Nitric Oxide Decreases the Expression of Endothelin-Converting Enzyme-1 Through mRNA Destabilization

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Objective—Endothelial function depends on the equilibrium in the synthesis of vasoactive endothelial factors. It is well known that endothelin and nitric oxide (NO) exhibit reciprocal regulation. We assessed the ability of NO to regulate endothelin-converting enzyme-1 (ECE-1) expression in vascular endothelial cells.

Methods and Results—Bovine aortic endothelial cells were incubated with 2 different NO donors as well as with a cyclic-GMP analog, dibutyryl-cGMP (dB-cGMP). ECE-1 protein content and mRNA expression were evaluated by Western blot and Northern blot, respectively, promoter activity by transfection experiments, ECE-1 activity by ELISA, and cGMP production by radioimmunoassay. Both NO donors decreased ECE-1 protein content, mRNA expression, and ECE-1 activity. ODQ, an inhibitor of soluble guanylyl cyclase, blocked those effects. NO donors raised cGMP levels, and dB-cGMP mimicked their effects on ECE-1 expression, which were blocked by KT5823, a nonspecific PKG inhibitor. The changes on ECE-1 expression were due to a destabilization on 3′-untranslated region (3′-UTR) of this mRNA, because the activity of a luciferase reporter construct containing the 3′-UTR of the ECE-1 gene was reduced by dB-cGMP in a PKG-dependent manner. The biological relevance of this regulation was confirmed in bovine aortic endothelial cells cocultured with macrophages in the presence of lipopolysaccharide, in eNOS-deficient mice, and in Wistar rats treated with NO donors. In every case, an inverse relationship was observed between NO and ECE-1 protein content.

Conclusion—Our results support that NO regulates ECE-1 expression through a cGMP/PKG-dependent regulatory mechanism at the post-transcriptional level via the 3′-UTR of the ECE-1 gene. (Arterioscler Thromb Vasc Biol. 2011; 31:2577-2585.)

Key Words: nitric oxide ■ cyclic GMP ■ endothelial cells ■ endothelin-converting enzyme-1 ■ protein kinase G

It is a well-known fact that endothelium exerts multiple functions to preserve vascular homeostasis. Vasoactive endothelial factors such as nitric oxide (NO) or endothelin-1 (ET-1) are involved in this regulation. An unbalanced production of these bioactive mediators results in endothelial dysfunction, a critical event in the development of renal and cardiovascular damage in some diseases like diabetes, hypertension, or atherosclerosis.1–4 ET-1, discovered in 1988,5 is the most powerful vasoconstrictor peptide known. Its synthesis is the result of 2 main steps. The first one converts the long precursor prepro-ET-1 into the inactive short precursor big ET-1, and the second one converts big ET-1 specifically into ET-1 through the action of the endothelin-converting enzyme-1 (ECE-1).6 ECE-1 is a zinc type II membrane metallopeptidase protein,7 an enzyme with 4 different isoforms generated by alternative splicing.8 NO is a gaseous metabolite with vasorelaxant properties, synthesized by a family of enzymes known as nitric oxide synthases.9,10 ET-1 and NO are synthesized by endothelial cells and are able to regulate each other to maintain a balanced vascular tone. Experimental studies have provided evidence that ET-1 may exert a bidirectional effect on NO production either enhancing its synthesis via ETB receptors or blunting it through ETA receptors.11,12 Conversely, NO was found to inhibit ET-1 synthesis in different cell types.13–15 Moreover, numerous conditions characterized by an impaired availability of NO have been found to be associated with enhanced
synthesis of ET-1,16 and vice versa,17 thereby suggesting that these 2 factors have a reciprocal regulation.

ECE-1 activity has been considered critical for ET-1 synthesis, and it has been demonstrated that the blockade of this protein exerts beneficial effect in some cardiovascular diseases,12,18 For this reason, its regulation has been actively investigated. Reactive oxygen species,19,20 oxidized low-density lipoproteins,21 angiotensin II,22 and big ET-1 itself23 may be involved in the regulation of its activity or content. However, the possibility that NO could modulate the synthesis or activity of ECE-1, a mechanism that could contribute to the cross-regulation between NO and ET-1, has not been adequately explored. Thus, this study was devoted to assess the effect of NO on ECE-1 levels in bovine aortic endothelial cells (BAEC), as well as to analyze the mechanisms involved in this regulation.

