Atrial Natriuretic Peptide Induces Mitogen-Activated Protein Kinase Phosphatase-1 in Human Endothelial Cells via Rac1 and NAD(P)H Oxidase/Nox2-Activation

Robert Fürst, Corinna Brueckl, Wolfgang M. Kuebler, Stefan Zahler, Florian Krötz, Agnes Görlach, Angelika M. Vollmar, Alexandra K. Kiemer

Abstract—The cardiovascular hormone atrial natriuretic peptide (ANP) exerts anti-inflammatory effects on tumor necrosis factor-α–activated endothelial cells by inducing mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1). The underlying mechanisms are as yet unknown. We aimed to elucidate the signaling pathways leading to an induction of MKP-1 by ANP in primary human endothelial cells. By using antioxidants, generation of reactive oxygen species (ROS) was shown to be crucially involved in MKP-1 upregulation. ANP was found to increase ROS formation in cultured cells as well as in the endothelium of intact rat lung vessels. We applied NAD(P)H oxidase (Nox) inhibitors (apocynin and gp91ds-*tat*) and revealed this enzyme complex to be crucial for superoxide generation and MKP-1 expression. Moreover, by performing Nox2/4 antisense experiments, we identified Nox2 as the critically involved Nox homologue. Pull-down assays and confocal microscopy showed that ANP activates the small Rho-GTPase Rac1. Transfection of a dominant-negative (RacN17) and constitutively active Rac1 mutant (RacV12) indicated that ANP-induced superoxide generation and MKP-1 expression are mediated via Rac1 activation. ANP-evoked production of superoxide was found to activate c-Jun N-terminal kinase (JNK). Using specific inhibitors, we linked ANP-induced JNK activation to MKP-1 expression and excluded an involvement of protein kinase C, extracellular signal-regulated kinase, and p38 MAPK. MKP-1 induction was shown to depend on activation of the transcription factor activator protein-1 (AP-1) by using electrophoretic mobility shift assay and AP-1 decoys. In summary, our work provides insights into the mechanisms by which ANP induces MKP-1 and shows that ANP is a novel endogenous activator of endothelial Rac1 and Nox/Nox2. **(*Circ Res*. 2005;96:43-53.)**

Key Words: endothelium ■ natriuretic peptides ■ signal transduction ■ Rac1 ■ Nox2

The cardiovascular hormone atrial natriuretic peptide (ANP) plays an important and well-investigated role in the cardiovascular system by participation in blood pressure regulation.[1] ANP mainly acts through binding to the guanylyl cyclase–coupled natriuretic peptide receptor-A (NPR-A), leading to generation of the second messenger cGMP.[1] ANP also binds to NPR-C, which generally acts as a clearance receptor[1] but is also able to mediate an inhibition of adenylyl cyclase activity and activation of phospholipase C.[2]

A growing number of studies highlight the profound anti-inflammatory and vasoprotective actions exerted by ANP.[3,4] In this context, we revealed recently that ANP is able to induce mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) in cultured endothelial cells (ECs).[5] MKP-1 inhibits tumor necrosis factor-α (TNF-α)–induced p38 MAPK activation, thus leading to a reduction of stress fiber formation,[5] endothelial permeability,[5] and expression of monocyte chemoattractant protein-1.[6] In summary, ANP protects TNF-α–activated ECs against structural and functional changes by inducing MKP-1.

MAPKs play a crucial role in a great variety of intracellular signaling cascades. Pathways of MAPK activation are quite well known compared with the mechanisms of their inactivation, which represent equally important steps in signal transduction. Dual specificity MAPK phosphatases such as MKP-1 inactivate MAPK by dephosphorylation of the two critical MAPK residues accountable for their activity. MKP-1 is constitutively expressed at a very low level and underlies a tight and rapid transcriptional induction by different stimuli.[7] Little is known about the signaling events leading to an MKP-1 induction besides reports describing that reactive oxygen species (ROS)[8,9] and different kinases (MAPK and protein kinase C [PKC])[10–13] might be involved.

Original received March 15, 2004; first resubmission received July 8, 2004; second resubmission received November 2, 2004; accepted November 18, 2004.

From the Department of Pharmacy (R.F., S.Z., A.M.V., A.K.K.), the Institute for Surgical Research (C.B.), and Institute of Physiology (F.K.), University of Munich, Germany; Institute of Physiology (W.M.K.), Campus Benjamin Franklin, Charité, Berlin, Germany; Experimental Pediatric Cardiology (A.G.), German Heart Center Munich, Technical University Munich, Germany; and The Scripps Research Institute (A.K.K.), La Jolla, Calif.

Correspondence to Alexandra K. Kiemer, PhD, The Scripps Research Institute, Molecular and Experimental Medicine, Mail MEM 131, 10550 N Torrey Pines Rd, La Jolla, CA 92037. E-mail kiemer@scripps.edu

Circulation Research **is available at http://www.circresaha.org** **DOI: 10.1161/01.RES.0000151983.01148.06**

For several decades, ROS were considered toxic byproducts of metabolic processes being able to damage cellular components. Many diseases have been implicated with oxidative stress, but in the last years, more and more studies revealed that ROS not only take part in pathological conditions but also as second messengers in cell signaling processes. The prototypical, well-known phagocyte NAD(P)H oxidase (Nox), a superoxide-producing enzyme complex, consists of six components: gp91^{phox}/Nox2, p22^{phox}, p47^{phox}, p67^{phox}, p40^{phox}, and Rac, which are all known to be present in ECs.[14] The low amounts of superoxide produced by endothelial Nox can stimulate a variety of transcription factors and signaling cascades without altering the cellular redox state.[15] In recent years, gp91^{phox}/Nox2 homologues (Nox1–Nox5) have been discovered. Besides gp91^{phox}/Nox2, Nox1 and Nox4 have been shown recently to be expressed in ECs,[16] suggesting that different types of Noxes coexist in the endothelium. The physiological significance of this fact is as yet only poorly investigated.

Because MKP-1 induction accounts for the above-mentioned protective effects of ANP on ECs,[5,6] we were interested in the molecular mechanisms underlying MKP-1 upregulation. We hypothesized that ANP-induced generation of ROS is an early event in the signal transduction pathway upregulating MKP-1 and aimed to characterize the enzymatic source of ROS generation. Moreover, we hypothesized that a ROS-activated kinase and transcription factor could be responsible for the increased MKP-1 expression.

Materials and Methods

Materials

Cell culture supplies were from PAN Biotech. Rat ANP was from Bachem and (Cys 18)-atrial natriuretic factor (4–18) amide (rat) from Saxon Biochemicals. 8-Br-cGMP, SB203580, calphostin C, PD98059, phorbol-12-myristate-13-acetate (PMA), and SP600125 were from Calbiochem. GTPγS was from Roche. gp91ds-*tat* was provided by Dr P. Pagano (Detroit, Mich). Anti–MKP-1 and anti-Nox4 antibodies were from Santa Cruz Biotechnology, anti–c-Jun N-terminal kinase (JNK) and anti–phospho-JNK from Cell Signaling, and anti-Rac from Upstate Biotechnology. Anti-Nox2 was provided by Dr D. Roos (Amsterdam, the Netherlands). AlexaFluor488 goat anti-mouse, dihydrofluorescein diacetate, and dihydrodichlorofluorescein diacetate were from Molecular Probes. Horseradish peroxidase–conjugated goat anti-rabbit and donkey anti-goat were from Dianova and goat anti-mouse from Cell Signaling. Activator protein-1 (AP-1) decoy/scrambled decoy and Nox2/4 antisense/sense phosphorothioate oligodesoxynucleotides and primers for MKP-1 and Nox1–Nox5 were from MWG Biotech or biomers.net. All other substances were from Sigma-Aldrich.

Cell Culture

Human umbilical vein ECs (HUVECs) were prepared, cultured, characterized, and used for studies as described.[5] Human microvascular ECs (HMECs), a cell line known to retain endothelial phenotypic and functional characteristics,[17] were from Centers for Disease Control and Prevention (Atlanta, Ga). HMECs were cultured in EC growth medium (Promocell).

Western Blot Analysis

In Vitro Samples

Immunoblotting was performed as described.[5] All experiments were performed in duplicates or triplicates, and one representative blot of two to four independent experiments is shown.

