

Endothelial Actions of ANP Enhance Myocardial Inflammatory Infiltration in the Early Phase After Acute Infarction

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ABSTRACT

Rationale: In patients after acute myocardial infarction (AMI), the initial extent of necrosis and inflammation determine clinical outcome. One early event in AMI is the increased cardiac expression of atrial (ANP) and B-type natriuretic peptides (BNP), their plasma levels correlating with severity of ischemia. It was shown that NPs, via their cGMP-forming guanylyl cyclase-A (GC-A) receptor and cGMP-dependent kinase I (cGKI), strengthen systemic endothelial barrier properties in acute inflammation.

Objective: We studied whether endothelial actions of local NPs modulate myocardial injury and early inflammation after AMI.

Methods and Results: Necrosis and inflammation after experimental AMI were compared between control mice and littermates with endothelial-restricted inactivation of GC-A (EC GC-A KO) or cGKI (EC cGKI KO). Unexpectedly, myocardial infarct size and neutrophil infiltration/activity 2-days after AMI were attenuated in EC GC-A KO and unaltered in EC cGKI KO animals. Molecular studies revealed that hypoxia and TNF- α , conditions accompanying AMI, reduce the endothelial expression of cGKI and enhance cGMP-stimulated phosphodiesterase (PDE)2A levels. Real-time cAMP measurements in endothelial microdomains using a novel FRET biosensor revealed that PDE2 mediates ANP/cGMP-driven decreases of submembrane cAMP levels. Finally, intravital microscopy studies of the mouse cremaster microcirculation showed that TNF α -induced endothelial NP/GC-A/cGMP/PDE2 signaling impairs endothelial barrier functions.

Conclusions: Hypoxia and cytokines such as TNF- α modify the endothelial postreceptor signaling pathways of NPs, with downregulation of cGKI, induction of PDE2A and altered cGMP/cAMP crosstalk. Increased expression of PDE2 can mediate hyperpermeability effects of paracrine endothelial NPs/GC-A/cGMP signaling and facilitate neutrophil extravasation during the early phase after MI.

Keywords:

Acute myocardial infarction, atrial natriuretic peptide, cyclic GMP, endothelial permeability, phosphodiesterase 2A, animal model cardiovascular disease, basic science, endothelial cell, guanylyl cyclase A.

Nonstandard Abbreviations and Acronyms:

GC-A	guanylyl cyclase A
Cgki	cGMP-dependent protein kinase I
PDE	phosphodiesterase
EC GC-A	KO mice with endothelial GC-A deletion
EC cGKI	KO mice with endothelial cGKI deletion
FRET	fluorescence resonance energy transfer

INTRODUCTION

In patients after acute myocardial infarction (AMI), the extent of myocardial necrosis and the degree of adverse remodeling of the infarcted and surrounding myocardium determine long-term prognosis (1). The infiltration of the ischemic tissue by inflammatory cells impacts initial injury and subsequent healing and remodeling processes (2). Hypoxia and cytokines released following AMI, such as tumor necrosis factor α (TNF- α), provoke pathological coronary endothelial hyperpermeability and facilitate leucocyte extravasation (3). In the absence of reperfusion, polymorphonuclear granulocytes (neutrophils) are the first inflammatory cells recruited to the infarct area (2). Neutrophils initiate the acute inflammatory response to engulf dead cells and tissue debris and facilitate post-MI repair. However, excessive neutrophil infiltration exacerbates tissue injury by release of inflammatory mediators and proteinases (1-3).

Another early event in AMI is the acute and marked increase of ventricular expression and release of atrial (ANP) and B-type natriuretic peptides (BNP) (4). Hypoxia (via HIF-1 α) and cytokines (TNF- α , interleukin-1 β) stimulate myocyte BNP expression (5). In fact, plasma N-terminal proBNP level measured 2 to 4 days after AMI is a clinical marker of the severity of ischemia and independently predicts left ventricular (LV) function and 2-year survival (6,7). Of course, ANP and BNP are much more than diagnostic markers of cardiovascular diseases. Via their shared cyclic GMP-forming transmembrane guanylyl cyclase (GC)-A receptor (also named natriuretic peptide receptor A, NPR-A), they stimulate diuresis/natriuresis and vasodilatation, and inhibit the renin-angiotensin-aldosterone (RAA) and sympathetic systems (reviewed in (8)). Even more, NPs exert cardioprotective functions not only as circulating hypotensive/hypovolemic hormones, but also as local auto/paracrine factors moderating pathological cardiomyocyte hypertrophy and interstitial fibrosis (9-11). Due to these systemic and cardiac protective effects, synthetic ANP and BNP were considered to be promising adjunctive treatments in patients with AMI (12).

Within the heart, the GC-A receptor is not only expressed on myocytes and fibroblasts but at even higher density in coronary endothelial cells (13). In vitro studies showed that synthetic ANP, via GC-A, reduces TNF- α -induced endothelial hyperpermeability (14) and limits neutrophil adhesion to hypoxic endothelia (15). In vivo, in the systemic microcirculation, ANP attenuates *acute, immediate* inflammatory effects of mast cells or histamine (16). These protective endothelial actions are mediated by the cGMP-dependent protein kinase I (cGKI) and inhibitory phosphorylation of TRPC6 cation channels, which prevents pathological calcium entry (16). These observations suggest that ANP and BNP (NPs), released in the very early phase of AMI, might improve the endothelial barrier and attenuate the inflammatory infiltration of the myocardium, thereby limiting the area of necrosis.

To follow this hypothesis, we studied myocardial inflammation after experimentally induced AMI in control mice and littermates with conditional, endothelial-restricted inactivation of GC-A (EC GC-A KO) or the downstream kinase cGKI (EC cGKI KO). Our observations indicate that hypoxia and cytokines, i.e. TNF- α , shift endothelial NP/GC-A/cGMP signaling towards activation of phosphodiesterase (PDE)2A. This diminishes subplasmalemmal cAMP levels and may contribute to disruption of the endothelial barrier and neutrophil activation and extravasation after AMI.

METHODS

Expanded methods are provided the Online Supplemental Material./

Animals.

The generation of mice with conditional, endothelial (EC)-restricted inactivation of either GC-A (*Tie2-Cre^{+/0};GC-A^{flox/flox}*; EC GC-A KO) or cGMP-dependent protein kinase I (*Tie2-Cre^{+/0};cGKI^{flox/flox}*; EC cGKI KO) and their control littermates (*GC-A^{flox/flox}*; *cGKI^{flox/flox}*) as well as of mice with green fluorescent granulocytes (LysM-eGFP^{TG} mice) has been described before (16,17). 2-3 months old mice were used for all experiments. The studies conformed to the regulations for animal experimentation and were approved by the local governments.

Experimental AMI and evaluation of the ischemic area at risk as well as infarct size.