Methods

Cell Culture

The culture of BAEC,19 EA.hy926 human endothelial cell line, murine-derived RAW 264.7 macrophage cell line (RAW),24 and human embryonic kidney 293T, as well as the BAEC and RAW cocultures22 were performed as described (please see Supplemental data, available online at http://atvb.ahajournals.org).

Animal Studies

Treatment of different animals were done as described (please see Supplemental data). The study design and the experimental protocols were performed in agreement with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No.85to 23, revised 1996) and with the European Union regulations.

Analytic Procedures

Immunoblot Analysis

Detection and quantification of ECE-1 protein from BAEC and tissues were done by immunoblotting as described previously,23 using a monoclonal anti-ECE-1 antibody (mAb AEC32-236 was provided by Dr. Kohei Shimada, Biological Research Laboratories, Sankyo Co, Ltd, Tokyo, Japan).

RNA Isolation and Northern Blot Analysis

Total RNA from BAEC was isolated as described.21,22 For Northern analysis, a 2900-bp fragment of bovine ECE-119 and an 18S RNA probe were radiolabeled (Ready to Go, GE Healthcare Bio-Sciences, Buckinghamshire, UK).

Measurement of ECE-1 Activity

Membrane proteins from BAEC treated with dB-cGMP were homogenized as described.23 Then, 30 µg of the homogenate were incubated with bovine big ET-1 (100 nmol/L) for 4 hours at 37°C in 50 mmol/L Tris-HCl buffer pH 7.0.20 ET-1 production from cultured cells or plasma from rats was measured by ELISA.

Measurement of Cyclic GMP

Cyclic GMP was extracted from BAEC following published methods26; cGMP production in each sample was measured by radioimmunoassay and corrected by protein amount (please see Supplemental data).

Nitrite Determination

Nitrite synthesis was evaluated by a fluorimetric assay using 2,3-diaminonaphthalene as described.27

Figure 1. Effect of NO donors on endothelin-converting enzyme-1 (ECE-1) protein content. Bovine aortic endothelial cells (BAEC) were incubated with sodium nitroprusside (SNP) (A and B) or diethylamine/nitric oxide complex sodium salt (C and D) as indicated. ECE-1 protein content was measured by Western blot. In the upper part of each panel a representative Western blot is shown, whereas in the lower part the densitometric analysis of 8 independent experiments is given (mean±SEM). Values are expressed as the percentage of control cells (C); *P<0.05 vs control.

Statistical Analysis

Unless otherwise specified, data are expressed as means±SEM and as a percentage of the control values. All experiments were performed at least 3 times in duplicate. Comparisons were made by nonparametric statistics, particularly the Wilcoxon (2 groups) or Friedman (more than 2 groups) tests. Animal studies were analyzed by ANOVA, followed by the Scheffe multiple comparison test, after confirming the normality of the data distribution. The level of statistical significance was defined as P<0.05.

Results

NO-Dependent Changes in ECE-1 Protein Content and its Dependence On Soluble Guanylyl Cyclase

Confluent BAEC monolayers were incubated with 2 different NO donors, sodium nitroprusside (SNP) and diethylamine/nitric oxide complex sodium salt (DEA-NO) at different times and dosage, to evaluate their effects on ECE-1 expression. Figure 1 shows how both NO donors were able to decrease ECE-1 protein content in a dose- and time-dependent fashion. The reduction was maximal at 24 hours (Figure 1B and 1D) and was attained with relatively low...
Role of PKG in the ECE-1 Downregulation Elicited by NO Donor and dB-cGMP

The role of PKG was studied by pretreating BAEC with a nonspecific PKG inhibitor, KT5823, and measuring ECE-1 protein content by Western blot. KT5823 completely blocked the downregulation of ECE-1 induced by DEA-NO and dB-cGMP (Figure 3A), at concentrations that inhibited phosphorylation of the vasodilator-stimulated phosphoprotein (P-VASP-Ser239) (Figure 3B). Besides, to confirm the involvement of PKG in the reduction of ECE-1 protein levels, BAEC were transfected with a dominant-negative PKG isofrom, active PKG, or an empty vector; and ECE-1 protein content was evaluated by Western blot after 6 hours of treatment with dB-cGMP (Figure 3C). ECE-1 protein content was reduced by treatment with dB-cGMP in BAEC transfected with empty vector, an effect that was not observed in cells transfected with a dominant-negative of PKG. In contrast, the constitutively active PKG construct itself was able to reduce ECE-1 protein content as dB-cGMP.