In Vivo Samples

In anesthetized Sprague-Dawley rats, a bolus of ANP (n=3) or an equivalent volume of NaCl (n=3) was injected via a central venous catheter. ANP dosage was calculated to reach a blood concentration of 1 μmol/L. Sixty minutes after bolus administration, lungs were excised and cryopreserved in liquid nitrogen. Lung tissue protein was extracted, and immunoblotting was performed as described.[5]

Detection of mRNA

RNA isolation, RT-PCR, agarose gel electrophoresis, and ethidium bromide staining were performed as described.[5] Primers for MKP-1: 5′-GCTGTGCAGCAACAGTC-3′ and 5′-TACCTTATGAGGACTAATCG-3′; Nox1: 5′-TGGCTAAATCCCATCCAGTC-3′ and 5′-AGTGGGAGTCACGATCATCC-3′; Nox2: 5′-TGGATAGTGGGTCCCATGTT-3′ and 5′-GCTTATCACAGCCACAAGCA-3′; Nox3: 5′-CCAGGGCAGTACATCTTGGT-3′ and 5′-CTGTGCCTCACTGCATTTGT-3′; Nox4: 5′-TGTTGGATGACTGGAAACCA-3′ and 5′-TGGGTCCACAACAGAAAACA-3′; and Nox5: 5′-CTACGTGGTAGTGGGGCTGT-3′ and 5′-AACAAGATTCCAG-GCACCAG-3′.

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared and electrophoretic mobility shift assay (EMSA) was performed as described.[18] SuperFect transfection reagent (Qiagen) was used to transfect AP-1 decoy (5′-cgctTGATGACTC-AGCCggaa-3′) and scrambled decoy (5′-cgctTGATGACTTGGCCggaa-3′; (lower case letter: phosphorothioate backbone). One representative image of 2 to 3 independent experiments is shown.

Detection of ROS in Cell Culture

Cells were loaded with 20 μmol/L dihydrofluorescein diacetate for 20 minutes. Fluorescence measurements (excitation, 485 nm; emission, 535 nm) were taken 30 minutes after treatment (except for ANP time course). Data were calculated as percent increase/decrease of fluorescence values of untreated cells. For each experiment, two to four independent cell preparations were used with at least six parallel samples.

Isolated-Perfused Rat Lung Preparation and In Situ Fluorescence Microscopy

Isolated-perfused rat (Sprague-Dawley) lung preparation was performed as described.[19,20] ROS production was determined in situ by digital fluorescence imaging of lung capillary ECs loaded with dihydrodichlorofluorescein diacetate,[21] which was infused into pulmonary venular capillaries using a venous microcatheter.[19,20] The procedure is proven for selective loading of fluorescent dyes to ECs. Fluorescence was excited at 488 nm. Emission was collected by a CCD camera and subjected to digital image analysis (TILL Photonics). Images of single subpleural capillaries were obtained in 5-second intervals and were corrected for background as determined over alveolar spaces. Capillaries were viewed at a focal plane corresponding to their maximum diameter (12 to 25 μm).

Assay of Rac1 Activity

The affinity precipitation assay detecting active Rac1 uses the p21-binding domain (PBD) of the Rac1 target p21-activated kinase 1 fused to glutathione *S*-transferase (GST) to isolate a complex containing GST-PBD bound to active Rac1 (Rac1-GTP). The plasmid encoding the GST-PBD fusion protein was provided by Dr U. Knaus (La Jolla, Calif). GST-PBD was expressed in *Escherichia coli*, purified, and bound to glutathione Sepharose beads (Amersham). Rac1 pull-down assays were performed as described.[22]

Nox2/4 Antisense Experiments

HUVECs were transfected with Nox2 antisense (5′-cctcatTCACAGCGcagtt-3′), Nox4 antisense (5′-ggacaCAGCCATGccgcc-3′), or the respective sense oligos (lower case letter: phosphorothioate backbone) by using

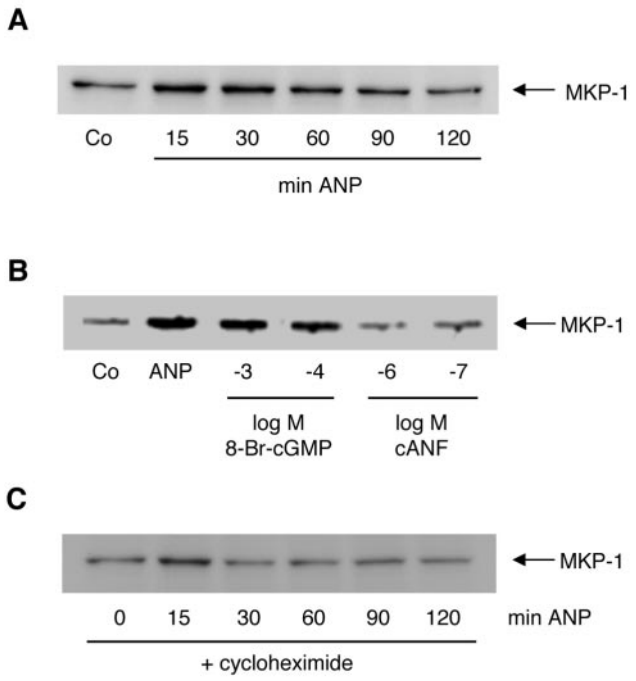

Figure 1. Effect of ANP, 8-Br-cGMP, cANF, and cycloheximide on MKP-1 protein expression. Control cells (Co) were left untreated. A, HUVECs were treated with ANP (1 μmol/L) as indicated. B, Cells were treated with ANP (1 μmol/L), 8-Br-cGMP, or cANF for 60 minutes. C, HUVECs were treated with ANP (1 μmol/L), as indicated in the presence of cycloheximide (10 μg/mL, 30 minutes before ANP). MKP-1 protein expression was determined by Western blot analysis.

jetPEI (Polyplus-Transfection). Experiments were started 24 to 48 hours after transfection.

Confocal Laser Scanning Microscopy

HUVECs were cultured on collagen-coated glass cover slips, treated, fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100, and incubated with primary and secondary antibody for 1 hour each. Images were obtained with Zeiss LSM 510 META.

Plasmid Constructs and Transfection of HMECs

The expression vectors for myc-tagged Rac1 were cloned by excision of the *Eco*RI fragment from the constructs RacN17pc-myc and RacV12pc-myc and ligation into the *Eco*RI site of pcDNA3.1. HMECs were transfected with the RacN17-, RacV12-construct, or control plasmid pcDNA3.1 by using SuperFect transfection reagent (Qiagen).

Statistical Analysis

Data are expressed as mean±SEM. Statistical analysis was performed with GraphPad Prism 3.03. A 5% significance level was used ($P \leq 0.05$). For ROS measurement in cultured cells, one-way ANOVA followed by Tukey's post test was used. For ROS measurement in rat lung ECs, data of independent groups were compared by Mann–Whitney *U* test.

Results

ANP Induces MKP-1 Expression via cGMP/NPR-A

ANP rapidly increased endothelial MKP-1 protein expression with a maximum at ≈30 minutes (Figure 1A). Because ANP may act via two different receptors, we investigated the receptor specificity of this effect. 8-Br-cGMP, a cell-permeable analogue of the NPR-A–dependent second messenger of ANP, mimicked the effect of ANP. MKP-1 levels were not altered by cANF, a specific ligand of NPR-C (Figure 1B). These data suggest that the induction of MKP-1 by ANP is mediated via cGMP/NPR-A without participation of NPR-C. Inhibition of protein biosynthesis by cycloheximide completely suppressed ANP-induced MKP-1 induction after 30 minutes (Figure 1C), indicating that ANP activates MKP-1 protein de novo synthesis within 30 minutes.

ANP Induces ROS, Which Are Responsible for MKP-1 Upregulation

We measured intracellular ROS generation by loading HUVECs with an ROS-sensitive fluorescent dye. ANP treatment augmented ROS production with a maximum dye fluorescence at ≈30 minutes (Figure 2A). The concentration dependency of this effect is shown in Figure 2B. We also tested whether 8-Br-cGMP and cANF affect ROS generation. cANF did not significantly change ROS levels, whereas 8-Br-cGMP clearly induced ROS production (Figure 2C), indicating that ANP induces ROS production via cGMP/NPR-A. We aimed to causally link ANP-induced ROS generation and MKP-1 expression. Treatment of HUVECs with tiron (superoxide scavenger), ebselen (scavenger of peroxynitrite and peroxides), and *N*-acetyl-L-cysteine (thiol-based antioxidant) abrogated ANP-induced MKP-1 expression (Figure 2D). We also confirmed that exogenous administration of an oxidant (hydrogen peroxide [H_2O_2]) increases MKP-1 levels (Figure 2E).

