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Activation of the Mitogen Activated Protein Kinase Extracellular Signal-Regulated Kinase 1 and 2 by the Nitric Oxide–cGMP–cGMP-Dependent Protein Kinase Axis Regulates the Expression of Matrix Metalloproteinase 13 in Vascular Endothelial Cells

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ABSTRACT

Matrix metalloproteinases (MMPs) are synthesized in response to diverse stimuli, including cytokines, growth factors, hormones, and oxidative stress. Here we show that the nitric oxide (NO) donor 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEA-NO) and NO from murine macrophages transcriptionally regulate MMP-13 expression in vascular endothelial cells (BAEC). The cGMP analog, 8-bromo-cGMP (8-Br-cGMP) mimicked the effect of NO, whereas incubation with the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one, or the cGMP-dependent protein kinase (PKG) inhibitor phenyl-1,N^2-etheno-8-bromoguanosine-3′,5′-cyclic monophosphorothioate, *R*p-isomer (PET) reduced the stimulatory effect of DEA-NO on the activation of the MMP-13 promoter. Overexpression of the catalytic subunit of PKG1-α resulted in a 5- to 6-fold increase of the MMP-13 regulatory region over control cells. On the other hand, incubation with the mitogen-activated protein/extracellular signal-regulated kinase inhibitor 2′-amino-3′-methoxyflavone (PD98059) significantly reduced DEA-NO and 8-Br-cGMP promoter activation and mRNA expression of MMP-13 in transfected BAEC. Moreover, a complex between PKG1-α and the G-protein Raf-1, an upstream activator of the extracellular signal-regulated kinase signaling pathway, was detected in cells overexpressing PKG1-α or treated either with DEA-NO or 8-Br-cGMP. Thus, we propose that the NO-cGMP-PKG pathway enhances MMP-13 expression by the activation of ERK 1,2. This effect of NO may be the result of pathophysiological importance in the context of inflammation or atherogenesis.

Matrix metalloproteinases (MMPs) or matrixins are enzymes implicated in the degradation of extracellular matrix components, a process involved in crucial physiological and pathophysiological events (Johnson et al., 1998; Nagase and Woessner, 1999). MMPs are synthesized in response to diverse stimuli including cytokines, growth factors, hormones, and oxidative stress (Damjanovski et al., 2000; Pendas et al., 2000; Siwik et al., 2000).

MMP-13 expression has been detected in human fibroblasts, keratinocytes (Johansson et al., 1997), chondrocytes (Mengshol et al., 2000), and osteoblasts (Quinn et al., 2000). MMP-13 cleaves collagens, gelatin, aggrecan, and fibronectin, thus playing an important role in keratinocyte migration, fetal ossification, bone formation, and osteoarthritis/rheumatoid arthritis (Nagase and Woessner, 1999).

Nitric oxide (NO) is produced by the activity of the family

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ABBREVIATIONS: MMP, matrix metalloproteinase; NO, nitric oxide; NOS, nitric oxide synthase; MAPK, mitogen activated protein kinase; BAEC, bovine aortic endothelial cells; RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; L-NAME, N^{ω}-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; AP-1, activator protein 1; OSE-2, osteoblast specific element 2; HUVEC, human umbilical vein endothelial cell(s); 8-Br-cGMP, 8-bromo-cGMP; DEA-NO, 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide; WT, wild-type; ODQ, 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; PKG, cGMP-dependent protein kinase; PET, phenyl-1,N^2- etheno-8-bromoguanosine-3′,5′-cyclic monophosphorothioate, *R*p-isomer; PD98059, 2′-amino-3′-methoxyflavone; MEK, mitogen activated protein kinase/extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase.

of enzymes nitric-oxide synthases (NOSs) (Nathan and Xie, 1994). NO is a signaling molecule, neurotransmitter (Jaffrey and Snyder, 1995), and immune effector (Zaragoza et al., 1998). In the vascular endothelium, it is an essential regulator of vascular tone (Moncada et al., 1988). NO regulates gene expression through its natural effector, cGMP (Pilz et al., 1995), or by the post-translational modification of proteins (Stamler et al., 2001). In pathophysiological contexts such as inflammation or atherogenesis, a plethora of signals and biological mediators are involved in a complex cross talk. In these settings, it is reasonable to assume that NO and MMPs may interact. We asked whether NO could regulate the expression of MMPs in the vascular endothelium. Among the several MMPs that can play a significant role in the vascular endothelium, we focused on MMP-13 because its presence has not been described in endothelium, to our knowledge, and it is involved in physiological and pathophysiological events that are closely related with the vascular endothelium (Sukhova et al., 1999; Seandel et al., 2001). We have found that in vascular endothelial cells, exogenously administered NO induces the expression and activity of MMP-13 (Zaragoza et al., 2002). In this work, we show that exogenously administered NO and NO from macrophages increase the expression of MMP-13 in endothelial cells by a process regulated at the transcriptional level. According to our data, the signal transduction cascade is proposed to be mediated by the interaction of the cGMP and MAPK signaling pathways.

Materials and Methods

Cells. Bovine aortic endothelial cells (BAEC) and the murine-derived RAW 264.7 macrophage cell line were incubated as described previously (Saura et al., 1999; Hernandez-Perera et al., 2000). Experiments were performed in cells grown in passage 4 with serum-free medium.

Reagents. Cell culture supplies were from Falcon (BD Biosciences, Europe, Erembodegem, Belgium), cell culture transwells from Costar (Corning B.V. Life Sciences, Schiphol-Rijk, The Netherlands), calf serum was from BioWhittaker (Verviers, Belgium), cell culture gelatin and antibiotics were from Sigma-Aldrich (St. Louis, MO). RT-PCR reagents were from Invitrogen (Carlsbad, CA), except *Taq* DNA polymerase, which was from Applied Biosystems (Foster City, CA). Klenow fragment, RNAase A, RNAase T1, G-50 Sephadex Columns for radiolabeled RNA purification, and proteinase K were from Roche (Productos Roche S.A., Madrid, Spain). T3 RNA polymerase, T7 RNA polymerase, RNAase inhibitor RNasin, dual luciferase reporter system, pGL3 plasmids, and restriction endonucleases were from Promega (Madison, WI). Radiolabeled nucleotides were from PerkinElmer Life Sciences (Boston, MA). Autoradiography film was from Eastman Kodak (Rochester, NY). Polyvinylidene difluoride protein transfer membranes were from Millipore (Millipore Ibérica S.A., Madrid, Spain), enhanced chemiluminescence-detecting immunoblot system was from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Transfection reagents OptiMEM and LipofectAMINE were from Invitrogen. ERK1,2 polyclonal antibodies were from Oncogene (CN Biosciences, Nottingham, UK), Phospho-ERK 1,2 antibodies were from New England Biolabs (Beverly, MA), Flag-tagged epitope monoclonal antibody was from Sigma (St Louis, MO), PKG polyclonal antibody was from Calbiochem (CN Biosciences, UK), Raf-1 antibody was from Santa Cruz Biotechnologies (Santa Cruz, CA), Phospho-Raf polyclonal anbibodies were from New England Biolabs (Beverly, MA), HRP-conjugated secondary antibodies were from DAKO (DAKO Diagnostics, Spain).