After anaesthesia with isoflurane (3%) and intubation, thoracotomy was performed and MI induced by permanent ligation of the proximal part of the left coronary artery (18). Buprenorphine was administered for analgesia after surgery. For sham operation, thoracotomy was performed without arterial ligation (18). Two days after AMI, the area at risk (AAR) and infarct area were evaluated (18). After thoracotomy Evans blue (Sigma, Deisenhofen, Germany) was injected from the cardiac apex to delineate the nonischemic tissue. The hearts were excised, washed with PBS, and cut into five transverse slices. The slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) to determine the infarct area, weighed, and photographed under a microscope (Olympus, Hamburg, Germany). LV area, AAR, and infarct were determined by computerized planimetry (18). Mice with area at risk below 30% were excluded from subsequent evaluations (18).

Analyses of myocardial inflammatory infiltration.

Myocardial slices were fixed in 4% paraformaldehyde and embedded in paraffin. Immunohistochemistry for neutrophils (clone 7/4, Linaris, Wertheim, Germany, # 550274) and myeloperoxidase (MPO antibody from Dako, Germany) was performed on 5- μ m sections. In separate experiments, hearts were digested with collagenase and suspended inflammatory cells were analyzed by Fluorescence-Activated Cell Sorting (FACS) (18). The specific antibodies are depicted in Online Table I.

Determination of MPO activity.

MPO activity in homogenates prepared from the LV infarct area and from corresponding right ventricles (RV) was determined as described (19).

RT-PCR analyses.

Extraction of mRNA from murine LV and reverse-transcription (RT) were performed as described (16). mRNA expression levels of PDE2A, CXCL1 (KC, keratinocyte-derived chemokine) and the adhesion proteins VCAM-1, ICAM-1 and E/P-selectins were analyzed by real time quantitative RT-PCR and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Detection of coronary endothelial glycocalyx by fluorescent hyaluronic acid staining.

Hyaluronic acid (HA), as one of the largest glycosaminoglycans (~1000 KDa), is an integral component of the endothelial glycocalyx. Fluorescent HA stainings were performed on cardiac cryosections (20 μ m) (20). Endothelial cells of myocardial arterioles and capillaries were visualized by confocal microscopy using CD31 and DAPI stainings. The glycocalyx was analyzed employing ImageJ 1.42. Resulting integrated densities were normalized to the number of endothelial cells. From each heart two sections were analyzed (3-6 vessels per section).

Culture of human umbilical vein endothelial cells (HUVECs), cGMP determinations and immunoblotting.

All experiments were performed with confluent HUVECs of passages 1 and 2. Cells were treated with human TNF- α (R&D Systems, Wiesbaden-Nordenstadt, Germany) or vehicle (PBS) during 24 h under normoxic or hypoxic conditions (1% O₂). Thereafter the cells were stimulated with ANP (Bachem, Bubendorf, Switzerland) or with the nitric oxide (NO) donor S-nitroso-N-acetyl-D-penicillamine (SNAP, Sigma) in the absence or presence of the specific PDE2 inhibitor Bay 60-7550 (Alexis). Intracellular cGMP content was determined by radioimmunoassay (16). The expression levels of cGKI (antibody from Cell Signaling, Frankfurt, Germany), PDE2A (FabGennix, Shreveport, LA, USA) and PDE3A (antibody was a gift from Chen Yan, Rochester, NY, USA) were determined by Western blotting (16). Levels were normalized to GAPDH.

Fluorescence resonance energy transfer (FRET) to monitor subsarcolemmal and cytosolic cAMP dynamics in single living HUVECs.

HUVECs were seeded onto 24 mm round glass coverslips, grown to 70-80 % confluency and infected with adenoviral vectors encoding the membrane-attached fluorescent cAMP sensor pmEpac2-camps or the (parental) cytosolic cAMP sensor Epac2-camps (21,22). A multiplicity of infection (MOI) of 30-50 was used to infect HUVECs 40-48 h prior to live-cell FRET measurements (21). 24 h before measurements cells were incubated with TNF- α or vehicle, and then subsequently with ANP, adenosine (Ado, Sigma) and isoprenaline (Iso, Sigma).

Intravital microscopy of the mouse cremaster microcirculation.

As a measure of microcirculatory endothelial permeability, leakage of the macromolecule fluorescein isothiocyanate-labeled (FITC)-Dextran (70 kDa; Sigma) from postcapillary venules within the mouse cremaster muscle was analyzed by intravital fluorescence microscopy (16). EC GC-A KO, EC cGKI KO and respective control mice were deeply anesthetized with intraperitoneal ketamine (100 mg/kg BW) and xylazine (10 mg/kg). After tail vein injection of FITC-Dextran, microscopy was performed using a fluorescence filter for FITC for epiillumination (Olympus). Topical application of ANP, BNP (mouse BNP, American Peptide Co., Sunnyvale, CA) or vehicle was always started 30 min after i.v. administration of FITC-Dextran and continued for additional 40 min. In a second series of experiments leucocyte extravasation was studied in transgenic LysM-eGFP reporter mice with green fluorescent granulocytes (17). FITC-Dextran and leucocyte extravasation in response to ANP or BNP were evaluated after intrascrotal injection of mouse TNF- α (23) or vehicle (PBS) and for ANP in the absence or presence of Bay 60-7550 (local superfusion). Quantitative off-line analysis of the microscopic images was performed with the computer-assisted image analysis system Cell-D (Olympus) (16).

Statistics.

Results are presented as the means $\pm$ standard error of mean (S.E.M.). The number of experiments (n) is indicated in the Figure legends. *P*-values were determined by Student's *t*-test if allowed or otherwise by ANOVA followed by non-parametric Mann-Whitney *U* test, with *P* < 0.05 considered significant.

Results

Decreased myocardial infarct size and inflammatory infiltration in mice with endothelial-restricted inactivation of the GC-A receptor for ANP.

In the absence of reperfusion, neutrophil infiltration occurs within hours post-MI and peaks at 1-3 days (2). To study whether endothelial effects of locally released ANP and/or BNP modulate acute inflammation post-MI, we subjected EC GC-A KO mice and control littermates to 48h of permanent coronary artery ligation (18). In sham-operated control and EC GC-A KO littermates, there were no detectable areas of myocardium at risk for ischemia (AAR) or with infarctions. In mice with coronary ligation, the AAR was not different between genotypes, indicating that ligation site was the same in both groups (Figure 1A). However, the ratios of infarct-to-AAR were mildly but significantly smaller in EC GC-A KO mice (Figure 1A). In other words, the amount of healthy myocardial tissue was larger in the absence of endothelial GC-A.

By immunohistochemistry, the numbers of neutrophils/mm² were determined in the infarct area, the border-zone and the septum (18). As shown in Figure 1B, almost no neutrophils were detectable in the myocardium of sham-operated mice. In mice with AMI, neutrophil density was tremendously increased in the infarct zone, and less in the border-zone and septum. Notably, the number of neutrophils infiltrating the infarcted myocardium was significantly diminished in EC GC-A KO mice (Figure 1B). FACS analyses (18) further revealed a smaller portion of neutrophils within the CD11b⁺ myeloid cells infiltrating the LV myocardium of EC GC-A KO as compared to control littermates (Figure 1C). Such analyses also revealed that the infiltration by other inflammatory cells, such as T-cells and monocytes/macrophages, was not different between genotypes (Online Table I). Accordingly, the number of MPO-stained neutrophils and MPO activity (a well known marker for neutrophil accumulation) were lower in the infarct area of EC GC-A KO mice (Figures 2A and B). Lastly, we determined the LV mRNA levels of CXCL1/KC, a chemokine which in the rodent postischemic myocardium is selectively expressed in infiltrating inflammatory cells (24). As shown in Figure 2C, LV CXCL1 levels were markedly lower in EC GC-A KO mice as compared to their control littermates.