Involvement of Nox/Nox2

We intended to clarify whether Nox is involved in ANP-induced ROS generation. Scavenging superoxide by a cell-permeable form of superoxide dismutase (SOD) (pegylated [PEG]-SOD) and inhibiting Nox by diphenyleneiodonium (DPI; nonspecific Nox inhibitor) completely abrogated the ANP-induced fluorescence signal and MKP-1 induction (Figure 3A and 3B). The highly specific Nox inhibitor peptide gp91ds-*tat* (inhibits interaction of Nox1/2/4 and $p47^{phox}$)[23] abolished the increase of ROS by ANP (Figure 3C), and apocynin (specific Nox inhibitor) reduced the ability of ANP to induce MKP-1 (Figure 3D). The treatment of HUVECs with SOD, catalase (degrades H_2O_2), and PEG-catalase did not influence ROS generation or MKP-1 induction by ANP (see the online data supplement, available at http://circres.ahajournals.org), pointing to intracellularly produced superoxide as the crucially involved ROS. Furthermore, we intended to clarify which Nox2 homologue (Nox1–5) is responsible for ROS production and subsequent MKP-1 induction. We examined the mRNA expression of Nox1–Nox5: Nox2 and Nox4 were found to be expressed (Figure 3E) in HUVECs and HMECs. Using a Nox2/4 antisense approach, we revealed that Nox2 (Figure 3F) but not Nox4 (Figure 3G) is crucial for the ANP-induced ROS generation and MKP-1 upregulation.

Rac1 Involvement

The small Rho-GTPase Rac plays an important role in the activation of phagocyte-type Nox and might also be involved

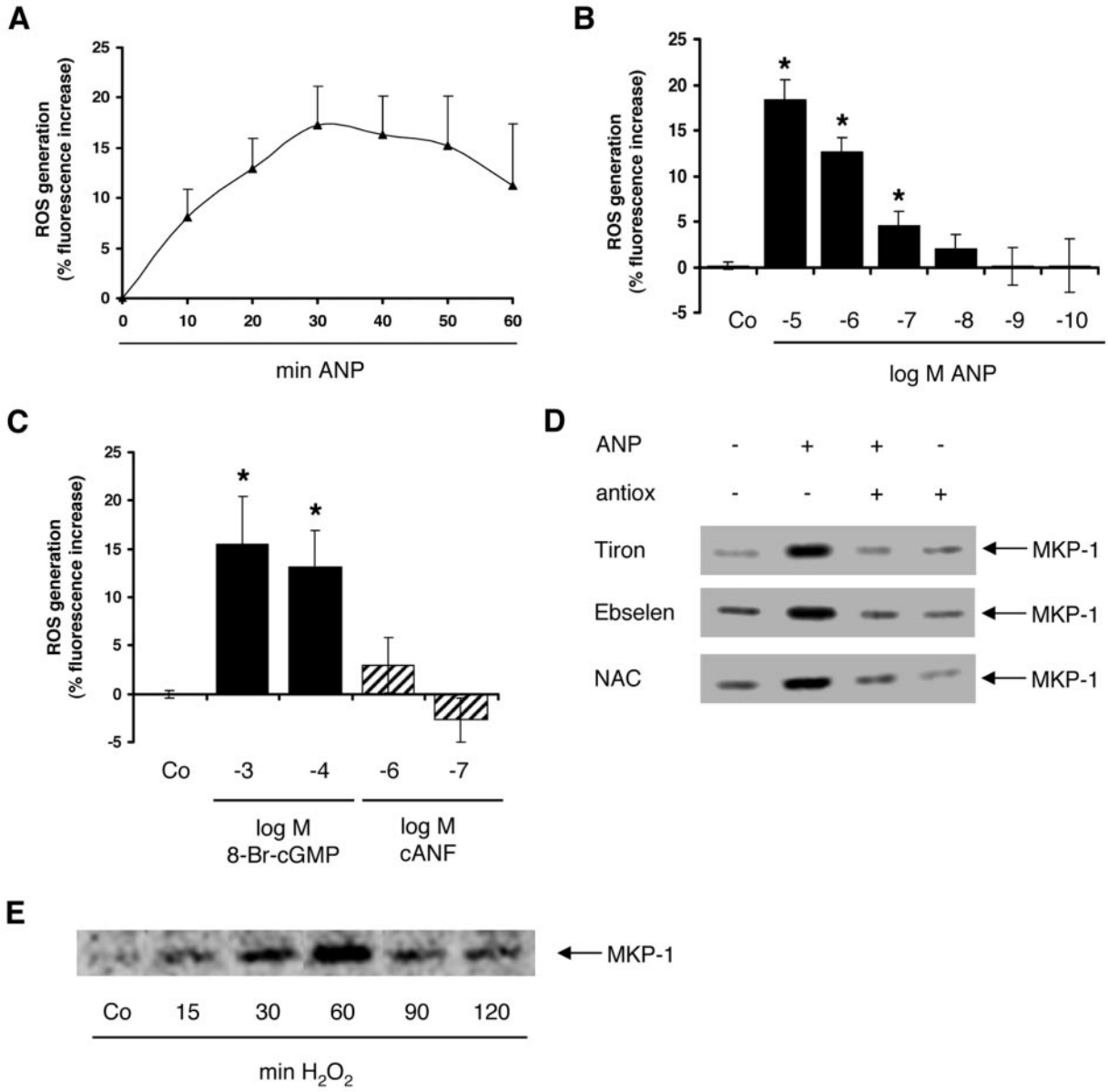

Figure 2. ROS generation in HUVECs. A, ANP (1 μmol/L) time course. B, Concentration-dependent action of ANP. C, Effect of 8-Br-cGMP and cANF (*$P \leq 0.05$ vs control [Co]). D and E, Involvement of ROS in the upregulation of MKP-1. D, HUVECs were preincubated with the antioxidants tiron (Ti; 10 mmol/L), ebselen (Eb; 40 μmol/L), and *N*-acetyl-L-cysteine (NAC; 10 mmol/L) for 60 minutes before adding ANP (1 μmol/L, 60 minutes). E, Cells were left untreated (Co) or were treated with H_2O_2 (50 μmol/L). MKP-1 protein expression was determined by Western blot analysis.

in the activation of endothelial Nox.[24] We performed Rac1 pull-down assays and found an increase of active Rac1 by ANP with a maximum at 2 to 5 minutes (Figure 4A). Further, we investigated Rac1 translocation by confocal microscopy. ANP mimicked the Rac1 translocation observed by treating HUVECs with the known Rac1 activator PMA[25] (Figure 4B). To causally link Rac1 activation to the effects of ANP on superoxide generation and MKP-1, we transfected HMECs (because of difficulties in transfecting plasmids into primary HUVECs[26]) with a dominant-negative (RacN17) and a constitutively active (RacV12) Rac1 mutant. ANP-induced superoxide production and MKP-1 expression were abolished by RacN17, whereas RacV12 mimicked the effects of ANP (Figure 4C), indicating that Rac1 is crucially involved in the generation of superoxide and expression of MKP-1 after ANP treatment.

MKP-1 Induction Is Not Mediated by PKC, Extracellular Signal-Regulated Kinase, or p38 MAPK

PKC and the MAPK extracellular signal-regulated kinase (ERK), p38, and JNK have been communicated to mediate MKP-1 induction after diverse stimuli.[10–13] We treated HUVECs with inhibitors of these kinases. The inhibitors calphostin C for PKC (Figure 5A), PD98059 for the ERK pathway (Figure 5B), and SB203580 for p38 MAPK (Figure 5C) did not affect ANP-induced MKP-1 expression, suggesting that PKC, ERK, and p38 MAPK do not mediate MKP-1 induction by ANP.