RNA Isolation, RT-PCR, and RNAase Protection Assays. Total RNA from BAEC was isolated by the guanidinium thiocyanate method as described previously (Zaragoza et al., 2002). The mRNA was detected by real-time quantitative RT-PCR (7700 Sequence Detection System; Applied Biosystems Hispania, Spain). The following primers based on the human MMP-13 mRNA were selected: sense primer, 5′-CCAAATTATGGAGGAGATGC-3′; antisense primer, 5′-CGCCAGAAGAATCTGTCTTTAAA-3′. We used the SYBR Green PCR Master Mix (Applied Biosystems) reagents to perform the amplifications, according to the manufacturer's instructions. The relative quantification of MMP-13 mRNA levels was measured using a comparative method according to the manufacturer's software analysis. In brief, this method is based on the threshold cycle of amplification, C_t, which correlates inversely with the mRNA levels, and measured as the cycle number at which the SYBR Green fluorescent emission increases above a threshold level. The amount of target mRNA, normalized to a mRNA reference is given by the following equation: $2^{-\Delta\Delta Ct}$ The ΔCt value is the result of subtracting the average reference mRNA C_t value from the average target mRNA C_t. The $\Delta\Delta$Ct value is the subtraction of the ΔCt value of the sample from the ΔCt value of the reference in case of equal efficiency of amplification of target and reference mRNAs. As a reference gene, we used glyceraldehyde-3 phosphate dehydrogenase (GAPDH). The following primers based on the bovine GAPDH mRNA were selected: sense primer, 5′-AGTGGGTGATGCTGGTGCTG-3′; antisense primer, 5′-CGCCTGCTTCACCACCTTCTT-3′. To verify the specificity of the amplification, the PCR products were resolved in ethidium bromide agarose gels, followed by Southern blot hybridization using the MMP-13 cDNA as a radiolabeled probe.

For the RNAase protection experiments we designed a riboprobe by the insertion of a 258 base-pair MMP-13 cDNA fragment into the *Bam*HI/*Xba*I sites of pBluescript (pBCol3). The MMP-13 cDNA was generated by PCR using the following primers: sense: 5′-CGCGGATTCATGCATCCAGGGGTCCTG-3′; antisense: 5′-TGCTCTAGATTTGCCAGTCACCTCTAA-3′. The riboprobe was generated by the use of T3 RNA polymerase using pBCol3 linearized with *Xho*I in the following reaction mixture: 5× transcription buffer (supplied by the T3 RNA-polymerase manufacturer), 100 mM dithiothreitol, 40 U/μl RNasin, 2.5 mM 3NTP mix, 10 mCi/ml α^{32}-UTP, 250 μg of DNA template, and 10 U of T3 RNA polymerase. The mixture was incubated 1 h at 37°C and the template removed with RNase-free–DNase-I; 5×10^5 cpm was used to hybridize with 10 μg of total RNA, in a 1× hybridization buffer (80 mM Tris-HCl, pH 7.6, 4 mM EDTA, 1.6 mol/l NaCl, and 0.4% SDS) and 3× formamide, for 16 h at 45°C. Dimers were cleaved with RNase A and RNase T1. The protected RNA duplexes were purified, resolved on 8% polyacrylamide gels, and exposed to film.

Coculture of BAEC and RAW Cells. BAEC were grown in six-well plates and RAW macrophages in 0.4-μm pore size "Costar" transwell plates. After treatment, RAW cells were placed on a BAEC monolayer. NO production was measured by the Griess assay as described previously (Saura et al., 1999), and total RNA from BAEC was isolated to evaluate MMP-13 expression.

DAPI Staining of BAEC. BAEC were grown in microscope cover slips. After treatment, the cells were fixed with 4% paraformaldehyde, permeabilized with cold acetone and stained with DAPI. Cover slips were washed with PBS, and mounted in microscope slides with the FluorSave reagent (Calbiochem). Nuclei were detected by fluorescence microscopy and the average number of apoptotic nuclei was determined.

Transient Transfection of BAEC. BAEC were grown in six-well plates and transfected with 1 μg of DNA and the LipofectAMINE reagent according to the manufacturer's instructions. Experiments were done using the Dual Luciferase Reporter system, cotransfecting BAEC with a pGL3 reporter plasmid expressing *Renilla reniformis* under the control of a CMV promoter (pCMV-*R. reniformis*).

Plasmids. Plasmid pCol3-WT contains a functional part of the 5′ regulatory region of the human MMP-13 (GenBank accession

NM_002427), located upstream of a luciferase reporter gene. Plasmids pCol3-mAP-1 and pCol3-mOSE-2 are identical to pCol3-WT except for mutations at the AP-1 and OSE-2 responsive elements, respectively (Pendas et al., 1997).

The plasmid fG1AC encodes the catalytic domain of human PKG1-α plus an epitope tag FLAG fused at the 5′ terminus and is essentially identical as one reported previously by Boerth and Lincoln (1994) in baculovirus. PKG phosphotransferase activity was previously monitored by using BPDEtide as substrate (Browning et al., 2000).

The 3× AP-1 plasmid was generated after an original construct that contained three AP-1 sites from the PAI-1 promoter were subcloned into the plasmid pGL-3 basic (Promega, Madison, WI) and used then for transfection.

Immunoblot Analysis. Cell lysate extraction and protein immunoblots were performed as described previously (Hernandez-Perera et al., 2000).

Immunoprecipitation. Cells were disrupted with radioimmunoprecipitation assay buffer and precleared with the appropriate control IgG together with protein A agarose. Precleared supernatants were incubated 16 h with the corresponding antibodies, and washed 4 times with cold PBS. The samples were boiled and analyzed by SDS-PAGE for immunoblot purposes.

Statistical Analysis. Unless otherwise specified, data are expressed as means ± S.D., and experiments were performed at least three times in duplicate. Comparisons were made with analysis of variance followed by Dunnett's modification of the *t* test, whenever comparisons were made with a common control. The level of statistical significance was defined as $p < 0.05$. Error bars represent S.D.