Mechanism Involved in the Modulation of ECE-1 by dB-cGMP

The ability of dB-cGMP to modulate ECE-1 mRNA expression was tested. A significant decrease of ECE-1 mRNA was found in cells incubated with several doses (Figure 4A) and for different times (Figure 4B) of dB-cGMP. To assess if this mRNA ECE-1 downregulation was due to transcriptional changes, the ECE-1 promoter activity was measured by transitory transfection assays using an ECE-1 promoter fragment (−682/+1 bp) linked to a luciferase expression reporter. The 5′-flanking region of the ECE-1 gene contains a CAAT box and potential binding sites for glucocorticoid receptors, NF-kappaB, PU-1, AP1, AP2, and c-ets1 transcription factors. The addition of dB-cGMP to BAEC produced no significant changes in ECE-1 promoter activity (Figure 4C). Phorbol myristate acetate (3×10−7 mol/L, 6 hours) was used as a positive control in these assays (Stimulation: 283±18% versus control cells), and SP-600125 (25 µmol/L, 6 hours), an AP-1 inhibitor that inhibits JNK, induced a moderate reduction around 25% (76±9% versus control cells). As no obvious effect was found at the level of ECE-1 gene transcription, ECE-1 mRNA stability in cells treated with dB-cGMP was checked using the transcriptional inhibitor 5, 6-dichloro-1-β-d-ribofuranosyl-benzimidazole (DRB). Figure 4D shows that the treatment of control cells with DRB produced no significant reduction in the levels of ECE-1 mRNA, a result suggesting that this mRNA is quite stable throughout the experimental time course. In contrast,
Figure 3. Role of PKG in the cyclic GMP-dependent endothelin-converting enzyme-1 (ECE-1) downregulation. A, Bovine aortic endothelial cells (BAEC) were incubated with 250 µmol/L diethylamine/nitric oxide complex sodium salt (DEA-NO) (closed bars) or 10 µmol/L dibutyryl-cGMP (dB) (open bars) for 6 hours in the presence or absence of the nonspecific PKG inhibitor, KT (KT5823, 250 nmol/L). Endothelin-converting enzyme-1 (ECE-1) protein content was measured by Western blot. B, BAEC were incubated with 250 µmol/L DEA-NO (DEA) (closed bars) or 10 µmol/L dB-cGMP (dB) (open bars) for 15 minutes in the presence or absence of 250 nmol/L KT. Phosphorylation of vasodilator-stimulated phosphoprotein (P-VASP-Ser239) was measured by Western blot. C, BAEC were transfected in the presence of a dominant-negative of PKG (PKG-DN), a constitutively active mutant of PKG (PKG-CA) or an empty vector (PKG-ND). Transfected cells were incubated with 10 µmol/L dB-cGMP (dB) for 6 hours, and ECE-1 protein content was evaluated by Western blot. In each panel, the upper part of each panel a representative Northern blot is shown, whereas in the lower part the densitometric analysis of 6 independent experiments is given (mean ± SEM). Values are expressed as the percentage of control cells (C); *P<0.05 vs control. C, BAEC were transfected with a human ECE-1 promoter/luciferase plasmid. Transfected BAECs were then incubated with 10 µmol/L dibutyryl-cGMP [dB-cGMP (dB)] at the indicated times, and the ECE-1 promoter activity was measured using a Luciferase Assay. Mean ± SEM of 3 independent experiments, in triplicate, are expressed as the percentage of control cells (C). D, BAEC were incubated with 10 µmol/L 6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB, transcriptional inhibitor), in the presence of 10 µmol/L of dB-cGMP (dB) or 250 µmol/L of diethylamine/nitric oxide complex sodium salt (DEA-NO) at different times. ECE-1 mRNA expression was analyzed by Northern blot. In the upper part of the panel a representative Northern blot is shown, whereas in the lower part the densitometric analysis of 9 independent experiments is given (mean ± SEM). Values are expressed as the percentage of control cells (C); *P<0.05 vs control.

ECE-1 mRNA content was diminished in cells treated with DRB plus dB-cGMP and DRB plus DEA-NO, an observation that suggests a reduction in mRNA stability.