To elucidate the mechanism(s) of diminished neutrophil activation in EC GC-A KO mice, the RNA levels of endothelial adhesion molecules were evaluated. Figures 2D-G illustrate that LV levels of ICAM-1, VCAM-1 and E/P-selectins were increased in control mice with AMI. These responses were drastically attenuated in EC GC-A KO littermates. Together our observations demonstrate that inactivation of the endothelial GC-A receptor attenuates early inflammation after AMI. They indicate that GC-A/cGMP-mediated actions of endogenous local NPs contribute to the impairment of the barrier functions of the coronary endothelium in the setting of myocardial ischemia, favouring the extravasation of neutrophils.

Unaltered myocardial infarct size and neutrophil infiltration in EC cGKI KO mice.

Endothelial cells express at least three effector molecules for cGMP: cGKI, cGMP-inhibited PDE3A and cGMP-stimulated PDE2A (13,25). To elucidate whether endothelial cGKI mediates the proinflammatory actions of NPs after AMI, we studied EC cGKI KO mice and respective control littermates (16,18). Figures 3A and B show that the myocardial infarct size, infarct-to-AAR and the number of neutrophils infiltrating the infarcted and surrounding myocardium were not different between genotypes. Therefore, cGKI does not mediate the proinflammatory effects of endothelial NP/GC-A/cGMP signaling.

Reduced coronary glycocalyx in control and EC GC-A KO mice after AMI.

Studies in isolated perfused hearts showed that exogenous, synthetic NPs cause shedding of the coronary glycocalyx (26). Because disruption of the glycocalyx favours postischemic neutrophil adhesion we hypothesized that shedding of the glycocalyx by endogenously released NPs contributes to myocardial inflammatory infiltration after AMI. By fluorescent hyaluronan staining the apical endothelial glycocalyx was clearly detectable in the hearts of sham-operated control mice and was indeed thinner and reduced $\approx 50\%$ after 2 days of ischemia (Online Figure I). If endothelial effects of NPs were involved in this damage, then EC GC-A KO mice should be protected. However, the glycocalyx of EC GC-A KO mice was similarly reduced after AMI (Online Figure I).

Phosphodiesterase 2A is upregulated in ischemic myocardium and in hypoxic and/or TNF- α -treated HUVECs.

TNF- α , an inflammatory cytokine induced by myocardial ischemia (27), increases the expression and activity of PDE2A3 in HUVECs (25,28). PDE2 is stimulated by cGMP, thereby increasing its rate of hydrolysis of both cGMP and cAMP. In general, decreases in cAMP levels impair endothelial barrier functions (25,28). Therefore we hypothesized that the proinflammatory endothelial effect of the NPs/GC-A pathway during AMI is mediated by cGMP-dependent activation of PDE2A. Figure 4A shows that baseline protein levels of PDE2A in HUVECs were very low. PDE2A expression levels significantly raised in response to TNF- α (10 ng/ml, 24h), hypoxia (1% O₂, 24h) or in response to a combination of both stressors (Figure 4A). The simultaneous exposure of HUVECs to both noxes exerted a greater effect on PDE2A induction, but the difference to separate exposures was not significant (Figure 4A). These increases of PDE2A were associated with significant decreases of cGKI levels (Figure 4A, right panel). Supporting the in vivo relevance of these findings, quantitative RT-PCR showed that LV PDE2A mRNA levels were increased by $\approx 30\%$ after 2 days of ischemia (Figure 4B).

Induction of PDE2A modifies the effects of ANP on endothelial cGMP and cAMP levels.

In contrast to PDE2A, PDE3 is inhibited by cGMP and therefore mediates a positive cGMP/cAMP crosstalk (25,28). Figure 5A (right side) confirms that treatment of HUVEC with TNF- α (10 ng/ml, 24h) induced PDE2A expression whereas PDE3A protein levels were unaltered. Because PDE2A is a dual substrate esterase, higher PDE2 expression following TNF- α treatment should affect both the cGMP- and cAMP-responses of HUVECs to ANP. Indeed, the cGMP-responses to ANP were significantly attenuated in TNF- α -treated HUVECs (Figure 5A, left). Specific inhibition of PDE2A with Bay 60-7550 (100 nM/L, added 15 minutes before ANP) did not affect the baseline responses to ANP (not shown) but fully rescued the cGMP responses of TNF- α -treated HUVECs to ANP (Figure 5A). Similarly the cGMP effects of the NO-donor SNAP (100 μ M/L SNAP provoked 3.1 ± 0.1 -fold increases of baseline cGMP) were significantly inhibited by TNF- α (1.8 ± 0.2 -fold increases; $P < 0.05$) and rescued by Bay 60-7550 (3.9 ± 0.3 -fold increases; $n=4$ per condition). These results confirm that PDE2A hydrolyses cGMP formed by either GC-A or NO-sensitive GC.

Next we studied the impact of ANP signaling on intracellular cAMP. Cyclic AMP signals within endothelial cells are highly compartmentalized and Sayner *et al.* suggested that cytosolic-produced cAMP decreases, whereas cAMP in submembrane compartments improves barrier functions (29). To analyze microdomain-specific cAMP dynamics, we transfected HUVEC either with pmEpac2-camps, a FRET cAMP sensor targeted to caveolae, or with the cytosolic sensor Epac2-camps (21,22). The cells were then treated with or without TNF- α during 24h prior to the acute FRET measurements. Single cell cAMP-responses to ANP (10 nmol/L) were compared with the responses to subsequent addition of Ado (10 μ mol/L) and Iso (1 μ mol/L). The effects of ANP and Ado were calculated as percent of the effect of the

β -adrenergic agonist Iso (FRET in % max), which fully activates both sensors in HUVEC. Figures 5B and C depict original tracings recorded with the membrane-attached sensor. As shown in Figures 5B and D, in resting HUVECs, ANP clearly increased submembrane cAMP levels, the effect being smaller than the effects of Ado and Iso. Figures 5C and D illustrate that TNF- α exposure and induction of PDE2A3 reversed the effect of ANP from an increase to a mild decrease of submembrane cAMP. The stimulatory cAMP effects of Ado were significantly inhibited by TNF- α , while the effects of Iso were not altered. Again the PDE2A inhibitor Bay 60-7550 (100 nM/L, 10 min) did not alter the cAMP effects of ANP and Ado in "resting" cells but fully rescued the stimulatory effects of ANP and Ado in TNF- α pretreated cells (Figure 5D, right bars). In other words, in TNF- α pretreated cells, inhibition of PDE2A prevented the decrease of submembrane cAMP levels by ANP. Figure 5E shows that ANP and Ado also enhanced the cytosolic cAMP levels of HUVECs, but these responses were not significantly altered by TNF- α . Together with published studies (28) these results indicate that TNF- α increases the endothelial expression and ANP/cGMP-stimulated activity of the membrane-anchored isoform PDE2A3. Ultimately, these changes can mediate a negative submembrane cross-talk between cGMP and cAMP which may weaken the endothelial barrier.