Results

Nitric Oxide Donors Induce MMP-13 Expression in BAEC. To investigate if exogenously administered NO could affect MMP-13 expression in endothelium, we treated BAEC with the NO donor DEA-NO (100 μM) and analyzed MMP-13 mRNA by RNAase protection experiments. We also included RNA from the human fibroblast cell line KMST, which constitutively expresses MMP-13 and RNA from BAEC treated with PMA (10 μM), because MMP-13 expression in other systems is AP-1 sensitive. We could detect a basal level of MMP-13 expression in resting cells. However, NO and PMA increased the steady-state level of MMP-13 RNA after 16 h of treatment (Fig. 1A). The effect of 100 μM of *S*-nitroso-aminopenicillamide, a different NO donor, was also tested in BAEC and similar results were obtained (data not shown).

We also performed real time quantitative RT-PCR experiments using RNA from BAEC and stimulated with DEA-NO (100 μM), with specific primers that amplify a 300 base-pair cDNA fragment of MMP-13. The results show the same range of increase at the steady state level of MMP-13 mRNA (Fig. 1B).

To test the potential cytotoxicity associated with the amount of DEA-NO used for the experiments, we incubated BAEC with different concentrations of DEA-NO, and evaluated total cell death by trypan-blue exclusion and apoptosis by DAPI staining, showing that the dose at which DEA-NO induces the effect in BAEC did not induce significant cell death in our system (Table 1).

Endogenous NO from Murine Macrophages Induces MMP-13 Expression in BAEC. To test whether NO produced by cells could also affect the expression of MMP-13 in BAEC, we cultured RAW macrophages in transwells (see *Materials and Methods* for details) in the presence or absence of the NOS inhibitor L-NAME, and after induction of inducible NOS with 0.5 μg of LPS, the transwells were placed for a period of 16 h over a monolayer of BAEC. After incubation, macrophage NO production was tested by measuring nitrite accumulation with the Griess reagent, and BAEC MMP-13 expression was tested by RT-PCR. Coincubation of BAEC either with RAW alone or RAW incubated with 500 μM L-NAME did not affect basal MMP-13 expression of BAEC. By contrast, MMP-13 expression was increased when BAEC were coincubated with LPS-stimulated RAW macrophages, whereas the presence of 500 μM L-NAME before stimulation with LPS partially reduced MMP-13 expression of BAEC (Fig. 1C). As we have shown with exogenous NO, micromolar amounts of NO produced by macrophages were able to recapitulate MMP-13 expression of BAEC, in a similar fashion and magnitude.

NO Regulates MMP-13 Expression at the Transcriptional Level in BAEC. To explore the possible transcriptional regulation of MMP-13 by NO, we transiently transfected BAEC with pCol3-WT, a construct containing the functional regulatory region of the human *MMP-13* gene, and also with pCol3-mAP-1 and pCol3-mOSE-2, two constructs that contain single point mutations at the AP-1 and OSE-2 responsive elements, respectively (Balbin et al., 1999). We detected a basal level of activity when BAEC were transfected with either pCol3-WT or pCol3-mOSE-2, whereas the addition of the NO donor DEA-NO (100 μM) increased the activity 2- to 3-fold with respect to the transfected-resting cells. However, DEA-NO was not effective when added to the pCol3-mAP-1 transfected cells (Fig. 2A). Thus, NO-mediated regulation of MMP-13 expression seems to occur at the transcriptional level, and our results suggest that it is highly dependent on the activation of AP-1, whereas the OSE-2 site does not contribute to the regulation of the MMP-13 promoter in BAEC.

In human umbilical endothelial cells (HUVEC), the behavior of the MMP-13 promoter was comparable with that of BAEC (data not shown). Therefore, we proceeded to perform the transfection experiments in BAEC, given the technical advantages provided by this system.

Involvement of cGMP in the Transcriptional Regulation of MMP-13 by NO in BAEC. To examine the role of the cGMP signaling pathway in the transcriptional regulation of MMP-13 by NO, we transiently transfected BAEC stimulated with the lipophilic cGMP analog 8-Br-cGMP. As was the case with DEA-NO, in pCol3-WT-containing cells stimulated with 8-Br-cGMP (10 μM), an increase in the activity of the MMP-13 promoter construct could be detected, an effect that was absent in the cells transfected with pCol3-mAP-1 (Fig. 2B).

To gain further insight into the mechanism exerted by NO, we also stimulated pCol3-WT–transfected cells with the guanylate cyclase inhibitor ODQ, showing that ODQ markedly reduced the DEA-NO-induced activation of the MMP-13 promoter (Fig. 2C). In addition, the treatment with the PKG inhibitor PET was also sufficient to reduce the stimulatory effect induced by DEA-NO (Fig. 2C). MMP-13 mRNA steady-state levels were also reduced in DEA-NO- and 8-Br-cGMP–treated cells when incubated with PET (Fig. 2D).

To confirm the involvement of the cGMP signaling pathway, we transfected BAEC with fG1AC, a construct coding for the catalytic subunit of PKG1-α, whose expression leads to high levels of PKG phosphorylation (Browning et al.,

2000). The overexpression of PKG1-α in cells transfected with pCol3-WT resulted in a marked increase of the MMP-13 promoter activity, 5- to 6-fold over the values of cells transfected with pCol3-WT alone (Fig. 3).

MAP Kinases ERK1,2 Are Involved in the DEA-NO–Mediated Transcriptional Regulation of MMP-13. Even when the NO-cGMP pathway has been shown to regulate gene expression through the AP-1 pathway (Pilz et al., 1995), the low basal activity of the pCol3-mAP-1 promoter precluded us from inferring a definitive interpretation of the experiments using this construct. Thus we explored alternative routes to link the effect of NO to the participation of AP-1 and that could simultaneously shed some light on specific signal transduction pathways. Others have demonstrated that in certain cell types, MMP-13 expression is transcriptionally regulated by kinases belonging to the mitogen-activated protein kinase (MAPK) family (Ravanti et al., 1999; Johansson et al., 2000). To evaluate whether MAPKs were

TABLE 1
Cell death induced by DEA-NO in BAEC

DEA-NO	Total Cell Death	Apoptosis
$\mu mol/l$	%	
0	0.5 ± 0.01	0
1	0.5 ± 0.01	0
10	0.5 ± 0.05	0
100	1.25 ± 0.01	0.5 ± 0.05
250	2.75 ± 0.1	1.25 ± 0.05
500	5.25 ± 0.1	3.25 ± 0.05