Many of the regulatory mechanisms at the post-transcriptional level take place within genetic elements present in the 3' untranslated region (3'UTR) of the genes.33 In order to assess if the 3'UTR of the ECE-1 mRNA was involved in the cGMP-mediated ECE-1 mRNA destabilization, we generated a luciferase reporter under
the control of a ribosomal protein L10 constitutive promoter, with the 3'-UTR of the human ECE-1 gene (full length 2707 bp fragment) cloned downstream of the reporter gene (pSGG-full length 3'-UTR-ECE). Transient transfection experiments in BAEC incubated with dB-cGMP for different time periods are shown in Figure 5A; dB-cGMP reduced the luciferase activity in the pSGG-full length 3'-UTR-ECE (UTR) construct, but not in the control plasmid pSGG-empty (ΔUTR). In order to study whether the cGMP-mediated destabilizing effect can be assigned to specific regions of the 3'-UTR, we divided the full-length sequence into 3 smaller fragments (1 to 639, 640 to 1425 and 1426 to 2707). Figure 5B shows that the plasmid containing the subfragment 640 to 1425 recapitulated the effect of the full length construct, a result indicating that regulatory elements involved in the action of cGMP are contained within this sequence.

To confirm this finding, the 3'-UTR of the ECE-1 gene was cloned downstream of the ECE-1 gene (pCMV6-UTR-ECE1) and the construct was expressed in a human embry-
Assessment of the Biological Relevance of the NO-Dependent ECE-1 Downregulation In Vivo

Lung tissues from 8-week-old eNOS deficient mice expressed higher levels of ECE-1 protein than their wild-type counterparts (Figure 6B). When these mice were treated with SNP, 2 μg/Kg via IP, for 24 hours, lung ECE-1 protein content decreased, not only in wild-type but also in eNOS deficient mice (Figure 6B). Wild-type mice treated with L-NAME, 50 mg/Kg/d dissolved in drink water for 72 hours, also exhibited an increased ECE-1 protein content in lungs (Figure 6C). Finally, rats treated with NO donors showed a significant reduction of ECE-1 protein content in aorta and lung tissues (please see Supplemental Figure III), as well as in circulating ET-1 levels (Control rats: 4.8 ± 0.8), or incubated with 250 μmol/L DEA-NO (2.3 ± 0.07) or 1 μmol/L L-NAME (0.1 ± 0.06).

Assessment of the Biological Relevance of the NO-Dependent ECE-1 Downregulation In Vivo

Lung tissues from 8-week-old eNOS deficient mice expressed higher levels of ECE-1 protein than their wild-type counterparts (Figure 6B). When these mice were treated with SNP, 2 μg/Kg via IP, for 24 hours, lung ECE-1 protein content decreased, not only in wild-type but also in eNOS deficient mice (Figure 6B). Wild-type mice treated with L-NAME, 50 mg/Kg/d dissolved in drink water for 72 hours, also exhibited an increased ECE-1 protein content in lungs (Figure 6C). Finally, rats treated with NO donors showed a significant reduction of ECE-1 protein content in aorta and lung tissues (please see Supplemental Figure III), as well as in circulating ET-1 levels (Control rats: 4.7 fmol/mL ET-1; SNP-treated rats: 3.4 fmol/mL ET-1, n=5 in each case, P<0.05).

Discussion

Our data suggest a new role for the NO/cGMP/PKG pathway in the vascular endothelium. Nitric oxide, through...
activation of the soluble guanylyl cyclase, cGMP synthesis, and PKG activation, downregulates ECE-1 expression. According to our results, the mechanism seems to involve a destabilization of ECE-1 mRNA, in which the 3′-UTR region plays a key role.

ECE-1 is the key enzyme in ET-1 synthesis, and this peptide is involved in the pathogenesis of different cardiovascular diseases. Our results demonstrate that NO donors decrease the ECE-1 protein content in vascular endothelial cells, suggesting that this enzyme could be considered as a target for NO in the local NO-ET-1 cross regulation within the vascular endothelium. One of the few studies that have previously evaluated the ability of NO to regulate ECE-1 expression was that of Kelly et al.13 This study was devoted to analyze the effect of diethylenetriamine/nitric oxide adduct, a long-acting nitric oxide donor, on ET-1 synthesis in ovine pulmonary arterial endothelial cells. They found that diethylenetriamine/nitric oxide adduct decreased ET-1 secretion and prepro-ET-1 mRNA without changes in ECE-1 expression. It is possible that differences in cell type could account for the divergent findings in our study regarding the effects on ECE-1 expression. Alternatively, the chemical nature of the selected NO donors could contribute to explain the observed differences, as DEA-NO releases NO more rapidly, in contrast to the more sustained and quantitatively lower action of diethylenetriamine/nitric oxide adduct. However, it is important to note that in our study NO action was mediated by its natural effector, cGMP. In this sense, Uchida et al.15 also revealed that the expression of ECE-1 and ECE-2 were downregulated by 8-bromo-cGMP through PKG-mediated signaling in glomerular endothelial cells.