TNF- α induces PDE2-mediated hyperpermeability effects of ANP/GC-A in the microcirculation.

The extravasation of neutrophils primarily occurs in post-capillary venules, where hemodynamic shear forces are diminished and the vessel wall is thin (2). We hypothesized that ANP/PDE2 signaling may increase transendothelial neutrophil migration partly by enhancing paracellular permeability and studied this possibility with intravital microscopy analyses of macromolecular FITC-Dextran leakage from post-capillary venules of the mouse cremaster muscle. Consistent with our previous studies (16), ANP or BNP (100 nmol/L, 40 min local superfusion) had no direct effects on the permeability for FITC-Dextran (Figures 6A and 6B). Pretreatment of the cremaster with a low dose of TNF- α (200 ng, intrascrotal injection (23)) enhanced the mRNA expression levels of PDE2A (Figure 6C) but only mildly affected baseline Dextran permeability (Figures 6A and B). However, after TNF- α pretreatment, ANP and BNP significantly enhanced FITC-Dextran extravasation, indicating augmented paracellular permeability (Figures 6A and B). Of note, this TNF- α -induced hyperpermeability effect of ANP was abolished in EC GC-A KO but preserved in EC cGKI KO mice (Figure 7A). Even more, in control mice pretreated with TNF- α the hyperpermeability effect of ANP was fully prevented by inhibition of PDE2 with Bay 60-7550 (100 nM/L, local superfusion of the cremaster during 15 min before addition of ANP) (Figure 7B).

Lastly we studied mice with fluorescent leucocytes (LysM-eGFP (17)) to visualize directly the effect of ANP on neutrophil extravasation. As shown in Figure 8, under basal conditions ANP (100 nmol/L, 40 min superfusion) did not change the number of perivascular leucocytes in the cremaster. TNF- α pretreatment (performed as above) significantly increased the number of interstitial leucocytes. In TNF- α pretreated mice, ANP further enhanced leucocyte extravasation and this ANP-effect was prevented by Bay 60-7550 (Figure 8).

We conclude that in the presence of TNF- α , ANP attenuates microcirculatory endothelial barrier functions and stimulates leucocyte transmigration. This effect involves endothelial GC-A/cGMP signaling and PDE2A3 as an effector molecule.

DISCUSSION

Principal findings.

Our study shows that mice with endothelial deletion of GC-A, the cGMP-producing receptor shared by ANP and BNP, are significantly protected from myocardial neutrophil activation and infiltration as well as from necrosis during the early phase after AMI. Attenuated neutrophil activation in the ischemic myocardium of EC GC-A KO mice is indicated by diminished MPO activity and decreased mRNA levels of CXCL1 (KC), a CXC chemokine which was previously shown to be induced in inflammatory cells but not in resident myocardial cells after experimental MI (24). Concomitantly, the induction of endothelial adhesion molecules (selectins, ICAM-1, VCAM-1) by MI was markedly attenuated in these mice. We conclude that NPs, which are induced and released within the ischemic myocardium, may contribute to endothelial barrier dysfunction and thereby to inflammation after AMI. Our functional and biochemical experiments in vitro together with intravital microscopy studies of the m. cremaster microcirculation in vivo demonstrate that hypoxia and cytokines such as TNF- α alter the immediate postreceptor signaling pathways of NPs/cGMP in endothelial cells, decreasing cGKI and inducing PDE2A expression. This molecular "remodeling" may reverse the effects of ANP/BNP from endothelial barrier protection (via cGKI (16)) to barrier disruption (via PDE2A3) (Online Figure II). It is well known that accentuation or prolongation of the inflammatory response in the early phase after AMI later on results in worse remodeling and dysfunction (30). Thus, our experimental studies may partly explain the clinical observations that sustained increases in ANP and, greater, BNP levels during the early phase of AMI correlate with progressive LV enlargement, decreased contractility, and increased stiffness (4,6,7).

Of note, EC GC-A KO mice have mild arterial hypertension and functionally well compensated subtle cardiac hypertrophy under baseline, resting conditions (16, 31). Because LV hypertrophy is a powerful risk factor for cardiovascular disease we expected that this intrinsic phenotype would exacerbate (not attenuate) ischemic myocardial necrosis of EC GC-A KO mice. This risk factor may explain why these mice exhibited only modest decreases in myocardial necrosis despite marked attenuation of inflammatory markers. In other words, the impact of the proinflammatory effects of NPs after MI might be underestimated in the here presented experimental studies.

Our observations of *acute* proinflammatory roles of ANP and BNP after AMI are in line with previous studies of permanent coronary occlusion or ischemia-reperfusion injury in mice with global, systemic inactivation (KO) of ANP or GC-A or with transgenic overexpression of BNP in the liver (32-34). However, these former studies have the limitation that systemic arterial hypertension, increased systemic sympathetic and renin-angiotensin activities as well as vascular abnormalities influence the cardiac responses of the global KO mice to ischemia. In the BNP^{tg} mice (34), the effects of very high systemic, pharmacological BNP concentrations were assessed, which may or may not resemble the local high endogenous BNP levels occurring during AMI. Another limitation of these studies is that they did not explore the specific cell types and signaling pathways through which NPs enhance neutrophil extravasation after myocardial ischemia. In particular, neutrophils express the GC-A receptor and in vitro studies revealed direct although controversial, either potentiating or inhibitory effects of synthetic NPs on neutrophil activation and their interactions with cultured endothelia (35,36,15). The present study in mice with EC-restricted inactivation of GC-A or cGKI addresses the clinically relevant question whether and how endothelial cell-specific signaling of NPs impacts inflammatory processes in the ischemic myocardium.

Dual regulation of endothelial cell barrier functions in vivo by ANP/cGMP via cGKI (enhancement) and PDE2 (impairment).

After their adhesion to endothelial cells, the subsequent extravasation of neutrophils occurs primarily in post-capillary venules, where hemodynamic shear forces are diminished and the vessel wall is thin (2). This transendothelial migration takes place by transcellular and, mainly, by paracellular trafficking (2). Endothelial barrier properties have an important role and a dysfunctional endothelium, with enhanced expression of neutrophil adhesion proteins and altered cell-cell contacts, is characteristic of many inflammatory diseases, including AMI. When a previously healthy, resting endothelium is involved in an *acute* allergic reaction mediated by mast cell histamine, the ANP/GC-A/cGMP pathway, via activation of cGKI, can attenuate pathological calcium entry and hyperpermeability (16). However, elegant *in vitro* studies in HUVEC showed that the effect of ANP/cGMP depends on the relative expression of different cGMP effector molecules (25). In particular, TNF- α , one of the main cytokines produced in AMI, induces the expression of PDE2A3, a membrane-anchored isoform (25,28). In HUVEC with high PDE2A3 levels, ANP/cGMP enhanced endothelial permeability (25). Our observations extend these studies in HUVEC, showing that TNF- α and also hypoxia enhance the expression and cGMP-hydrolysing activities of PDE2A and, even more, attenuate cGKI levels. The later observation is consistent with published findings of diminished cGKI levels in hypoxic smooth muscle cells (37). However, whereas a loss of endothelial cGKI prevents the acute antihistaminergic action of ANP (16), this is not sufficient to "reverse" this protective effect into a proinflammatory action since EC cGKI KO mice did not show enhanced (but unaltered) myocardial inflammation after AMI.