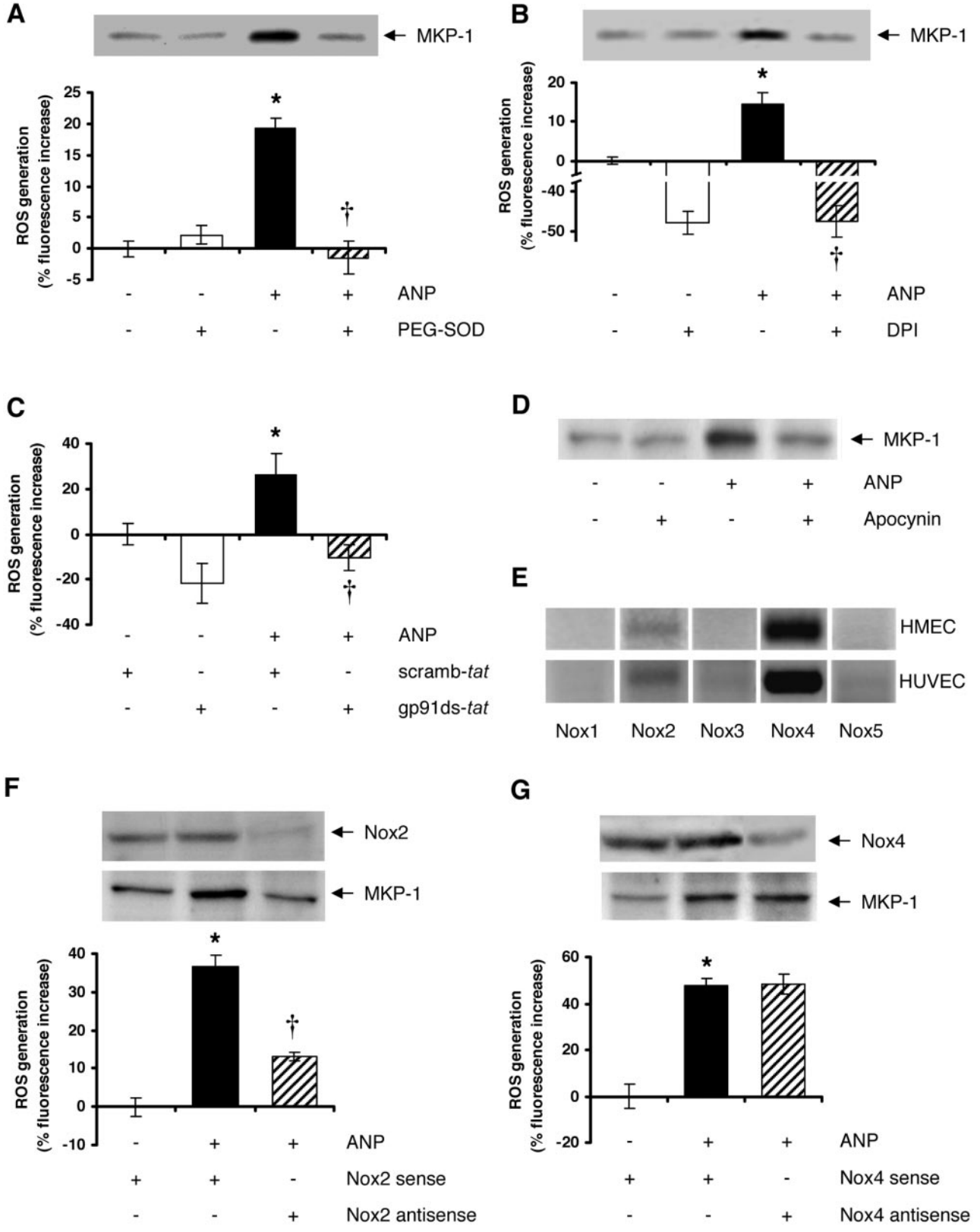

Figure 3. Involvement of Nox. A through D, HUVECs were pretreated with PEG-SOD (300 U/mL; 60 minutes), DPI (10 μmol/L; 60 minutes), gp91ds-*tat* (or scrambled scramb-*tat*; 100 μmol/L; 30 minutes), or apocynin (0.5 mmol/L; 30 minutes) before ANP (1 μmol/L). ROS generation was measured and MKP-1 protein expression (60 minutes ANP) was determined by Western blot analysis (*$P \leq 0.05$ vs Co; †$P \leq 0.05$ vs ANP). E, Expression of Nox1–Nox5 mRNA in untreated HMECs and HUVECs was investigated by RT-PCR. Nox2/4 antisense experiments. F and G, HUVECs were transfected with Nox2 or Nox4 antisense oligos. Nox2/4 downregulation is shown in the top panel. Cells were treated with ANP (1 μmol/L), and ROS generation was measured. Nox2, Nox4, and MKP-1 protein expression (60 minutes ANP) was determined by Western blot analysis (*$P \leq 0.05$ vs Co; †$P \leq 0.05$ vs ANP).

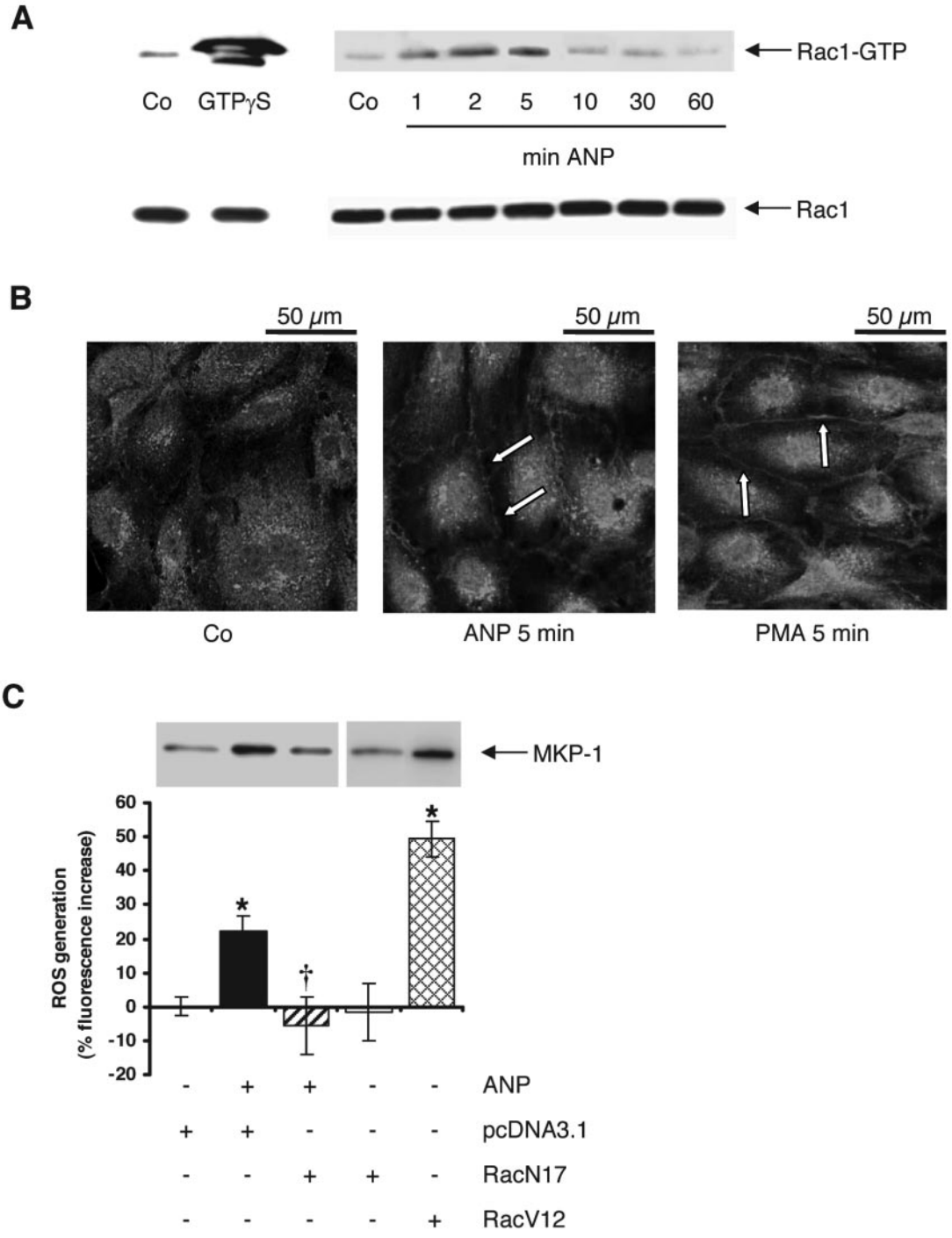

Figure 4. Involvement of Rac1. A, Pull-down assays of Rac1-GTP. HUVECs were treated with ANP (1 μmol/L) as indicated. GTPγS serves as positive control (Co). Bottom panel shows uniformity of total Rac1 content. B, Confocal images of Rac1 translocation. HUVECs were treated with ANP (1 μmol/L) and PMA (1 μmol/L) for the indicated times. Arrows show Rac1 translocation to the membranes. C, ROS generation was measured, and MKP-1 protein expression was determined. HMECs were transfected with the control plasmid pcDNA3.1, the dominant-negative (RacN17), or the constitutively active (RacV12) Rac1 mutant, and were treated with ANP (1 μmol/L) as indicated (*$P \leq 0.05$ vs untreated cells; †$P \leq 0.05$ vs ANP-treated cells).

