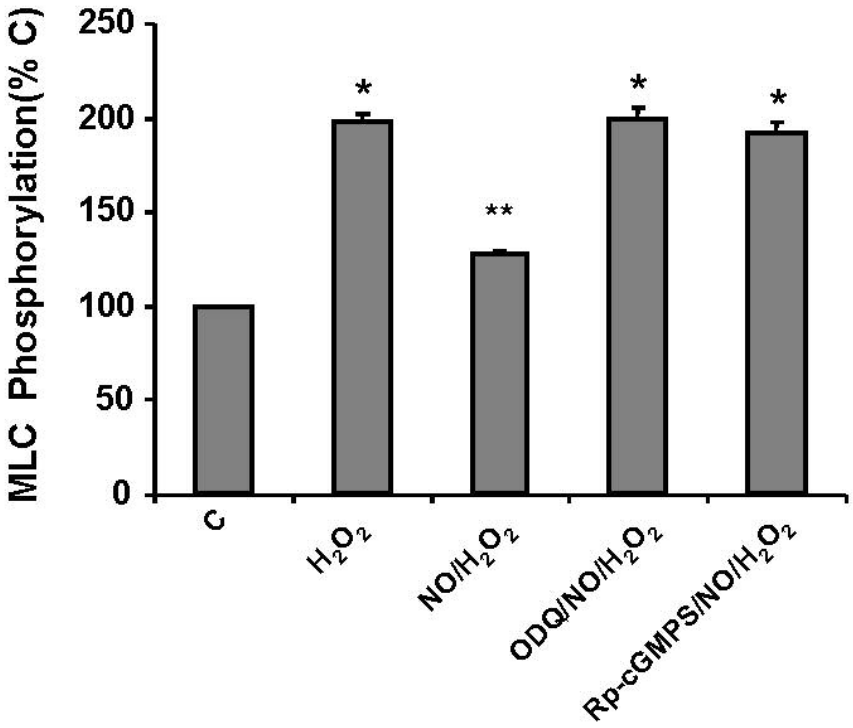

A.
cGMP Production
(fmoles/µg prot)
Time (minutes)
C
H2O2
NO
NO/H2O2
B.
cGMP Production
(fmoles/µg prot)
Time (minutes)
C
H2O2
CNP
CNP/H2O2
C.
cGMP production
(fmoles /µg prot)
C
H2O2
NO
NO/H2O2
CNP
CNP/H2O2

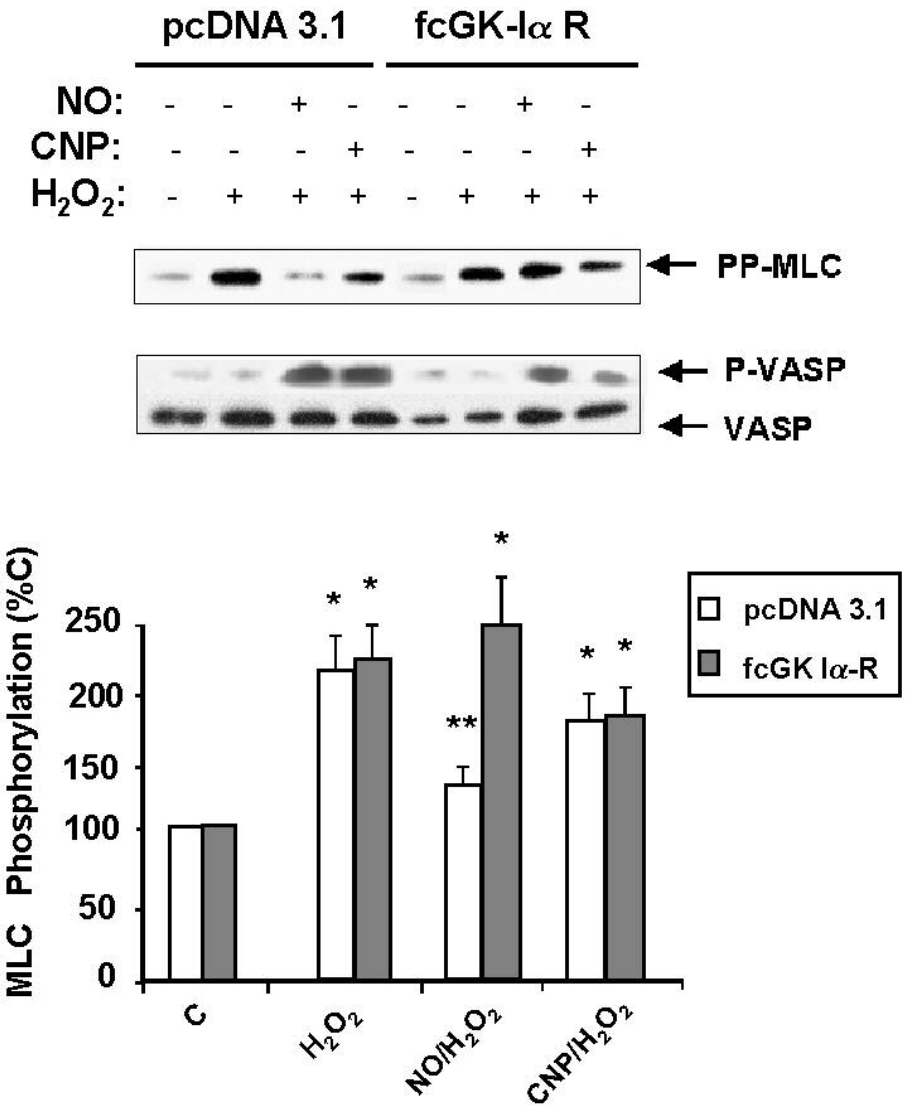

C
H_2O_2
NO/H_2O_2
CNP/H_2O_2
^{32}P-MLC


MLC Phosphorylation (%C)
250
200
150
100
50
0
*
**
*
C
H_2O_2
NO/H_2O_2
CNP/H_2O_2


PCSA (% C)
120
100
80
60
40
20
0
**
**
*
*
*
C
H2O2
NO/ H2O2
Br-cGMP/ H2O2
ODQ/NO/ H2O2
Rp-cGMPS/NO/ H2O2


C
H_2O_2
NO/H_2O_2
$ODQ/NO/H_2O_2$
$Rp\text{-}cGMPS/NO/H_2O_2$
P^{32}-MLC


MLC Phosphorylation(% C)
250
200
150
100
50
0
*
**
*
*
C
H_2O_2
NO/H_2O_2
$ODQ/NO/H_2O_2$
$Rp\text{-}cGMPS/NO/H_2O_2$


pcDNA 3.1
fcGK-Iα R
NO:
CNP:
H2O2:
PP-MLC
P-VASP
VASP
MLC Phosphorylation (%C)
250
200
150
100
50
0
pcDNA 3.1
fcGK Iα-R
C
H2O2
NO/H2O2
CNP/H2O2