In general, cAMP has been shown to stabilize the endothelial barrier. However, it is becoming increasingly apparent that cAMP signals within cells are compartmentalized (29,38). Studies suggest that elevation of cytosolic cAMP disrupts the endothelial barrier (38,39). In contrast, subplasmalemmal cAMP enhances the phosphorylation of effectors that promote junctional integrity, such as filamin A (39). In HUVECs the NP/GC-A/cGMP pathway may have dual effects on cAMP, depending on the relative expression levels of the cAMP-degrading enzymes PDE3A (cGMP-inhibited) and PDE2A (cGMP-stimulated), mediating positive vs negative cGMP/cAMP cross talks (25). TNF- α does not alter PDE3 but markedly enhances PDE2A3 expression (25,28 and present study). To analyze the impact on the regulation of submembrane vs cytosolic cAMP levels by ANP, we performed comparative FRET studies with a membrane-targeted and a cytosolic cAMP sensor. In resting HUVECs, ANP increased submembrane and cytosolic cAMP, indicating inhibition of PDE3A. In contrast, in cells pretreated with TNF- α , we observed an ANP/cGMP-driven, PDE2A-mediated mild but clear decrease of submembrane cAMP, while the effect of ANP on cytosolic cAMP was unchanged. The observation that this cAMP decrease is confined to the submembrane compartment supports the involvement of the membrane-anchored PDE2A3 isoform previously shown to be induced by TNF- α (28).

Of course HUVEC just provide a more or less valid *in vitro* model system. Since we observed a significant induction of PDE2A expression *in vivo*, in hearts after AMI, our major goal was to study whether such molecular changes impact the microcirculatory actions of NPs. Limiting our studies, up-to-now it is technically impossible to measure dynamic cAMP changes in the microcirculatory endothelium *in situ*. Instead we applied intravital microscopy to directly visualize the effects of synthetic ANP or BNP on the endothelial barrier of post-capillary venules within the cremaster muscle of monogenetic mouse models dissecting endothelial NP/GC-A signaling. By monitoring the extravasation of FITC-Dextran and of fluorescent leucocytes as indices of endothelial barrier functions we confirmed that NPs do not alter endothelial permeability under baseline, resting conditions (16). However, in cremaster preparations pretreated with TNF- α to induce PDE2 expression, both ANP and BNP exerted striking proinflammatory effects, with macromolecule and leucocyte extravasation. A combination of specific genetic and

pharmacological tools demonstrated that this effect was mediated by endothelial GC-A/cGMP/PDE2 signaling and was independent of cGKI.

Downstream pathways contributing to proinflammatory endothelial NP/GC-A signalling.

Decreases in endothelial submembrane cAMP might enhance the recruitment and transmigration of neutrophils by different mechanisms such as endothelial production of reactive oxygen species, expression of adhesion molecules and weakening of endothelial junctions (25,40,41). In particular it was shown that increased endothelial cAMP down-regulates the expression of the cell surface adhesion proteins VCAM-1, ICAM-1 and E-selectin (40). Notably, the induction of these endothelial proinflammatory proteins by AMI was drastically downregulated in EC GC-A KO as compared to control littermates. This supports our concept that endothelial NP/GC-A signaling, possibly through PDE2-mediated cAMP decreases, contributes to the induction of the proinflammatory features of coronary endothelial cells during AMI.

Such adhesion molecules are normally harboured within the glycocalyx and are exposed after ischemia-induced shedding of larger constituents, particularly hyaluronan, which enhances leukocyte adhesion. Studies in isolated perfused hearts showed that intracoronary infusion of synthetic ANP or BNP caused shedding of the coronary glycocalyx, resulting in enhanced permeability (26). However, the pathophysiological significance in vivo was not investigated. Indeed, our fluorescent stainings of myocardial vessels revealed loss of hyaluronan from the apical surface of the endothelium 48h after AMI. However, we observed similar alterations in control and EC GC-A KO littermates, indicating that endogenous endothelial NP/GC-A signaling does not provoke this damage.

Summary and conclusions.

Our in vivo studies extend previous in vitro findings (25) that the effect of the NP/GC-A/cGMP signaling pathway in endothelial cells is highly dependent on the molecular equipment of the cells, i.e. the expression levels of the cGMP-modulated regulatory proteins cGKI and PDE2 vs PDE3. To our knowledge, this is the first study showing that cGMP/cAMP crosstalk is compartmentalized in endothelial cells and that this dual NP/cGMP-dependent regulation of cAMP and microvascular endothelial cell permeability has pathophysiological relevance. When a previously healthy endothelium is involved in an acute allergic reaction, endothelial NP/GC-A/cGMP/cGKI signaling can counterbalance calcium-linked hyperpermeability (16) (Online Figure II). However, when endothelial cells are exposed to hypoxia and cytokines such as TNF- α , conditions which enhance PDE2A and impair cGKI expression, NP/GC-A/cGMP signaling may increase PDE2 activity, resulting in a decrease of submembrane cAMP with breakdown of endothelial barrier functions and enhanced expression of cell surface adhesion proteins. Such changes ultimately facilitate leucocyte activation and transmigration (Online Figure II). Our findings indicate that this pathway may contribute to inflammation in the early phase after MI, and suggest that inhibition of endothelial PDE2A could have a beneficial effect in this setting.

Clinical relevance.

Our experimental studies unravel a novel endothelial signaling mechanism of NPs. Although previous (25) and here presented experiments confirm this pathway in cultured human EC, the relevance for the pathophysiology of MI in patients of course requires further investigations. In fact, clinical evaluation of intravenous infusions of synthetic ANP (carperitide) or BNP (nesiritide) indicate that NPs may be an effective adjunctive therapy for cardiac function protection in AMI patients (12,42,43). Recent insights from the Meta-analysis of 20 different trials showed that additional ANP or BNP treatment (low doses of carperitide or nesiritide infused during 2 to 7 days after AMI) was significantly superior to standard medical therapy. LV ejection fraction was improved by synthetic NPs both in the short-term (1

month) and long-term follow-up, with a trend towards lower infarct sizes and reduced risk of major acute cardiovascular events (12). However, all of these studies only mentioned that the synthetic NPs were given at the acute phase after AMI, but the real interval between the onset of AMI and start of infusion remained unclear (12). Such protective long-term effects of short-term administered synthetic NPs likely involve their favourable systemic actions including inhibition of the RAAs and vasodilation, as well as local cardiac antihypertrophic and antifibrotic actions (44), actions which obviously predominate over the unwarranted acute proinflammatory actions described here. In addition, two clinical aspects may counter regulate possible acute proinflammatory cardiac actions of local NPs: 1) AMI routine therapy includes anti-inflammatory drugs such as aspirin and statins (12); and 2) concurrent plasma concentrations of the endogenous NPs (NT-proBNP and NT-proANP) fell with the introduction of nesiritide infusions and cardiac unloading, indicating diminished local, cardiac (endogenous) NP levels in the presence of enhanced systemic (exogenous) NP plasma levels (45). The here presented experimental data illustrate a novel pathway which could be a target for therapies improving NPs actions.