Role of JNK and AP-1

The JNK inhibitor SP600125 abolished the ANP-induced expression of MKP-1 protein and mRNA (Figure 6A and 6B), suggesting an involvement of JNK in MKP-1 upregulation by ANP. Consistently, ANP increased the active form of JNK (phospho-JNK) with a maximum at ≈5 minutes (Figure 6C). Because we found that ANP induces superoxide generation, we proved the role of superoxide in the activation of JNK by ANP. The superoxide scavengers tiron and PEG-SOD decreased ANP-induced JNK phosphorylation (Figure 6D and 6E). Because the transcription factor AP-1 is an important target of JNK, we assessed AP-1 DNA-binding activity after treatment with ANP and SP600125. Inhibition of JNK completely blocked the ANP-induced activity of AP-1 (Figure

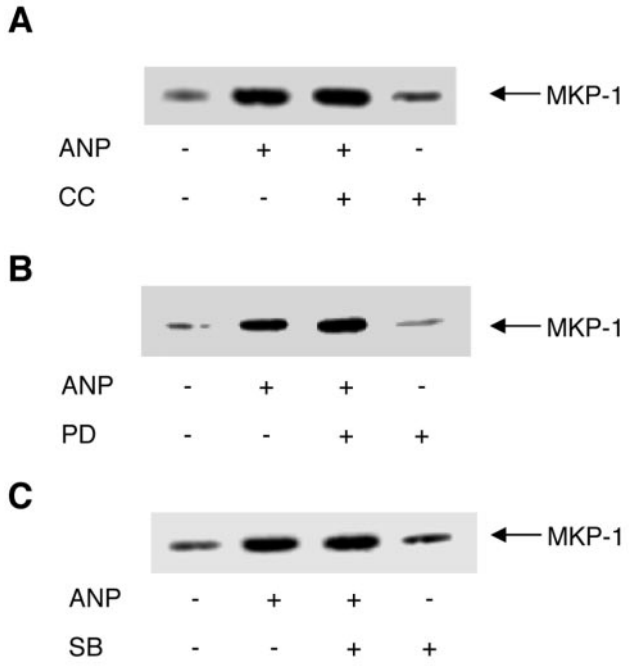

Figure 5. Role of PKC, ERK, and p38 MAPK. HUVECs were left untreated (control [Co]), treated with ANP alone (1 μmol/L; 60 minutes), in combination with the PKC inhibitor calphostin C (CC; 100 nmol/L; A), the ERK pathway inhibitor PD98059 (PD; 10 μmol/L; B), or the p38 MAPK inhibitor SB203580 (SB; 5 μmol/L; C), or were treated with the respective inhibitor alone (60 minutes). MKP-1 protein expression was determined by Western blot analysis.

6F). To causally link AP-1 to the induction of MKP-1, we used an AP-1 decoy approach.[27] Figure 6G demonstrates the functionality of the transfected AP-1 decoy. The decoy completely abrogated the increase of MKP-1 expression by ANP (Figure 6H). These results indicate that MKP-1 induction by ANP is mediated via the JNK/AP-1 pathway.

ROS Generation in Intact Blood Vessels

To clarify whether the ANP-induced ROS generation measured in cultured ECs is also relevant in vivo (ie, in intact blood vessels), we loaded rat lung ECs in isolated perfused organs with an ROS-sensitive fluorescent dye. Intracellular ROS generation was measured by in situ fluorescence microscopy. Resting ECs showed no alteration of dye fluorescence over 2 hours (Figure 7A), but ANP treatment increased endothelial ROS formation (Figure 7B). This increase was abolished by addition of DPI (Figure 7C and 7D). Importantly, we proved that ANP causes an increase in MKP-1 protein levels in vivo (ie, in the rat lung [Figure 7E]). Together, our findings demonstrate that ANP induces ROS not only in cultured ECs but also in the endothelium of intact rat lung vessels and that MKP-1 is also induced by ANP in vivo.

Discussion

In recent years, more and more studies emerged that present profound data suggesting a pivotal role for MKP-1 in different signaling pathways leading to anti-inflammatory,[28] antiatherogenic,[29] and cytoprotective[30] actions. Accordingly, we have shown recently that ANP exerts its anti-inflammatory effects on TNF-α–activated ECs via induction of MKP-1.[5,6] Therefore, it seemed of special interest to obtain information about the as yet completely unknown signaling mechanisms involved in MKP-1 induction by ANP in ECs. We revealed that ANP activated Rac1, leading to the production of superoxide by a Nox2-containing Nox. Superoxide activates the JNK/AP-1 pathway, resulting in transcriptional upregulation of MKP-1. The proposed pathway is schematically depicted in Figure 8.

MKP-1 induction was found to be mediated via NPR-A/cGMP. Supporting this finding, cGMP-dependent induction of MKP-1 was described in vascular smooth muscle cells (VSMCs) and renal cells.[8,31] Blockade of protein biosynthesis did not inhibit the early increase of MKP-1 at 15 minutes, suggesting that other effects than transcriptional upregulation are responsible for this early induction. MKP-1 is known to be rapidly degraded by the ubiquitin/proteasome pathway.[32] It might be speculated that ANP interferes with MKP-1 catabolism, accounting for the early increase of MKP-1. However, the increased MKP-1 protein levels at later time points definitely represent newly synthesized protein.

We found that ANP concentration-dependently increases ROS formation (Figure 8). This increase is mediated via NPR-A/cGMP. Nothing has yet been known about the potency of ANP to induce endothelial ROS. However, ANP is known to induce ROS in macrophages[33] and in renal cells,[8] and cGMP was reported to induce ROS in cardiomyocytes.[34] A causal link between ROS generation and MKP-1 induction was established, and we corroborated the ability of ROS to induce MKP-1 by exogenously supplying H_2O_2, which is known to induce MKP-1 in VSMCs and epithelial cells.[9,35] Most important, we found that these in vitro results were approved by in situ ROS measurement in ECs of intact lung vessels. Moreover, we confirmed that ANP induces MKP-1 not only in cell culture but also in the isolated perfused rat lung, pointing to an in vivo relevance of our in vitro findings.

Nox was identified as the crucial ANP-induced ROS source responsible for MKP-1 induction (Figure 8) by using different inhibitors. Experiments with catalase and SOD (see the online data supplement) corroborated that superoxide but not H_2O_2 is the crucially involved oxidant species mediating the effects of ANP. SOD converts superoxide to H_2O_2. Interestingly, we did not observe an augmented MKP-1 induction during ANP/SOD treatment, although exogenously supplied H_2O_2 leads to an upregulation of MKP-1. This might be attributable to the fact that endothelial Nox produces only low amounts of superoxide, which may consequently lead to SOD-converted H_2O_2 levels unable to induce MKP-1. In contrast, the exogenously supplied H_2O_2 amounts are incomparably higher.