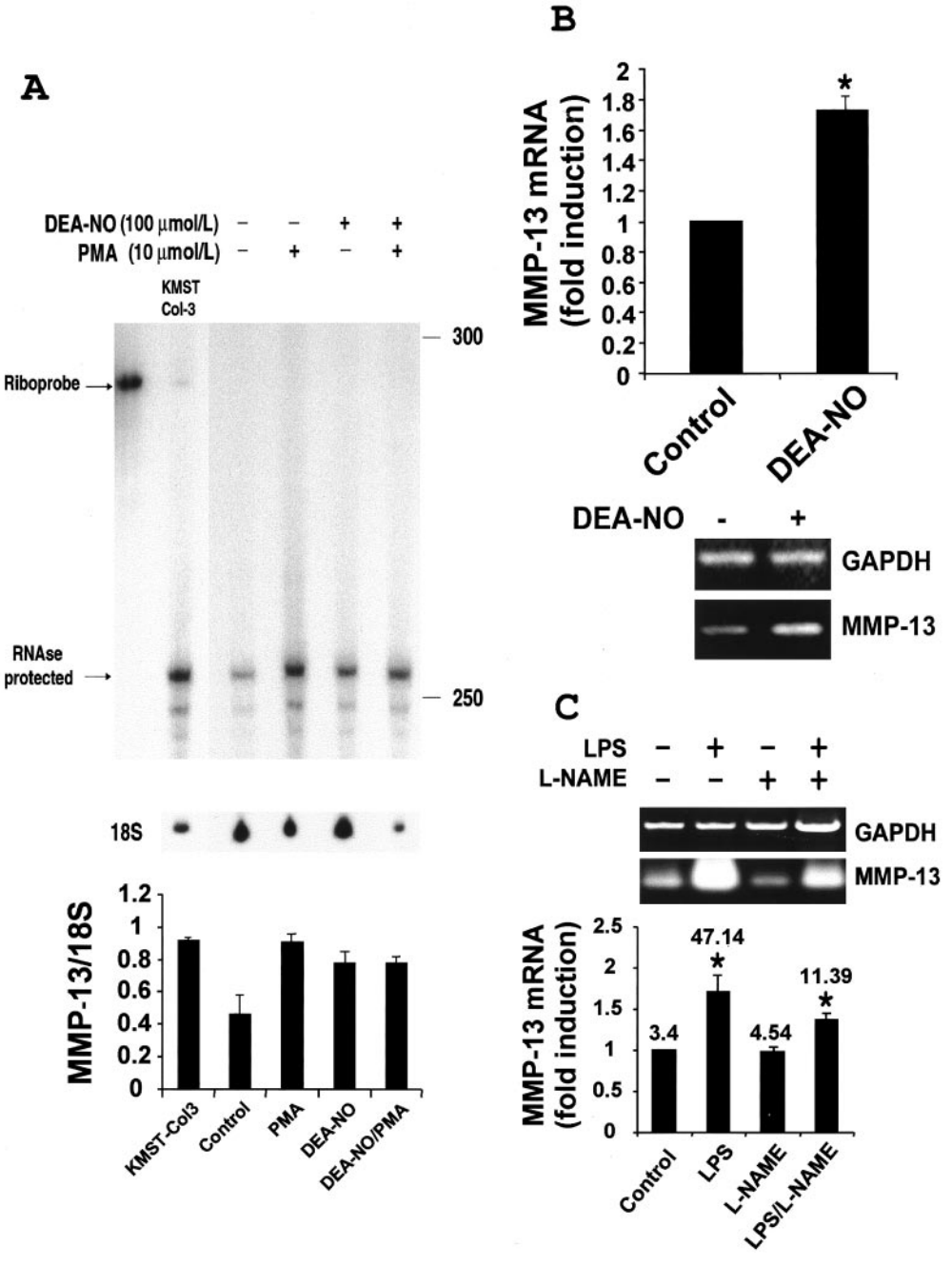

Fig. 1. MMP-13 expression is increased upon induction with NO. A, RNAase protection assay from BAEC and KMST-Col3 (stably transfected fibroblast cell line with a full-length cDNA from human MMP-13) hybridized with an MMP-13 antisense riboprobe. 18S RNA was included as a control. Results are representative of two independent experiments (mean ± S.D.). B, real time quantitative RT-PCR assay from BAEC stimulated with DEA-NO (100 μM) (see *Materials and Methods* for details). Values are represented as -fold induction of MMP-13 mRNA respect to the resting cells (n = 3, for every experiment each sample was assayed in duplicate (*, $p <$ 0.05 versus control). Bottom, an aliquot from the reaction mixture of one representative experiment electrophoresed in ethidium bromide stained agarose gel. C, RT-PCR assay from BAEC cocultured with RAW 264.7 macrophages in transwells (see *Materials and Methods* for details), in the presence or absence of 500 μM of L-NAME, and stimulated with 0.5 μg of LPS. Macrophage NO production was assayed by the Griess reaction and values of NO_2^- (expressed in micromoles per liter) are shown at the top of the bars. MMP-13 expression levels are represented as -fold induction of MMP-13 mRNA respect to the resting cells (n = 3, for every experiment each sample was assayed in duplicate (*, $p <$ 0.05 versus control). Top, result of one representative experiment from a total of 3.

also controlling MMP-13 expression in BAEC, we did dose- and time-response experiments to test the involvement of DEA-NO in the phosphorylation of specific MAPK representatives. Immunoblot experiments with specific anti-phospho-MAPK antibodies had shown that DEA-NO induces in BAEC the phosphorylation of ERK1,2 (Fig. 4A) and p38, the latter to a much lower extent (data not shown). We could detect phospho-ERK1,2 in cells treated with 8-Br-cGMP, whereas the well known MEK inhibitor PD98059 blocked basal, NO, and 8-Br-cGMP-induced ERK 1,2 phosphorylation (Fig. 4B). The MEK inhibitor PD98059 did not affect the steady state levels of MMP-13 mRNA, although it was sufficient to reduce the stimulatory effect of DEA-NO and 8-Br-cGMP (Fig. 5A). PD98059 blocked the activation of the MMP-13 promoter in cells transfected with pCol3-WT induced with either DEA-NO or 8-Br-cGMP (Fig. 5B). This result implies that ERK may function as a downstream effector of the NO-cGMP signaling in BAEC.