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DISCLOSURES

None.

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Circulation Research

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FIGURE LEGENDS

Figure 1. Evaluation of myocardial infarct size and neutrophil infiltration in control and EC GC-A KO mice 2 days after AMI. (A) The ratios of infarct/AAR were diminished in EC GC-A KO mice, despite identical AAR (n=16 per genotype). Right: Representative photographs of midventricular myocardial tissue from control and EC GC-A KO mice (magnification: x16). The infarct area is in white color, AAR is red and non-ischemic area is blue. (B and C) Neutrophil infiltration was determined by immunohistochemistry in the infarct and surrounding areas (B; n=8 per genotype) and by FACS analyses of whole myocardial LV tissue (C; n=6 CTR and 7 KO mice). Both the absolute number of neutrophils in the infarct zone (B) and the relative amount of neutrophils in the LV (C) were significantly lower in EC GC-A KO mice. Right panels: Representative pictures and plots. Ly6G stains neutrophils (in Q2) and CD11b stains myeloid cells including granulocytes, natural killer (NK) cells, monocytes/macrophages and dendritic cells. *P < 0.05 vs. controls.

Figure 2. Diminished neutrophil activity and reduced expression of endothelial adhesion proteins in hearts from EC GC-A KO mice with AMI. (A, B) Myeloperoxidase (MPO) expression (A; immunohistochemistry) and activity (B; RFU: relative fluorescence units) in the infarct zone and right ventricle (RV) 2 days after induction of MI (n=5). (C-G) LV mRNA expression (by qRT PCR) of the chemokine CXCL1 and the endothelial adhesion proteins ICAM-1, VCAM-1 and E/P-selectin in sham and MI mice (n=8-9 mice); *P < 0.05. Together these results demonstrate diminished myocardial inflammation in EC GC-A KO mice with experimental MI.

Figure 3. Evaluation of infarct size and neutrophil infiltration in hearts from control and EC cGKI KO mice subjected to AMI. (A) The ratios of infarct/AAR were not different between genotypes (n=16 CTR and 13 KO mice). Right: Representative photographs (x16) of midventricular myocardial tissue. The infarct area is white, AAR is red and non-ischemic area is blue. (B) Neutrophil infiltration was determined by immunohistochemistry. Neutrophil numbers in the infarct and surrounding areas were not different between genotypes (n=8). Right panel: Representative pictures.

Figure 4. Increased expression of PDE2A in HUVECs subjected to hypoxia and/or TNF- α as well as in the myocardium of mice with AMI. (A) HUVEC were incubated with TNF- α (10 ng/mL) or vehicle (PBS) under normoxic or hypoxic (1% O₂) conditions for 24h. Western blot analyses demonstrated that TNF- α , hypoxia or a combination of both attenuate the expression of cGKI (right panel) and increase the expression of PDE2A (left panel). However, the combination of both stressors did not exert significant additional effects. Top: Representative western blot. Bottom: Results from 3 independent experiments. *P < 0.05 vs. normoxia/PBS. (B) Real-time RT-PCR demonstrates increased LV PDE2A expression after AMI (n = 6; *P < 0.05 vs. sham).

Figure 5. TNF- α alters ANP-induced cGMP/cAMP crosstalk in HUVEC. (A) ANP-induced cGMP accumulation is reduced by TNF- α and rescued by Bay 60-7550. HUVECs were incubated with TNF- α (10 ng/mL) or vehicle for 24h followed by ANP $\pm$ Bay 60-7550 (100 nM/L). Right side: Immunoblotting confirmed increased PDE2A and revealed unaltered PDE3 levels in TNF- α pretreated HUVECs. (B-E) FRET-based measurements of submembrane (B-D) vs cytosolic (E) cAMP in HUVEC pretreated with vehicle (B) or 10 ng/mL TNF- α (C) for 24h. Increase of CFP/YFP ratio indicates an increase in cAMP levels. Cells were stimulated with 10 nmol/L ANP and subsequently with subsaturating concentrations of adenosine (Ado, 1 μ mol/L) and isoproterenol (Iso, 1 μ mol/L). D and E: data are shown for ANP and Ado as a % of maximal Iso response (*P<0.05; n=8-9 cells from 2 independent experiments).

Figure 6. TNF- α induces endothelial hyperpermeability effects of ANP and BNP in postcapillary venules of the m. cremaster. (A, B) Time course of changes in net integrated optical intensity (IOI; an index of FITC-Dextran extravasation) during 40 min of local ANP, BNP (100 nM/L) or vehicle superfusion after intrascrotal injection of vehicle (200 μ l PBS) or TNF- α (200 ng) (n = 6; *P < 0.05 vs. baseline at -10 min). (C) Real-time RT-PCR: Pretreatment of the m. cremaster with TNF- α enhanced the mRNA expression levels of PDE2A. Top in A,B: Representative original photographs.

Figure 7. The TNF- α -induced hyperpermeability effect of ANP in cremaster venules is mediated by endothelial GC-A/cGMP/PDE2 signaling and independent from cGKI. Time course of changes in net integrated optical intensity (IOI) during 40 min of continuous local ANP (100 nM/L) superfusion 2h after intrascrotal injection of TNF- α (200 ng): (A) in mice with conditional, endothelial deletion of (EC GC-A KO) or cGKI (EC cGKI KO); and (B) in control mice after local superfusion with vehicle or with Bay 60-7550 (100 nmol/L, 15 min before addition of ANP). Endothelial GC-A inactivation (A) or pretreatment with Bay 60-7550 for 15 min (B) prevented TNF- α /ANP-induced hyperpermeability (n = 6; *P < 0.05 vs baseline at -10 min). Top in A,B: Representative original photographs.

Figure 8. After pretreatment with TNF- α , ANP stimulates the extravasation of leucocytes from postcapillary venules of the m. cremaster venules. Transgenic LysM-eGFP mice were used to observe neutrophils. Top: Representative original pictures of intra- and extravascular neutrophils (green). Bottom: Changes in perivascular leukocytes during 40 min of continuous local ANP (100 nM/L) superfusion, after intrascrotal injection of TNF- α (200 ng) or PBS. Local superfusion of Bay 60-7550 (100 nmol/L, 15 min before addition of ANP) prevented this proinflammatory ANP effect. Interstitial leucocytes are illustrated as number per square millimeter (n = 4; *P < 0.05).