Most of cellular actions of NO are mediated by a classical pathway in which the activation of soluble guanylyl cyclase, the synthesis of cGMP, and the activation of PKG are involved.13,36–39 We tested the possibility that this pathway could be responsible for the decrease in ECE-1 protein content observed in cells incubated with NO donors. As expected, NO donors increased cGMP, and this stimulation was blocked by ODQ, a soluble guanylyl cyclase inhibitor. ODQ also inhibited the effect of NO donors on ECE-1 protein content. Moreover, exogenously added cGMP reproduced the effects of NO on ECE-1 by activating PKG, as the blockade of this enzyme abrogated the inhibitory effect of cGMP on ECE-1 protein content. Taken together, these results strongly support that the regulation of ECE-1 by NO is regulated by the canonical NO/sGC/cGMP/PKG pathway.

Our data are consistent with a post-transcriptional mechanism of regulation of ECE-1 mRNA, mediated by PKG and involving a reduction in its stability. We also provide insight about the specific region of the 3′-UTR responsible for this effect. Our results indicate that the central portion between positions 640 to 1425 with respect to the stop codon contains the elements required for this downregulation. Previous reports in the bibliography have already shown that the cGMP pathway can modulate message stability of different mRNAs, including the α1 and β1 subunits of soluble guanylyl cyclase, the inducible NO synthase, COX-1, TNF-α, and TGF-β3.40 Of interest, the contribution of the 3′-UTR of ECE-1 to the regulation of ECE-1 protein content has also been reported in a model of hepatic wound healing.41 These authors demonstrated that the increased ECE-1 content observed in this model was the consequence of increased mRNA stability, and that the 3′-UTR was critical in the maintenance of this stability.41 It has been proposed that these effects may involve the downregulation of the RNA-stabilizing factor HuR, a protein that binds preferably to adenine- and uridine-rich regions. However, it is not obvious that adenine and uridine-rich elements are present in the subfragment 640 to 1425 of the 3′-UTR ECE-1 mRNA. On the other hand, there is accumulating evidence that microRNAs (miRNA) control multiple aspects of cell biology by silencing gene expression.42 In most cases silencing occurs by inhibition of translation or mRNA degradation through a specific interaction with the 3′-UTR of the target mRNA. Different specific miRNAs have been shown to influence endothelial function, including proliferation, migration and morphogenesis.43 For example, miR-296 has been shown to be a critical component of the angiogenic process. The miRNAs miR-221 and miR-222, on the contrary, have been shown to inhibit endothelial cell migration, proliferation and angiogenesis in vitro by targeting stem cell factor receptor.44 Computer-assisted analysis of putative miRNA binding sites in the fragment 640 to 1425 of ECE-1 3′-UTR (Microinspector algorithm, http://bioinfo.uni-provdiv.bg/microinspector/) have revealed the existence of potential binding sites for miRNAs of endothelial interest, miR-296, miR-221, or miR-20a/b. Whether any of these miRNAs are regulated by NO/cGMP and interact with ECE-1 3′-UTR are specific objectives of our current investigation.

The addition of NO donors is a pharmacological intervention that attempts to mimic the effect of endogenous NO. To assess the biological relevance of the NO-dependent ECE-1 regulation, we checked ECE-1 protein levels in BAEC cocultured with a macrophage cell line treated with LPS for 24 hours. It is well known that LPS induces NO production in macrophages.45 BAEC incubated with LPS alone showed increased ET-1 production and ECE-1 expression. In contrast, BAEC cocultured with macrophages plus LPS showed significantly decreased ET-1 production and ECE-1 expression, an effect that was blocked by ODQ and 1400W. The biological relevance of the in vitro studies was confirmed in aorta and lung tissues of SNP-treated rats, which showed reduced ECE-1 content and lower circulating ET-1 levels. Furthermore, lungs extracted not only from eNOS-deficient mice but also from L-NAME-treated wild-type mice presented higher expression of ECE-1 than their wild-type counterparts, supporting the relevance of NO in this effect.