The phagocyte-type Nox contains a transmembrane electron transport chain. Oxygen is reduced to superoxide in the extracellular space or in phagocytic vacuoles.[36] Neither the precise structure nor the exact localization of functionally active endothelial Noxes has yet been clarified. The question arises whether endothelial Nox releases superoxide intracellularly or extracellularly. Our data suggest an intracellular superoxide release because the cell-permeable PEG-SOD

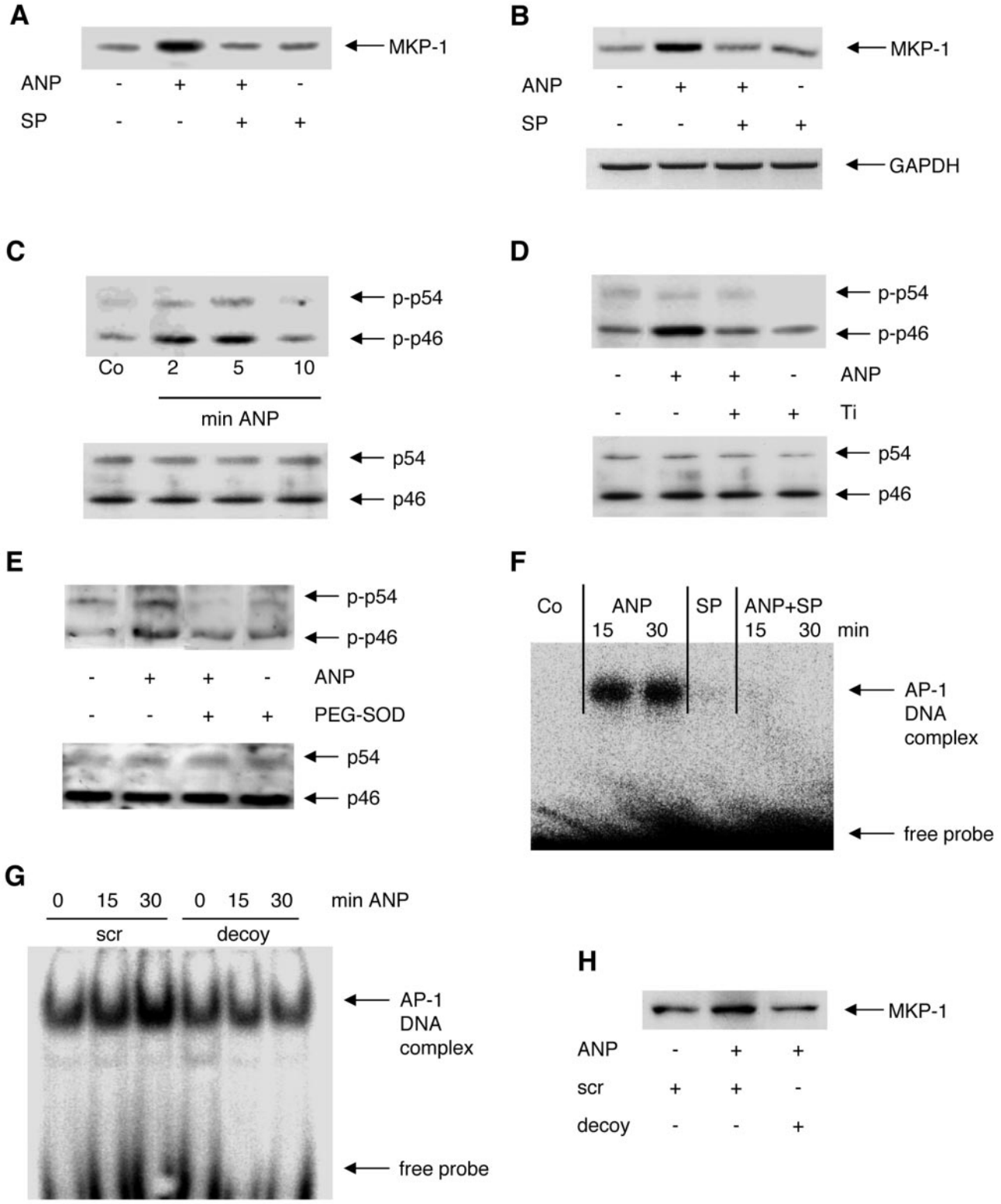

Figure 6. Involvement of JNK. A, HUVECs were preincubated with the JNK inhibitor SP600125 (SP; 10 μmol/L) 60 minutes before ANP (1 μmol/L; 60 minutes). MKP-1 protein expression was determined by Western blot analysis. B, Cells were preincubated with SP (10 μmol/L) 60 minutes before ANP (1 μmol/L; 30 minutes). MKP-1 and GAPDH mRNA expression was assessed by RT-PCR. C, HUVECs were treated with ANP (1 μmol/L) as indicated. D and E, HUVECs were left untreated, were treated with ANP alone (1 μmol/L; 5 minutes) or in combination with the superoxide scavenger tiron (Ti; 10 mmol/L; 60 minutes pretreatment) or PEG-SOD (300 U/mL; 60 minutes pretreatment), or were treated with tiron or PEG-SOD alone (60 minutes). Phospho-JNK Western blots were performed (top panels). Bottom panels show uniformity of total JNK content. Involvement of AP-1. F, HUVECs were left untreated (control [Co]), treated with ANP (1 μmol/L) or SP (10 μmol/L) alone, or with a combination of both (SP 60 minutes pretreatment). G and H, HUVECs were transfected with AP-1 decoy or scrambled decoy and were treated with ANP (1 μmol/L; 60 minutes; H). AP-1 DNA-binding activity was investigated by EMSA, and MKP-1 protein expression was determined by Western blot analysis.

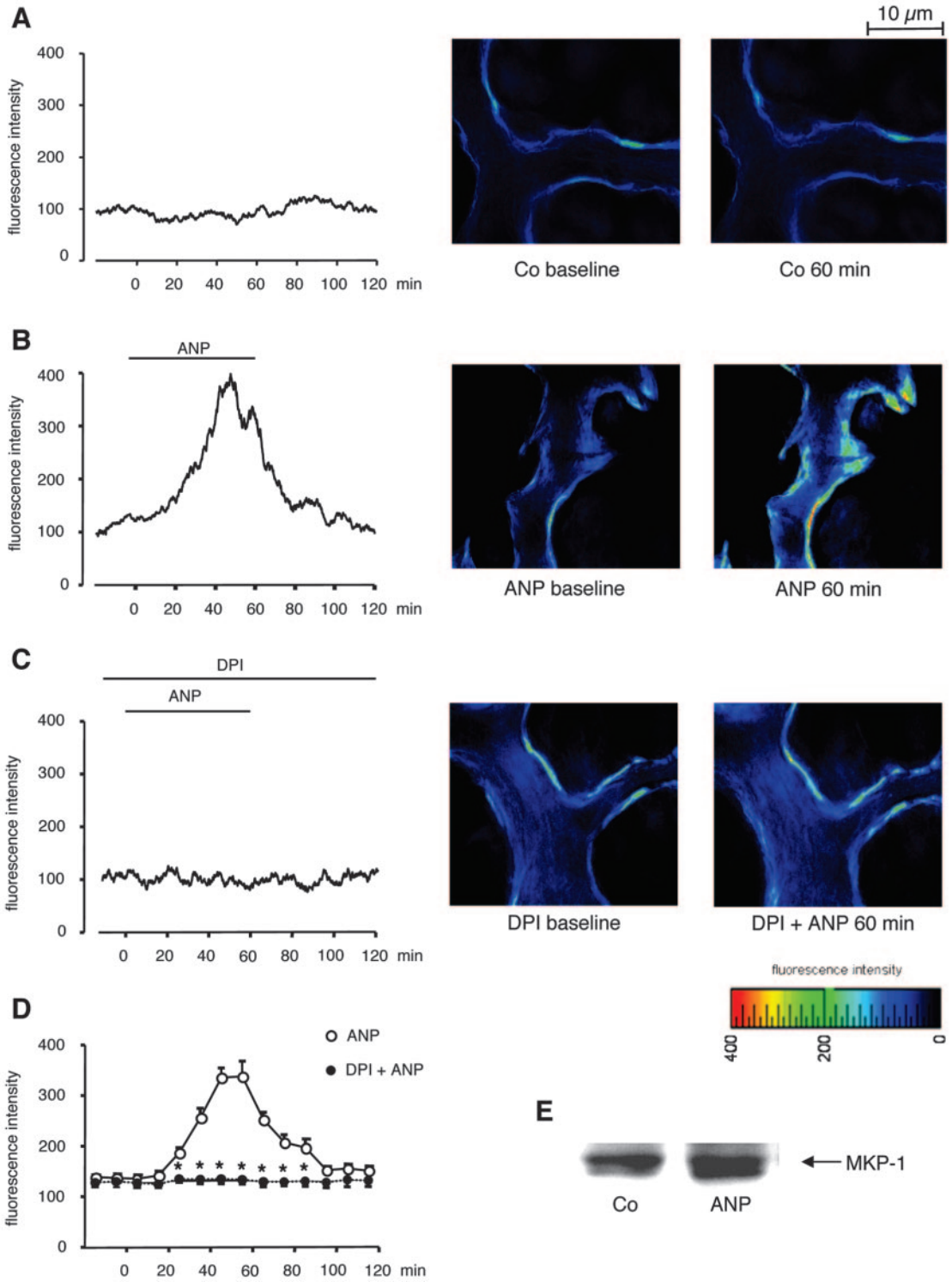

Figure 7. ROS generation in intact rat lung blood vessels. Baseline fluorescence is shown in each panel. A, Regular HEPES buffer was continuously infused over 120 minutes. B, ANP (1 μmol/L) was infused for 60 minutes, followed by 60 minutes of buffer perfusion. C, Vessels were pretreated with DPI (20 μmol/L) for 20 minutes, followed by 60 minutes ANP and 60 minutes buffer perfusion. One representative graph and image of five independent experiments is shown. D, Data of ANP-treated vs ANP- and DPI-treated cells of all five experiments are shown as means±SEM (*$P \leq 0.05$ vs data from lungs treated with ANP alone). E, Rats were anesthesized, and NaCl solution (control [Co]) or ANP (≈1 μmol/L plasma concentration) was parenterally applied. After 1 hour, lungs were excised. Rat lung lysates were investigated for MKP-1 protein expression by Western blot analysis.