Research

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Novelty and Significance

What Is Known?

- In acute myocardial infarction (AMI), the infiltration of the ischemic tissue by inflammatory cells impacts initial injury and subsequent healing and remodelling processes.
- Another early event in AMI is the acute and marked increase of ventricular expression and release of the cardiac hormones atrial (ANP) and B-type natriuretic peptide (BNP), but the pathophysiological significance is unknown.

What New Information Does This Article Contribute?

- Normally, ANP and BNP, via their shared guanylyl cyclase A (GC-A) receptor and enhanced intracellular levels of the cyclic nucleotides cGMP and cAMP, exert protective endothelial actions improving vascular barrier functions.
- In AMI, tissue hypoxia and cytokines induce the expression of phosphodiesterase (PDE) type 2A in endothelial cells, a cGMP-stimulated PDE which degrades submembrane cAMP in response to natriuretic peptide/GC-A/cGMP signalling.
- In mice, genetic deletion of the endothelial GC-A receptor attenuates myocardial inflammation in the early phase of AMI, suggesting the presence of an ANP/BNP-mediated proinflammatory crosstalk between cardiomyocytes and endothelial cells in this situation.

Plasma proBNP level after AMI is a clinical marker of the severity of ischemia and independently predicts cardiac function and 2-year survival. Of course, ANP and BNP are much more than diagnostic markers of cardiovascular diseases. Via their GC-A receptor, they exert cardioprotective functions as circulating hypotensive hormones, and also as local factors moderating pathological cardiac remodelling. Therefore, synthetic ANP and BNP were considered promising adjunctive treatments in patients with AMI. In the early phase of AMI endothelial dysfunction and myocardial inflammation are critical determinants of injury. To dissect the endothelial actions of NPs, we studied mice with endothelial-specific knockout of GC-A. To our surprise, we found that such mice exhibit less myocardial necrosis and inflammation after experimental AMI. Moreover, the induction of proinflammatory endothelial adhesion proteins was markedly attenuated. Molecular and intravital microscopy studies revealed that hypoxia and cytokines such as TNF- α , induce the expression of the cGMP-stimulated PDE type 2A in endothelial cells. NP/GC-A/cGMP signalling increases PDE2 activity, resulting in a decrease of submembrane cAMP with breakdown of endothelial barrier functions and enhanced leucocyte transmigration. This pathway may contribute to inflammation after MI, suggesting that inhibition of endothelial PDE2A could have a beneficial effect in this clinical setting.