In consequence, the present results clearly demonstrated that NO downregulates ECE-1 in cultured cells and in animals, through a mechanism in which the sGC/cGMP/PKG pathway is involved. The activation of this system induces a destabilization of the ECE-1 mRNA with participation of the 3′-UTR of the gene. These findings support the biological
relevance of the cross-regulation between NO and the endothelin system and point to ECE-1 as a target for NO.

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Disclosures

None.

References


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SUPPLEMENTAL MATERIAL

I- DETAILED MATERIAL AND METHODS SECTIONS

Materials

NO donors, 2-(N,N-Diethyl-amino)-diazenolate 2-oxide (DEA-NO) and sodium nitroprusside (SNP), as well as an active analogue of cyclic GMP, dibutyryl-GMP (dB-cGMP) were from Sigma-Aldrich-Fluka Chemical Co. (St. Louis, MO, USA). PKG inhibitor, KT5823, was from Cayman Chemical (Ann Arbor, MI, USA). A selective, irreversible, inhibitor of inducible nitric oxide synthase, 1400W was from Calbiochem (Merck Chemicals, Nottingham, UK). Culture plates, Supersignal detection system and secondary horseradish peroxidase-conjugated goat anti-mouse IgG were from Cultek (Pierce, Rockford, USA). Dual Luciferase Reporter Assay System, pGL3 vector and pRL-SV40 vector were from Promega (Walkersville, MD, USA). Lipofectamine reagent and OptiMEM I media were from GIBCO-Invitrogen (Barcelona, SPAIN). Acrylamide-bisacrylamide was from Hispanlab-Pronadisa (Madrid, Spain). MXB films were from Kodak (Rochester, NY, USA). Protein markers, BioRad protein assay kit, plates and electrophoresis equipment were from Bio-Rad Laboratories (Richmond, CA, USA). Protease inhibitor cocktail tablets were from Roche Diagnostics (Madrid, Spain). The alpha-[\(^{32}\)P]-dCTP were from GE Healthcare Bio-Sciences (Buckinghamshire, UK). The ET-1 ELISA system was from R&D Systems (Abingdon, United Kingdom). Cyclic GMP \([^{125}\text{I}]\) RIA kit was from PerkinElmer Life Sciences, INC. (Boston, USA). Unless otherwise indicated, the rest of drugs, culture media, antibodies and reagents were from Sigma-Aldrich-Fluka Chemical Co. (St. Louis, MO, USA).

Cell culture

BAEC were isolated from bovine thoracic aortas, using previously described methods (1). Characterization was based on their typical cobblestone appearance and uniform uptake of fluorescent acetylated LDL. Cells were maintained in Roswell Park Memorial Institute 1640 (RPMI 1640) supplemented with 15 % calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin in an atmosphere of 95% air and 5% CO\(_2\). Experiments were routinely performed on confluent monolayers between passages 2-5, made quiescent by serum deprivation. Cellular toxicity was evaluated in all
experimental conditions using the trypan blue dye exclusion method and by measurement of lactic dehydrogenase (LDH) activity in the incubation media, without finding any toxicity with all drugs studied.

Confluent BAEC were treated with different times and doses of two different NO donors, Diethylamine/nitric oxide complex sodium salt: 2-(N,N-Diethyl-amino)-diazenolate 2-oxide (DEA-NO) and sodium nitroprusside (SNP), as well as an active analogue of cyclic GMP, dibutyryl-GMP (dB-cGMP), in order to study the effect of NO on ECE-1 regulation.

RAW 264.7 (RAW), a macrophage cell line was from American Type Culture Collection (Manassas, VA, USA). RAW were grown in Roswell Park Memorial Institute 1640 (RPMI 1640) supplemented with 10 % fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin in an atmosphere of 95% air and 5% CO₂.

EA.hy926 (EA), a human endothelial cell line was from American Type Culture Collection (Manassas, VA, USA). EA were grown in Dulbeco’s Modified Eagle Media (DMEM) containing 4.5 g/L glucose and supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin in an atmosphere of 95% air and 5% CO₂.

Human embryonic kidney cells 293T (HEK 293) were from American Type Culture Collection (Manassas, VA, USA). HEK 293 were grown in DMEM Ham’s F-12 containing 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin in an atmosphere of 95% air and 5% CO₂.

Coculture of BAEC and RAW Cells.