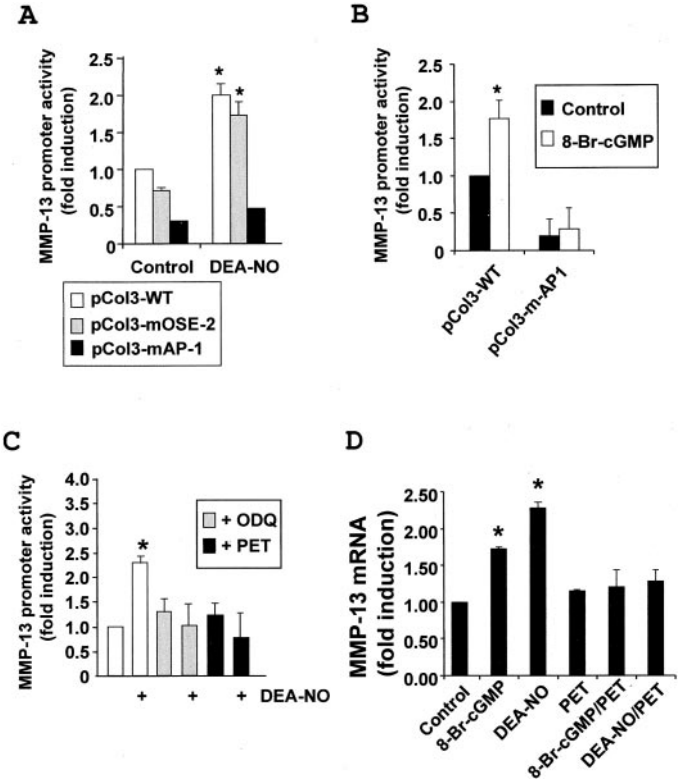

Fig. 2. DEA-NO transcriptional regulation of MMP-13 is mediated through the cGMP signaling pathway. A, transient transfection experiments of BAEC with different human MMP-13 promoter constructs. Cells were transiently transfected with either pCol3-WT, pCol3-mAP-1, or pCol3-mOSE-2 (see *Materials and Methods* for details) and stimulated with DEA-NO (100 μM) for 16 h. Single data points were normalized by cotransfecting with pCMV-*R. reniformis* (see *Materials and Methods* for details). Values are represented as -fold induction with respect to pCol3-WT–transfected resting cells (n = 3, mean ± S.D.; *, $p < 0.05$ versus control). BAEC were transiently transfected with pCol3-WT or pCol3-mAP-1 (see *Materials and Methods* for details) and treated with: 8-Br-cGMP (10 μM), (n = 3, mean ± S.D.) (B) or DEA-NO (100 μM), DEA-NO/ODQ (100/0.2 μM) (n = 4, mean ± S.D.), and DEA-NO/PET (100/0.5 μM) (n = 3, mean ± S.D.) (C) for 16 h. Single data points were normalized by cotransfecting with pCMV-*R. reniformis* (see *Materials and Methods* for details). Values are represented as -fold induction with respect to pCol3-WT–transfected resting cells. D, effect of PET on the MMP-13 mRNA steady-state levels, after stimulation with DEA-NO (100 μM) or 8-Br-cGMP (10 μM) for 16 h. RT-PCR of BAEC using specific MMP-13 oligonucleotides. Amplification of a GAPDH cDNA fragment was used as control. Results are representative of three independent experiments (*, $p < 0.05$ versus control for B, C, and D).

To probe the downstream ERK signaling contribution to the MMP-13 expression induced by NO, we cotransfected BAEC with pCol3-WT and the expression plasmid for PKG1-α, fG1AC. When cells were incubated with the MEK inhibitor PD98059, a 60% reduction in the basal MMP-13 promoter activity and a 50% reduction in cells treated with 8-Br-cGMP was achieved (Fig. 6). Of note, cotransfection of fG1AC resulted in a significantly increased basal activity of the pCol-3-WT promoter, implying that PKG may be important for the expression of MMP-13.

In HUVEC, the serine/threonine kinase Raf, upstream activator of ERK, is one of the targets of PKG (Hood and Granger, 1998). To test this phenomenon in BAEC, we immunoprecipitated cell lysates treated with 8-Br-cGMP, DEA-NO, or cells transiently transfected with fG1AC (overexpressing PKG1-α), with Raf-1 antibodies and then immunoblotted these same lysates with an anti-PKG antibody. Treated and transfected BAEC showed increased amounts of PKG compared with control cells, and consistent results were obtained with the crossed immunoprecipitation, suggesting that the NO-cGMP pathway promotes the association of Raf-1 with PKG (Fig. 7A). The functionality of the complex was tested by the use of specific phospho-Raf antibodies, which detect the phosphorylation of Raf. Immunoprecipitated BAEC lysates with PKG antibodies and treated with DEA-NO, 8-Br-cGMP or expressing the dom-

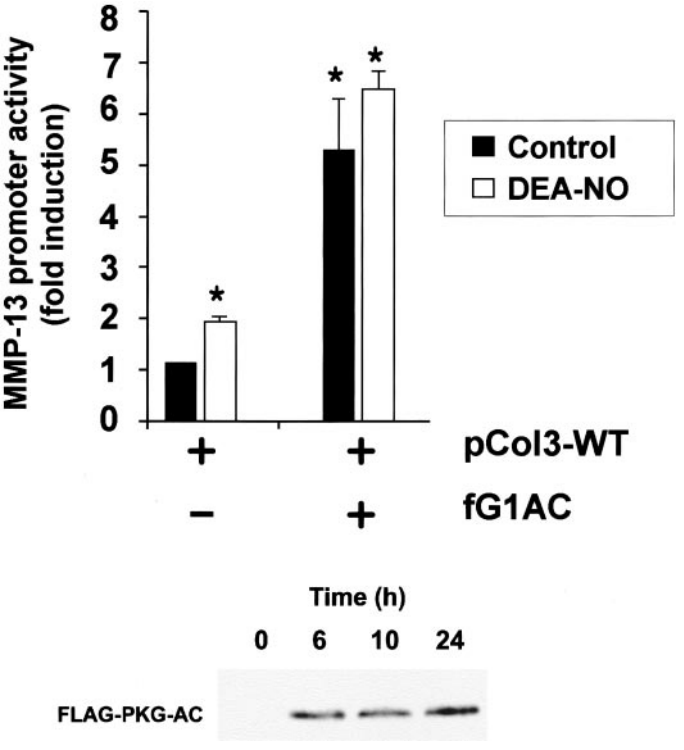

Fig. 3. Overexpression of PKG increases the activity of the human MMP-13 promoter in transiently transfected BAEC. MMP-13 promoter activity in BAEC transfected with fG1AC, a construct containing the catalytic subunit of PKG1-α plus an epitope tag FLAG fused at the 5′-terminus, and pCol3-WT. Single data points were normalized by co-transfecting with pCMV-*R. reniformis* (see *Materials and Methods* for details). Values are represented as -fold induction with respect to pCol3-WT–transfected resting cells (n = 3, mean ± S.D.; *, $p < 0.05$ versus control without fG1AC). Bottom, time course of PKG1-α expression in transiently transfected BAEC. Cells were transfected with fG1AC. Cell lysates were immunoblotted and PKG1-α was detected with a mouse monoclonal anti-FLAG antibody. The experiment shown is representative of two independent experiments.

inant positive PKG construct contain phospho-Raf, as shown by Western blot (Fig. 7A, bottom). Although a nonspecific interaction between PKG and Raf-1 cannot be definitively excluded, the capacity of the catalytic region of PKG1α to interact with the full-length and the regulatory regions has been previously shown (Browning et al., 2001). In addition, the complex is inducible and time-sensitive, supporting the idea that Raf-1 is a target of PKG in the aortic endothelium (Fig. 7B).