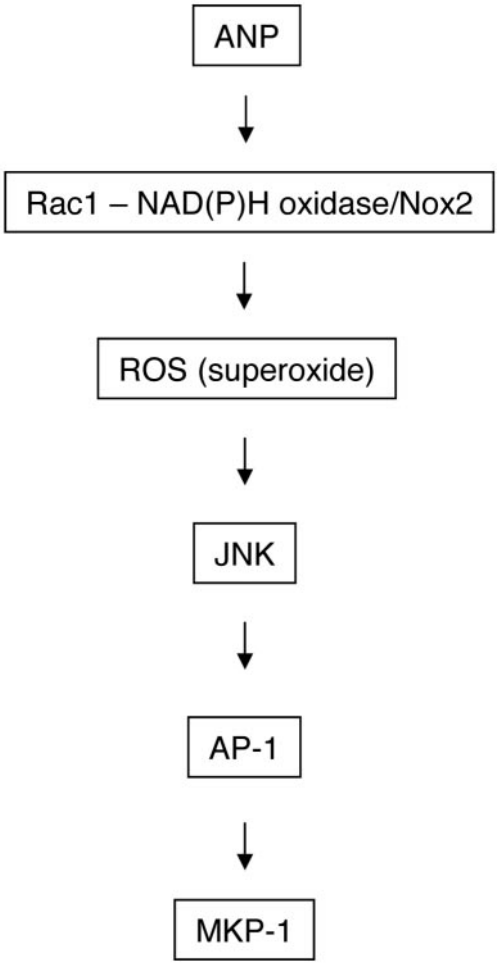

Figure 8. Schematic overview of the proposed signaling pathway.

completely blocked the fluorescence signal, whereas only extracellularly present SOD did not alter the signal.

Rac, a well-known component of the phagocyte-type Nox, was revealed to be rapidly activated by ANP (Figure 8). The precise role of Rac in endothelial Nox assembly and activation is not yet completely understood, and only few studies suggest a role for Rac in Nox-derived endothelial ROS generation.[37,38] Therefore, it might be of special interest that our results show that Rac1 plays a pivotal role in the generation of superoxide and in the upregulation of MKP-1. Rac1 is known to translocate to the plasma membrane during activation,[39] which could mean that Nox also is localized there. However, the pure microscopic localization of Rac at the plasma membrane does not allow any conclusions about the direction of superoxide release (cytosolic versus extracellular).

To clarify which Nox isoform is responsible for superoxide production, we characterized the Nox spectrum expressed in HUVECs and HMECs and found Nox2 and Nox4 mRNA and protein to be present. Ago et al communicated similar findings for HUVECs and rat aortic ECs,[16] but they also described an extremely low expression of Nox1, which we could not confirm and which might be attributable to different cell culture conditions or experimental settings. By using an antisense approach, only Nox2 was found to be responsible for ANP-induced superoxide generation and subsequent MKP-1 induction (Figure 8). Interestingly, DPI and gp91ds-*tat* reduced superoxide production below control levels. This suggests a constitutive production of superoxide in resting cells. DPI is known to inhibit several other ROS sources (flavin-containing enzymes), which could in part account for basal ROS/superoxide production. The fact that the inhibitor peptide gp91ds-*tat* targets Nox2 and Nox4 suggests that Nox4 might be a constitutively active Nox isoform responsible for basal levels of superoxide production because Nox2 but not Nox4 antisense reverses the effect of ANP. In summary, we found that ANP-induced superoxide generation and MKP-1 expression are mediated via Rac1 and a Nox2-containing Nox.

Downstream of Nox2-mediated superoxide formation, we causally linked JNK to the ANP-induced expression of MKP-1 and excluded a role for PKC, ERK, and p38 MAPK. Accordingly, numerous studies exist demonstrating that JNK is regulated by ROS.[40,41] The transcription factor AP-1 is a central target of JNK. As reported previously by ourselves[42] and confirmed in the present study, ANP induces AP-1 activity. This finding is supported by a study showing cGMP, the second messenger of ANP, as an activator of AP-1.[43] By using AP-1 decoys, we could causally link the activation of AP-1 to an upregulation of MKP-1. In conclusion, our data suggest that JNK-mediated AP-1 activation is crucially involved in MKP-1 induction by ANP.

In the present study, we, for the first time, provide evidence that Nox/Nox2 is activated by ANP in human ECs. It should be noted that other known endogenous Nox activators, such as thrombin, angiotensin II, or TNF-α,[15] are generally regarded as proinflammatory agents. Therefore, it seems of special interest that the functional outcome of Nox2 activation by ANP is the induction of MKP-1, a protein that exerts cytoprotective effects on ECs. The potential of ANP to induce an increase in superoxide generation could also be regarded as a form of preconditioning leading to protection of ECs against an inflammatory response. A similar observation was reported in HUVECs after short-time treatment with H_2O_2.[44]

In conclusion, we describe here ANP as a novel endogenous activator of Nox2-containing endothelial Nox, which mediates anti-inflammatory actions by inducing formation of ROS/superoxide. We showed that ANP enhances production of ROS/superoxide in ECs in vitro and in intact blood vessels, and we revealed that ANP activates Rac1. The Rac1-dependent increase of superoxide formation via Nox2 and the superoxide-activated JNK/AP-1 pathway were presented as key events in the induction of MKP-1 by ANP (Figure 8).

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (FOR 440/1 TP 5) and the Bavaria California Technology Center (A.K.K.). A.K.K. is supported by the Alexander von Humboldt Foundation. The staff of the Frauenklinik vom Roten Kreuz (Munich, Germany) is gratefully acknowledged for providing umbilical cords and Ulla G. Knaus for providing the GST-PBD plasmid.