BAEC were grown in gelatine-coated six-well plates, and RAW macrophages were grown on 24 mm coverslips, until confluence. The coverslips were placed upside down over an adequate insert placed in BAEC six-well plates, so that both cells were near each other (< 2mm) but not in contact, sharing the same incubation media (RPMI without serum). Cocultured cells were maintained for 24 h in the presence or absence of 0.1 µg/mL lipopolysaccharide (LPS). Supernatants were collected to measure ET-1 production by ELISA (2), nitric oxide synthesis was evaluated by a fluorimetric assay (3, 4), and proteins from BAEC were extracted to evaluate ECE-1 expression.
Measurement of cyclic GMP

BAEC were grown to confluence, and then cells were washed twice with PBS in the presence of 1 μmol/L IBMX, a phosphodiesterase inhibitor in order to avoid cyclic GMP degradation. After that, cells were treated for 30 min with or without 10 μmol/L ODQ, a soluble guanylyl cyclase inhibitor, and then NO donors were added for 15 min. Cells were washed twice with PBS and 300 μL 65 % ethanol was added to extract cGMP for 30 min on ice. Cells were scraped and after centrifugation at 3,000 rpm for 10 min, supernatants were lyophilized. The dry residues were reconstituted with assay buffer, and cyclic GMP production in each sample was measured by radioimmunoassay assay (5), and then corrected by protein amount.

Animal studies

Two-month old male Wistar rats were treated with NO donors, 300 mg/Kg body weight of isosorbide dinitrate (ISDN) in the drinking water or 7.5 μg/Kg body weight of SNP intraperitoneally for different periods. Male homozygous eNOS-deficient (eNOS KO) and C57BL/6J control (WT) mice were obtained from The Jackson Laboratory (Charles River España, Barcelona, Spain). Two-month old male mice were treated with buffer or SNP 2 μg/Kg body weight i.p., and other group were treated with buffer or 50 mg/Kg/day L-NAME dissolved in drink water for 72 h. All animals had free access to water, were maintained at 24°C, and kept at a 12 h light/dark cycle. Twenty four hours after treatment, animals were anaesthetized with pentobarbital (50 mg/kg i.p.), and aortas and lungs were removed and stored until analysis. In a group of rats, plasma was also obtained.

Transient transfection experiments

To determine whether the effect of NO donors on ECE-1 gene expression was mediated by the 5’-flanking region of the gene, a human ECE-1 promoter/luciferase reporter gene plasmid was constructed (pGL3-ECE-1) (1, 6). We used HeLa cell genomic DNA to generate by PCR the human ECE-1 gene promoter with the 5’ end at nucleotide -682 and the 3’ end nucleotide +1 using the Advantage Genomic PCR Kit (Clontech Lab., Palo Alto, CA, USA). The whole promoter was subcloned in the Xho I-Hind III site of pGL3 vector (Promega Co., Walkersville, MD, USA), upstream from a luciferase reporter gene.
To determine whether the effect of NO donors on ECE-1 gene expression was mediated by the 3'-UTR of the ECE-1 mRNA, a 2707 bp fragment containing the full length sequence of the 3'-UTR of the human ECE-1 was generated by PCR and cloned downstream the luciferase reporter of the pSGG vector (reporter cassette under the control of a ribosomal protein L10 constitutive promoter, Switch Genomics, CA, USA). In order to generate luciferase reporters containing deleitional fragments of the 3'-UTR, full length sequence was digested with XcmI restriction enzyme, to yield three subfragments (1-639, 640-1425, and 1426-2707, numbered relative to the first nucleotide of the 3'-UTR, GenBank accession number NM 001 397), blunt ends were obtained by treatment with Klenow fragment of DNA polymerase, and subfragments were then cloned back in pSGG empty vector (7, 8).

To confirm the role of the 3'-UTR, a human ECE-1 transgene plasmid was constructed containing the 3'-UTR intact region (pCMV6-UTR-ECE1) or lacking it (pCMV6-ECE1). HEK 293 cell line does not express ECE-1 (9); HEK 293 cells were transfected with ECE-1 transgenes and then treated with dB-cGMP for 6h. ECE-1 protein content was analyzed in protein extracts from these cells.

To evaluate the role of PKG on ECE-1 gene expression, plasmids containing the Flag-tagged cGMP-dependent protein kinase 1α regulatory region, a dominant negative (PKG-DN), and a cGMP-dependent protein kinase 1α constitutively active catalytic region, (PKG-CA) were used (10, 11) (kindly donated by Dr. D. Browning, Medical College of Georgia, Augusta). Plasmids were co-transfected with the 3'-UTR-ECE/luciferase reporter or with an empty vector in the presence or absence of dB-cGMP.