To further support the involvement of PKG in the AP-1 activation through the ERK signaling pathway, we transfected BAEC with a construct containing three AP-1 responsive elements repeated in tandem, controlling the luciferase gene (p3XAP-1-luc). After transfection with p3XAP-1-luc, cells were treated with 100 μM DEA-NO, 10 μM 8-Br-cGMP, or cotransfected with fG1AC. To elucidate the involvement of ERK in the activation of AP-1, cells were also treated with 20 μM PD98059. The promoter activity was tested by measuring luciferase activity detecting a significant reduction of the activity when PD98059 was present (Fig. 8).

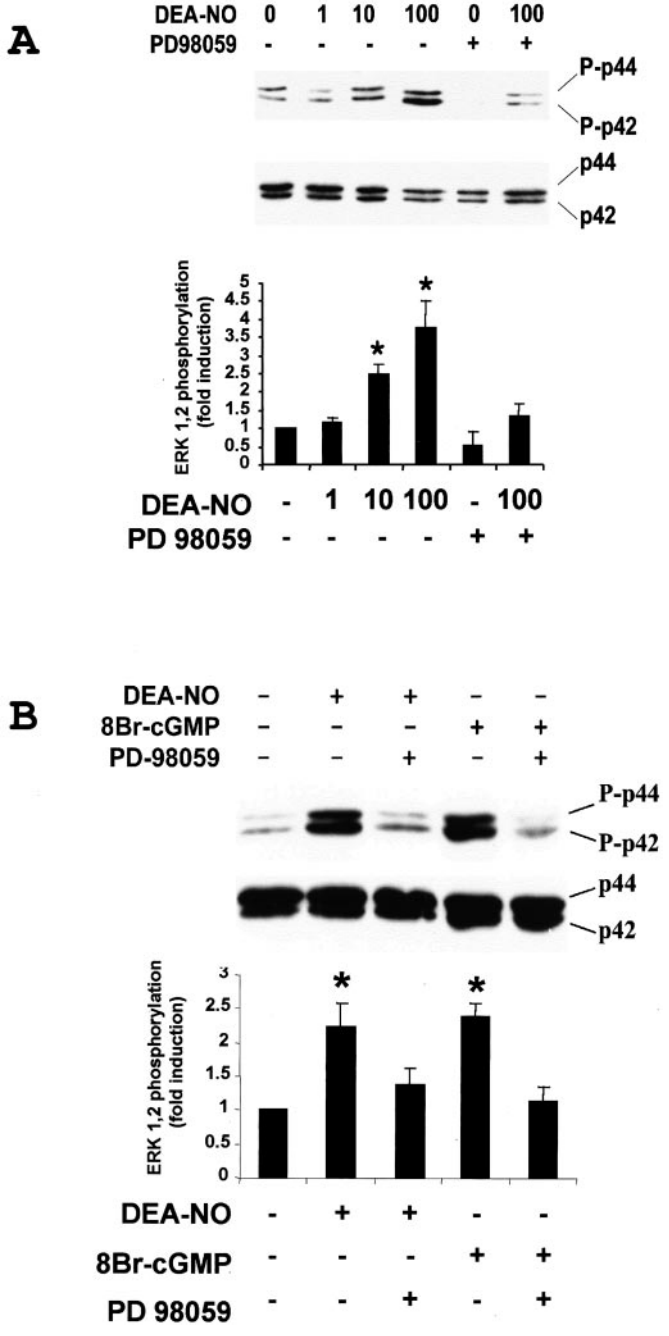

Fig. 4. NO-cGMP signaling phosphorylates the MAPK ERK1, 2. Cell lysates from BAEC treated with either DEA-NO (1, 10, and 100 μM, 16 h), and/or PD98059 (20 μM, preincubated 1 h) immunoblotted with phospho-ERK1,2 antibodies (A) or DEA-NO (100 μM, 16 h), 8-Br-cGMP (10 μM, 16 h), and/or PD98059 (20 μM, preincubated 1 h) (B) and immunoblotted with phospho-ERK1,2 (top) and total ERK (bottom) antibodies (n = 3, mean ± S.D. for A and B; *, $p < 0.05$ versus control).

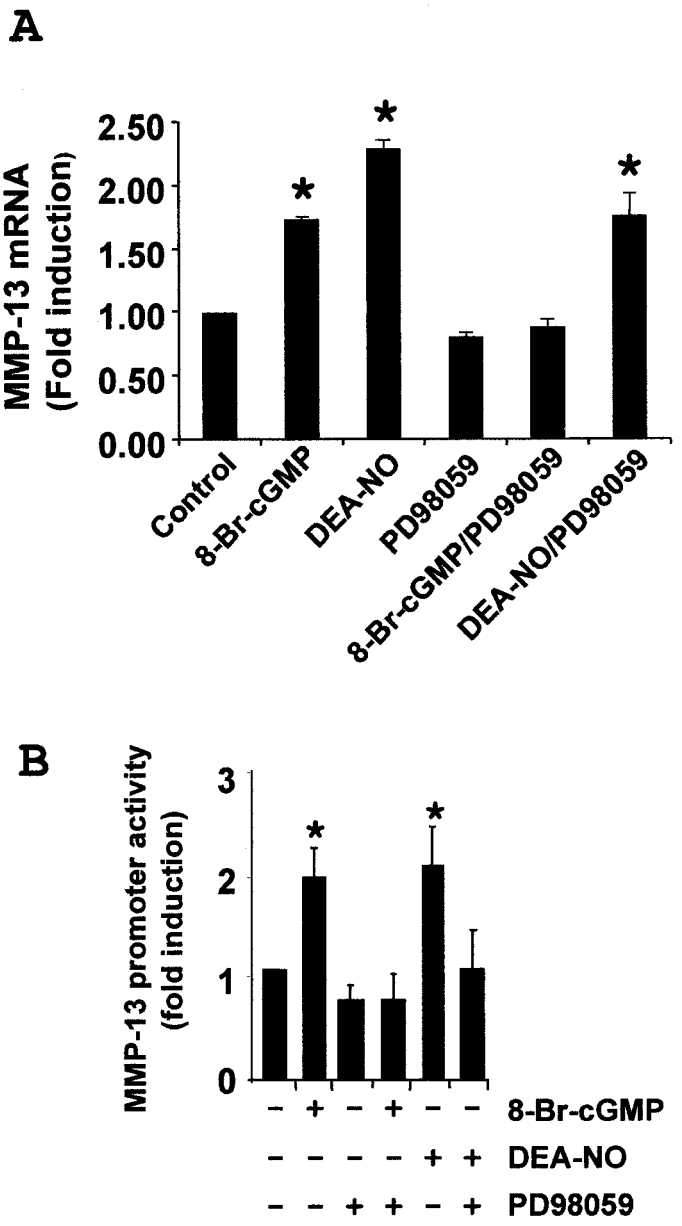

Fig. 5. PD98059 reduces the expression of MMP-13 in DEA-NO- and 8-Br-cGMP–stimulated BAEC. A, RT-PCR with specific MMP-13 oligonucleotides, treated with PD98059 (20 μM, preincubated 1 h) and challenged with DEA-NO (100 μM, 16 h) or 8-Br-cGMP (10 μM, 16 h). Amplification of a GAPDH cDNA fragment was used as control (n = 3, mean ± S.D.; *, $p < 0.05$ versus control). B, cells were transfected with pCol3-WT and incubated with PD98059 alone (20 μM) or in combination with DEA-NO (100 μM, 16 h) and 8-Br-cGMP (10 μM, 16 h). Single data points were normalized by cotransfecting with pCMV-*R. reniformis* (see *Materials and Methods* for details). Values are represented as -fold induction with respect to pCol3-WT–transfected resting cells (n = 3, mean ± S.D.; *, $p < 0.05$ versus control).