References

1. Levin ER, Gardner DG, Samson WK. Natriuretic peptides. *N Engl J Med.* 1998;339:321–328.

2. Murthy KS, Teng BQ, Zhou H, Jin JG, Grider JR, Makhlouf GM. G_{i-1}/G_{i-2}-dependent signaling by single-transmembrane natriuretic peptide clearance receptor. *Am J Physiol Gastrointest Liver Physiol*. 2000;278: G974–G980.
3. Kiemer AK, Vollmar AM. The atrial natriuretic peptide regulates the production of inflammatory mediators in macrophages. *Ann Rheum Dis*. 2001;60(suppl 3):iii68–iii70.
4. Kiemer AK, Fürst R, Vollmar AM. Vasoprotective actions of the atrial natriuretic peptide. *Curr Med Chem Cardiovasc Hematol Agents*. In press.
5. Kiemer AK, Weber NC, Fürst R, Bildner N, Kulhanek-Heinze S, Vollmar AM. Inhibition of p38 MAPK activation via induction of MKP-1: atrial natriuretic peptide reduces TNF-α-induced actin polymerization and endothelial permeability. *Circ Res*. 2002;90:874–881.
6. Weber NC, Blumenthal SB, Hartung T, Vollmar AM, Kiemer AK. ANP inhibits TNF-α-induced endothelial MCP-1 expression—involvement of p38 MAPK and MKP-1. *J Leukoc Biol*. 2003;74:932–941.
7. Camps M, Nichols A, Arkinstall S. Dual specificity phosphatases: a gene family for control of MAP kinase function. *FASEB J*. 2000;14:6–16.
8. Hannken T, Schroeder R, Stahl RA, Wolf G. Atrial natriuretic peptide attenuates Ang II-induced hypertrophy of renal tubular cells. *Am J Physiol Renal Physiol*. 2001;281:F81–F90.
9. Yoneda K, Peck K, Chang MM, Chmiel K, Sher YP, Chen J, Yang PC, Chen Y, Wu R. Development of high-density DNA microarray membrane for profiling smoke- and hydrogen peroxide-induced genes in a human bronchial epithelial cell line. *Am J Respir Crit Care Med*. 2001;164: S85–S89.
10. Bokemeyer D, Sorokin A, Yan M, Ahn NG, Templeton DJ, Dunn MJ. Induction of mitogen-activated protein kinase phosphatase 1 by the stress-activated protein kinase signaling pathway but not by extracellular signal-regulated kinase in fibroblasts. *J Biol Chem*. 1996;271:639–642.
11. Bokemeyer D, Lindemann M, Kramer HJ. Regulation of mitogen-activated protein kinase phosphatase-1 in vascular smooth muscle cells. *Hypertension*. 1998;32:661–667.
12. Kim F, Corson MA. Adhesion to fibronectin enhances MKP-1 activation in human endothelial cells. *Biochem Biophys Res Commun*. 2000;273: 539–545.
13. Valledor AF, Xaus J, Marques L, Celada A. Macrophage colony-stimulating factor induces the expression of mitogen-activated protein kinase phosphatase-1 through a protein kinase C-dependent pathway. *J Immunol*. 1999;163:2452–2462.
14. Jones SA, O'Donnell VB, Wood JD, Broughton JP, Hughes EJ, Jones OT. Expression of phagocyte NADPH oxidase components in human endothelial cells. *Am J Physiol*. 1996;271:H1626–H1634.
15. Lassegue B, Clempus RE. Vascular NAD(P)H oxidases: specific features, expression, and regulation. *Am J Physiol Regul Integr Comp Physiol*. 2003;285:R277–R297.
16. Ago T, Kitazono T, Ooboshi H, Iyama T, Han YH, Takada J, Wakisaka M, Ibayashi S, Utsumi H, Iida M. Nox4 as the major catalytic component of an endothelial NAD(P)H oxidase. *Circulation*. 2004;109:227–233.
17. Ades EW, Candal FJ, Swerlick RA, George VG, Summers S, Bosse DC, Lawley TJ. HMEC-1: establishment of an immortalized human microvascular endothelial cell line. *J Invest Dermatol*. 1992;99:683–690.
18. Kiemer AK, Weber NC, Vollmar AM. Induction of IκB: atrial natriuretic peptide as a regulator of the NF-κB pathway. *Biochem Biophys Res Commun*. 2002;295:1068–1076.
19. Kuebler WM, Ying X, Singh B, Issekutz AC, Bhattacharya J. Pressure is proinflammatory in lung venular capillaries. *J Clin Invest*. 1999;104: 495–502.
20. Kuebler WM, Parthasarathi K, Wang PM, Bhattacharya J. A novel signaling mechanism between gas and blood compartments of the lung. *J Clin Invest*. 2000;105:905–913.
21. Parthasarathi K, Ichimura H, Quadri S, Issekutz A, Bhattacharya J. Mitochondrial reactive oxygen species regulate spatial profile of proinflammatory responses in lung venular capillaries. *J Immunol*. 2002;169: 7078–7086.
22. Benard V, Bohl BP, Bokoch GM. Characterization of Rac and cdc42 activation in chemoattractant-stimulated human neutrophils using a novel assay for active GTPases. *J Biol Chem*. 1999;274:13198–13204.
23. Rey FE, Cifuentes ME, Kiarash A, Quinn MT, Pagano PJ. Novel competitive inhibitor of NAD(P)H oxidase assembly attenuates vascular O_2^- and systolic blood pressure in mice. *Circ Res*. 2001;89:408–414.
24. Bokoch GM, Knaus UG. NADPH oxidases: not just for leukocytes anymore! *Trends Biochem Sci*. 2003;28:502–508.
25. Price LS, Langeslag M, ten Klooster JP, Hordijk PL, Jalink K, Collard JG. Calcium signaling regulates translocation and activation of Rac. *J Biol Chem*. 2003;278:39413–39421.
26. Teifel M, Heine LT, Milbredt S, Friedl P. Optimization of transfection of human endothelial cells. *Endothelium*. 1997;5:21–35.
27. Park SK, Yang WS, Han NJ, Lee SK, Ahn H, Lee IK, Park JY, Lee KU, Lee JD. Dexamethasone regulates AP-1 to repress TNF-α induced MCP-1 production in human glomerular endothelial cells. *Nephrol Dial Transplant*. 2004;19:312–319.
28. Clark AR. MAP kinase phosphatase 1: a novel mediator of biological effects of glucocorticoids? *J Endocrinol*. 2003;178:5–12.
29. Begum N, Song Y, Rienzie J, Ragolia L. Vascular smooth muscle cell growth and insulin regulation of mitogen-activated protein kinase in hypertension. *Am J Physiol*. 1998;275:C42–C49.
30. Kaiser RA, Bueno OF, Lips DJ, Doevendans PA, Jones F, Kimball TF, Molkentin JD. Targeted inhibition of p38 mitogen-activated protein kinase antagonizes cardiac injury and cell death following ischemia-reperfusion in vivo. *J Biol Chem*. 2004;279:15524–15530.
31. Jacob A, Molkentin JD, Smolenski A, Lohmann SM, Begum N. Insulin inhibits PDGF-directed VSMC migration via NO/cGMP increase of MKP-1 and its inactivation of MAPKs. *Am J Physiol Cell Physiol*. 2002;283:C704–C713.
32. Brondello JM, Pouyssegur J, McKenzie FR. Reduced MAP kinase phosphatase-1 degradation after p42/p44MAPK-dependent phosphorylation. *Science*. 1999;286:2514–2517.
33. Vollmar AM, Förster R, Schulz R. Effects of atrial natriuretic peptide on phagocytosis and respiratory burst in murine macrophages. *Eur J Pharmacol*. 1997;319:279–285.
34. Xu Z, Ji X, Boysen PG. Exogenous nitric oxide generates ROS and induces cardioprotection: involvement of PKG, mitochondrial K_{ATP} channels, and ERK. *Am J Physiol Heart Circ Physiol*. 2004;286: H1433–H1440.
35. Baas AS, Berk BC. Differential activation of mitogen-activated protein kinases by H_2O_2 and O_2^- in vascular smooth muscle cells. *Circ Res*. 1995;77:29–36.
36. Cross AR, Segal AW. The NADPH oxidase of professional phagocyte—prototype of the NOX electron transport chain systems. *Biochim Biophys Acta*. 2004;1657:1–22.
37. Lopes NH, Vasudevan SS, Gregg D, Selvakumar B, Pagano PJ, Kovacic H, Goldschmidt-Clermont PJ. Rac-dependent monocyte chemoattractant protein-1 production is induced by nutrient deprivation. *Circ Res*. 2002; 91:798–805.
38. Wung BS, Cheng JJ, Shyue SK, Wang DL. NO modulates monocyte chemotactic protein-1 expression in endothelial cells under cyclic strain. *Arterioscler Thromb Vasc Biol*. 2001;21:1941–1947.
39. Gregg D, Rauscher FM, Goldschmidt-Clermont PJ. Rac regulates cardiovascular superoxide through diverse molecular interactions: more than a binary GTP switch. *Am J Physiol Cell Physiol*. 2003;285:C723–C734.
40. Adler V, Yin Z, Tew KD, Ronai Z. Role of redox potential and reactive oxygen species in stress signaling. *Oncogene*. 1999;18:6104–6111.
41. Dröge W. Free radicals in the physiological control of cell function. *Physiol Rev*. 2002;82:47–95.
42. Kiemer AK, Bildner N, Weber NC, Vollmar AM. Characterization of heme oxygenase 1 (heat shock protein 32) induction by atrial natriuretic peptide in human endothelial cells. *Endocrinology*. 2003;144:802–812.
43. Sciorati C, Nistico G, Meldolesi J, Clementi E. Nitric oxide effects on cell growth: GMP-dependent stimulation of the AP-1 transcription complex and cyclic GMP-independent slowing of cell cycling. *Br J Pharmacol*. 1997;122:687–697.
44. Zahler S, Kupatt C, Becker BF. Endothelial preconditioning by transient oxidative stress reduces inflammatory responses of cultured endothelial cells to TNF-α. *FASEB J*. 2000;14:555–564.