In all cases, BAEC were grown in RPMI 1640 supplemented with 15% serum and antibiotics, kept in 5% CO₂, plated 24 h before transfection at a density of 60-80% of confluence in 12-well plates and transfected with each luciferase construct, by mixing plasmid DNA (0.1 µg/µL of luciferase reporter, 1 µg/µL PKG expression vector, 1 ng/µL of plasmid control from *Renilla* luciferase, pRL-SV40 vector, Promega Co.) and 4 µg/mL of Lipofectamine into OptiMEM I media. Cells were washed with PBS 24 h after transfection, and then dB-cGMP was added or not at different times using RPMI without serum. Cells were harvested and assayed for luciferase activity using a Dual-Luciferase Reporter Assay System (Promega Co.). Luciferase activity was expressed as relative light units of each plasmid DNA per relative light units of plasmid control (*Renilla*) per mg protein of each well.
References


II- NON-STANDARD ABBREVIATIONS AND ACRONYMS:

ECE-1: Endothelin-converting enzyme-1
BAEC: Bovine aortic endothelial cells
dB-cGMP: N’2-O-Dibutyrylguanosine 3’, 5’-cyclic monophosphate sodium salt hydrate
DEA-NO: Diethylamine/nitric oxide complex sodium salt, 2-(N,N-Diethyl-amino)-diazenolate 2-oxide
DRB: 5, 6-Dichloro-1-beta-D-ribofuranosyl-benzoimidazole
IBMX: 3-Isobutyl-1-methylxanthine
ISDN: Isosorbide dinitrate
LPS: lipopolysaccharide
NO: Nitric oxide
ODQ: 1H-(1,2,4) Oxadiazolo (4,3-□) quinoxalin-1-one
PKG: Protein kinase G
RAW: Murine derived RAW 264.7 macrophage cell line
SNP: Sodium nitroprusside
TTBS: Tween Tris buffer saline
3’-UTR: 3’-untranslated region
VASP: Vasodilator-stimulated phosphoprotein

III- SUBJECT CODES:

[95] Endothelium/vascular type/nitric oxide;
[137] Cell biology/structural biology;
[138] Cell signaling/signal transduction;
[155] Physiological and pathological control of gene expression.

IV- LEGENDS AND SUPPLEMENTARY FIGURES:
Supplementary Figure I. Effect of nitric oxide donors and cyclic GMP on ECE-1 protein content in basal and stimulated conditions. A) Bovine (BAEC, closed bars) and human (EA, open bars) endothelial cells were incubated with 1μmol/L L-NAME, a non-selective nitric oxide synthase inhibitor, or with high doses of DEA-NO (500 μmol/L, DEA) and dB-cGMP (100 μmol/L, dB) alone, or DEA+dB together for 24 h, to evaluate their potential effect to stimulate the pathway. ECE-1
protein content was measured by Western blot. Values are expressed as the percentage of control cells (C); *p<0.05 vs. control. B) BAEC were incubated with 10 μmol/L dB, or 250 μmol/L DEA for the last 8h of incubation with media containing high (22 mmol/L) or low (5.5 mmol/L) levels of glucose. Values are expressed as relative densitometric units, *p<0.05 vs. control exposed to low levels of glucose. In the upper part of each panel a representative Western-blot is shown, whereas in the lower part the densitometric analysis of 3 independent experiments is given (mean ± SEM).
**Supplementary Figure II. PKG expression levels.** BAEC were transfected with a PKG negative dominant (PKG-ND), a constitutively active mutant of PKG (PKG-CA) or empty vector (PGK-E) as control plasmid. Cells were treated in the presence or absence of 10 μmol/L dB-cGMP (dB) for 6h, and the expression levels of the Flag tagged constructs were evaluated by anti-Flag immunoblotting. β–tubulin expression was used to determine differences in protein loading. Transfection efficiency was ~60%.
Supplementary Figure III. In vivo effect of NO donors on ECE-1 protein content. Wistar rats were treated with SNP (7.5 μg/Kg) or isosorbide dinitrate (ISDN, 300 mg/Kg) for the indicated times. ECE-1 protein content was studied in aorta (A, B) and lung (C, D) tissues by Western blot. In the upper part of each panel a representative immunoblot is shown whereas the lower part shows the mean ± SEM of the densitometric analysis of 12 animals per group. *p<0.05 vs. control group (C).