The results presented here suggest that NO-induced MMP-13 expression is mediated downstream by the NO-cGMP and the MAPK-ERK signaling pathways, which leads to the binding of AP-1 to the corresponding *cis*-acting element located in the promoter of the gene.

Discussion

Matrix metalloproteinases and vasoactive factors are important homeostatic elements within the vascular wall. Balance in the build-up of matrix proteins and vascular tone are fundamental processes that are perturbed in such conditions as atherogenesis or hypertension. Investigation of their cross-talk and interaction is thus of interest. We previously have found that in the vascular endothelium, exogenously administered NO induces the expression and activity of MMP-13 (Zaragoza et al., 2002). In this work, we show that both exogenous and endogenous NO are able to increase MMP-13 expression in vascular endothelial cells by increasing the promoter activity of its gene. In addition, our data are consistent with the involvement of the cGMP-PKG axis, which in turn phosphorylates Raf and leads to the activation of the MAPK pathway, in particular ERK 1,2.

NO has been shown to regulate the expression of several matrix components and matrix metabolizing enzymes (Pfeilschifter et al., 2001). These include collagen, MMP-2, MMP-9, and tissue inhibitors of metalloproteinases. In most cases, however, the molecular mechanisms underlying these observations remain unclear. The MAPK pathway is the one of the signaling cascades that links external stimuli to the transcriptional regulation of MMPs. In particular, p38 activation successfully mediates MMP-13 expression in transforming growth factor-β–stimulated fibroblasts, or tumor necrosis factor-α– and transforming growth factor-β–stimulated keratinocytes (Johansson et al., 2000). In endothelial cells, the MAPK-ERK signaling pathway may represent an important route by which MMP-13 is regulated. In keeping with this concept, it has been shown that MMP-9 expression is regulated in endothelial cells precisely by the ERK signaling cascade (Genersch et al., 2000). Here, we show that in endothelial cells, NO may enhance MMP-13 by way of the interaction of two distinct molecular pathways: the NO-cGMP-PKG and the MAPK ERK. This is based upon the following observations: 1) PKG overexpression results in MMP-13 promoter activation; 2) NO and cGMP promote ERK phosphorylation; 3) the MAPK inhibitor PD98059 inhibits NO-and cGMP-induced ERK phosphorylation and cGMP-induced MMP-13 expression; 4) PD98059 prevents the NO- and cGMP-mediated effect in the presence and absence of overexpressed PKG1-α; and 5) we detected in BAEC the phosphorylation of Raf, an upstream effector of MEK, by PKG. The inter-relationship between these two pathways has been reported in vascular smooth muscle cells (Komalavilas et al., 1999), fibroblasts (Gu et al., 2000), and human endothelial cells (Hood and Granger, 1998).

Studies using the yeast-two hybrid technique have shown

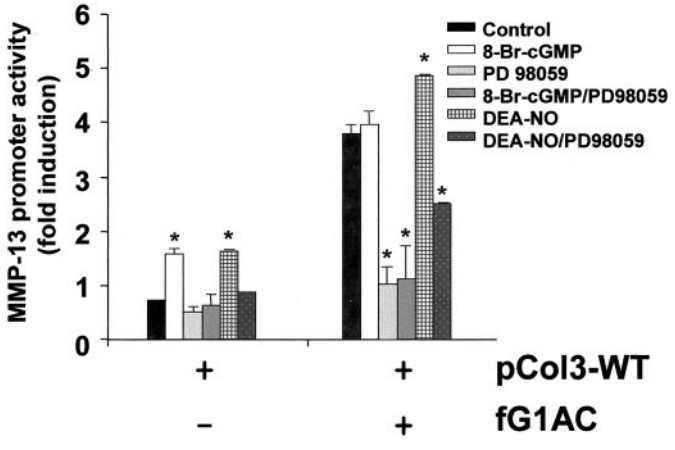

Fig. 6. The MEK inhibitor PD98059 reduces PKG-induced activity of MMP-13 promoter in transiently transfected BAEC. Transiently transfected BAEC with pCol-3WT, in the absence or presence of fG1AC, assayed for MMP-13 promoter activity after treatment with DEA-NO (100 μM, 16 h), 8-Br-cGMP (10 μM, 16 h), or in combination with PD98059 (20 μM, preincubated 1 h). Single data points were normalized by cotransfecting with pCMV-*R. reniformis* (see *Materials and Methods* for details). Values are represented as -fold induction with respect to pCol3-WT–transfected resting cells (n = 3, mean ± S.D.; *, $p < 0.001$ versus each respective control).

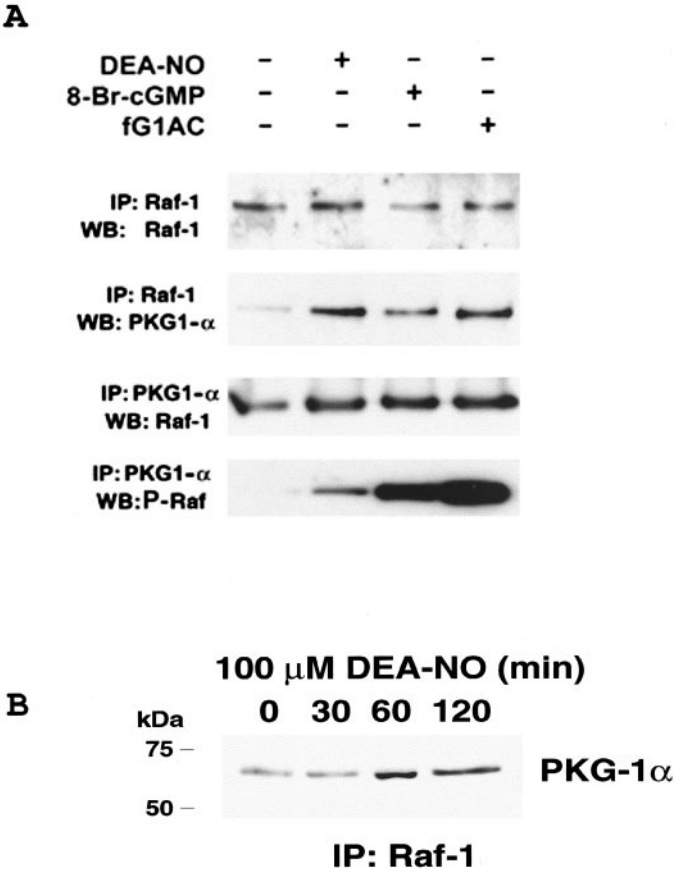

Fig. 7. NO and cGMP promote Raf phosphorylation when associated with PKG in BAEC. A, BAEC were treated with DEA-NO (100 μM), 8-Br-cGMP (10 μM), or transiently transfected with fG1AC, a dominant positive construct that overexpresses the catalytic subunit of PKG-1α. Cellular extracts were immunoprecipitated with anti-Raf-1 polyclonal antibodies (top) or with anti-PKG polyclonal antibodies (bottom). Raf-1 immunoprecipitated extracts were immunobloted with the same antibody as a control (top) or with anti-PKG polyclonal antibodies (top middle). In addition, cellular extracts immunoprecipitated with PKG were immunobloted with anti-Raf (bottom middle) and with anti phospho-Raf polyclonal antibodies (bottom). Results are representative of three independent experiments. B, time course of the PKG/Raf-1 complex formation after stimulation with DEA-NO. Cells were treated with DEA-NO for the indicated times and then immunoprecipitated with polyclonal anti-Raf-1 antibodies. Immunoprecipitated extracts were immunoblotted and PKG was detected with a polyclonal anti-PKG antibody. Results are representative of three independent experiments.

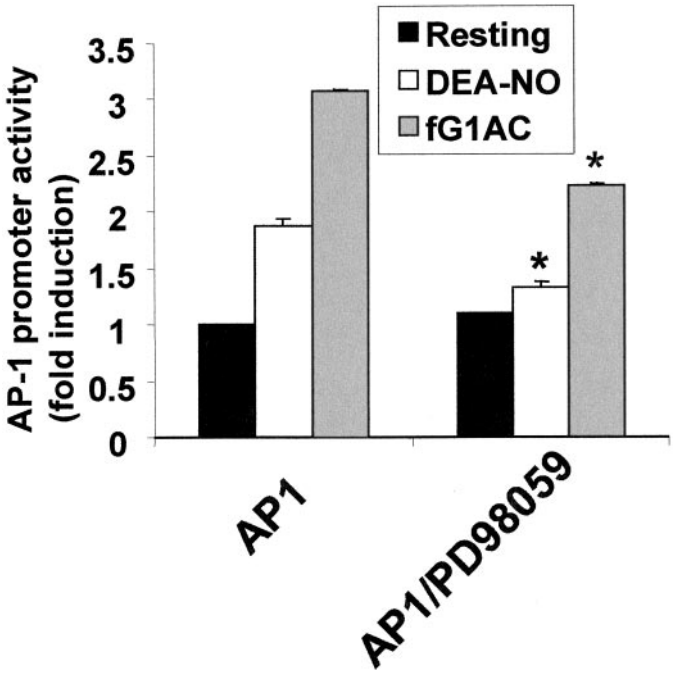

Fig. 8. Effect of PKG on the MAPK-induced AP-1 activity in BAEC. BAEC transiently transfected with p3XAP-1 (see *Materials and Methods* for details) were treated with DEA-NO (100 μM), 8-Br-cGMP (10 μM), or transiently transfected with fG1AC, and treated or not with PD98059 (20 μM, preincubated 1 h) or vehicle. Single data points were normalized by cotransfecting with pCMV-*R. reniformis* (see *Materials and Methods* for details). Values are represented as -fold induction with respect to p3XAP-1–transfected resting cells (one experiment representative of two).

that PKG-1α interaction is mediated by the N-terminal domain of the kinase (Yuasa et al., 2000). However, it is possible that other domains may participate. In the present study, the construct encoded by fG1AC lacks the N-terminal domain, although it still interacts with Raf-1, and the same complex can be detected when endogenous PKG was immunoprecipitated. Besides, after activation, the complex is time sensitive, as shown in Fig. 7B.

We also attempted to render specificity to the phosphorylation of Raf mediated by PKG in endothelial cells. In preliminary experiments, the use of a dominant negative PKG construct (T516A), which was previously shown to reduce NO-cGMP–mediated p38 phosphorylation in human embryonic kidney 293 fibroblasts (Browning et al., 2000), significantly reduced the amount of Raf phosphorylation in BAEC, compared with the amount detected after transfection of BAEC with fG1AC (data not shown). Although we cannot exclude a nonspecific phosphorylation of Raf by the NO-cGMP pathway in BAEC, the data presented here support the specificity of the reaction as demonstrated previously in HUVEC (Hood and Granger, 1998), as well as in other systems, such as the phosphorylation of the myosin-binding protein of myosin phosphatase (Surks et al., 1999).

This is the first time, to our knowledge, that the involvement of both signaling mechanisms triggered by NO in the expression of MMP-13 by endothelial cells has been reported. Although we have not directly addressed the pathways leading from ERK 1,2 activation to MMP-13 expression, the downstream activation of AP-1 through the ERK 1,2 signaling pathway is well established (El-Dahr et al., 1998). Hence, it is likely that this mechanism could underlie the enhancement of MMP-13 expression by the NO-cGMP-PKG axis.

Endothelial cells synthesize MMPs during angiogenesis as a result of their interaction with immune cells (Hojo et al., 2000), during atherosclerosis (Huang et al., 2001), in response to blood flow changes (Tronc et al., 2000) and also to facilitate the shedding of different molecules, including soluble adhesion molecules, and growth factors (Silletti et al., 2001). Late stages of atherosclerosis are associated with increased synthesis of MMPs involved in plaque disruption. Enhanced expression of MMP-13 has been detected in advanced atherosclerotic lesions of aortas from apolipoprotein E deficient mice (Jeng et al., 1999). It might be speculated that NO generated by macrophage foam cells could be in part responsible for MMP-13 expression during atherosclerosis. Besides, endothelial cells present at the atherosclerotic lesion might also contribute to plaque rupture. It has been shown that oxLDL induces MMP-1 expression in human aortic endothelial cells (Huang et al., 1999). In this context, oxidized low-density lipoprotein, but also NO from macrophage foam cells, may enhance MMP-13 production by endothelial cells, thus contributing to destabilization or rupture of the plaque. Corroboration of this hypothesis will require approaches using in vivo models of atherogenesis.

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