FIGURE 1

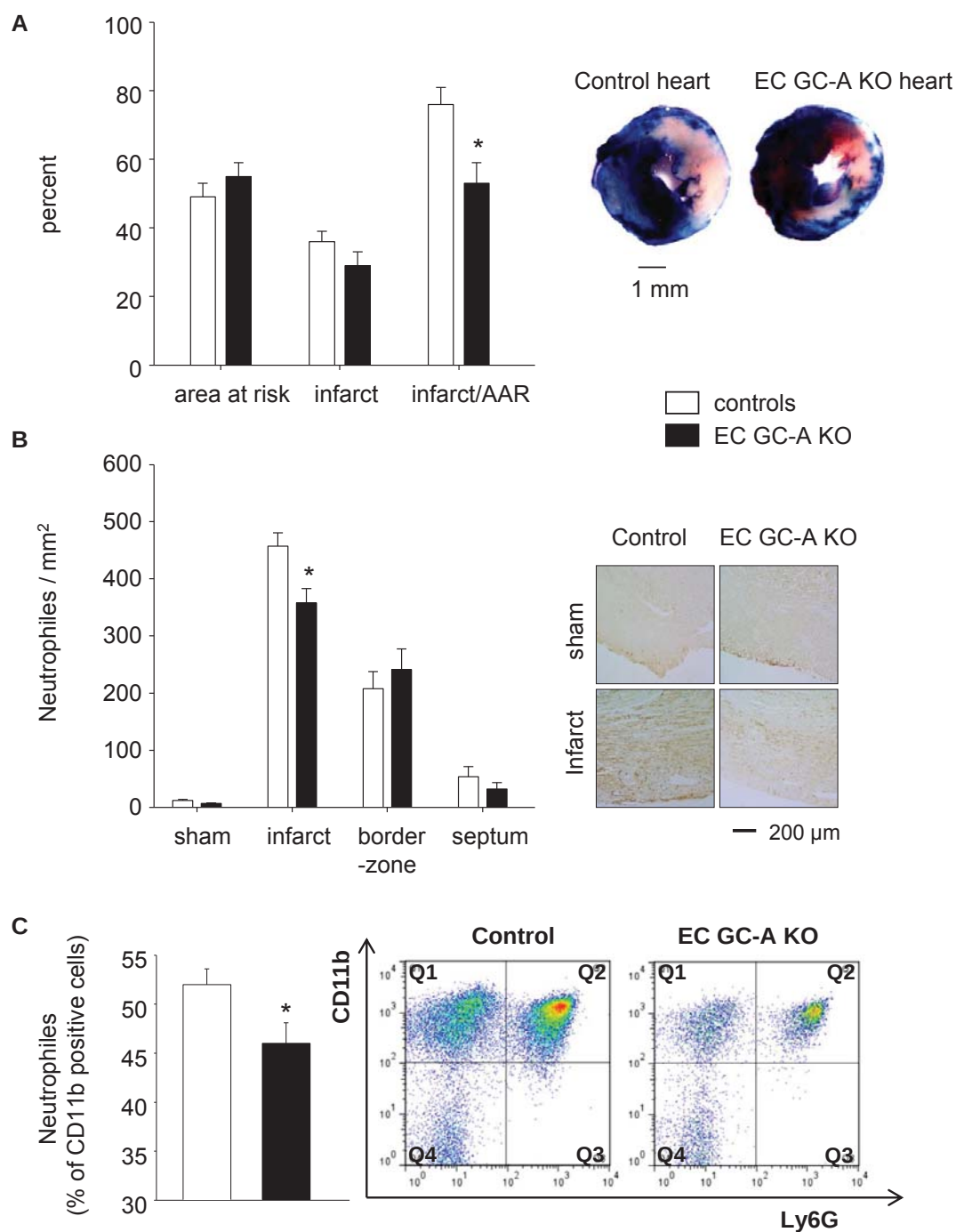


FIGURE 2

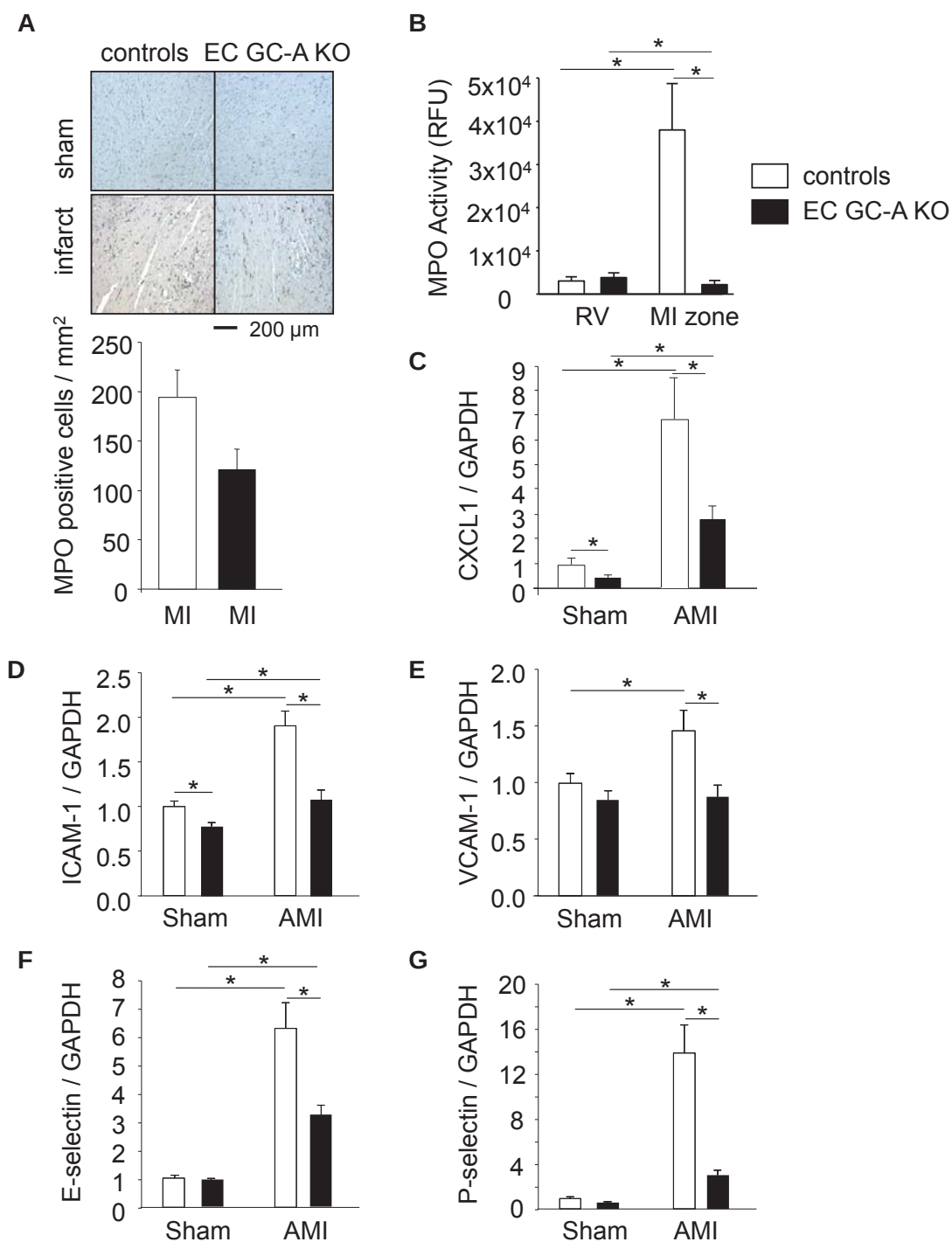
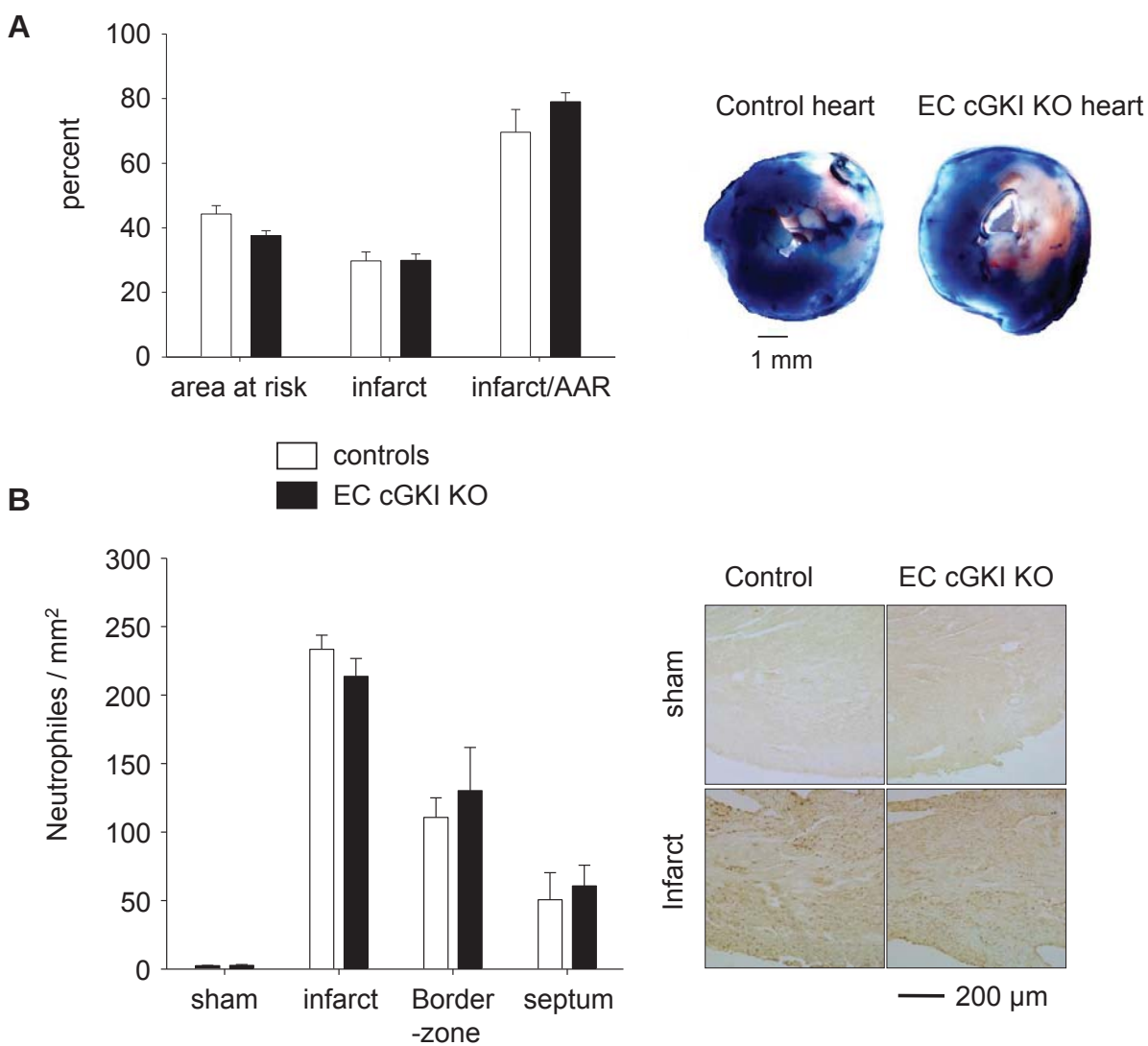
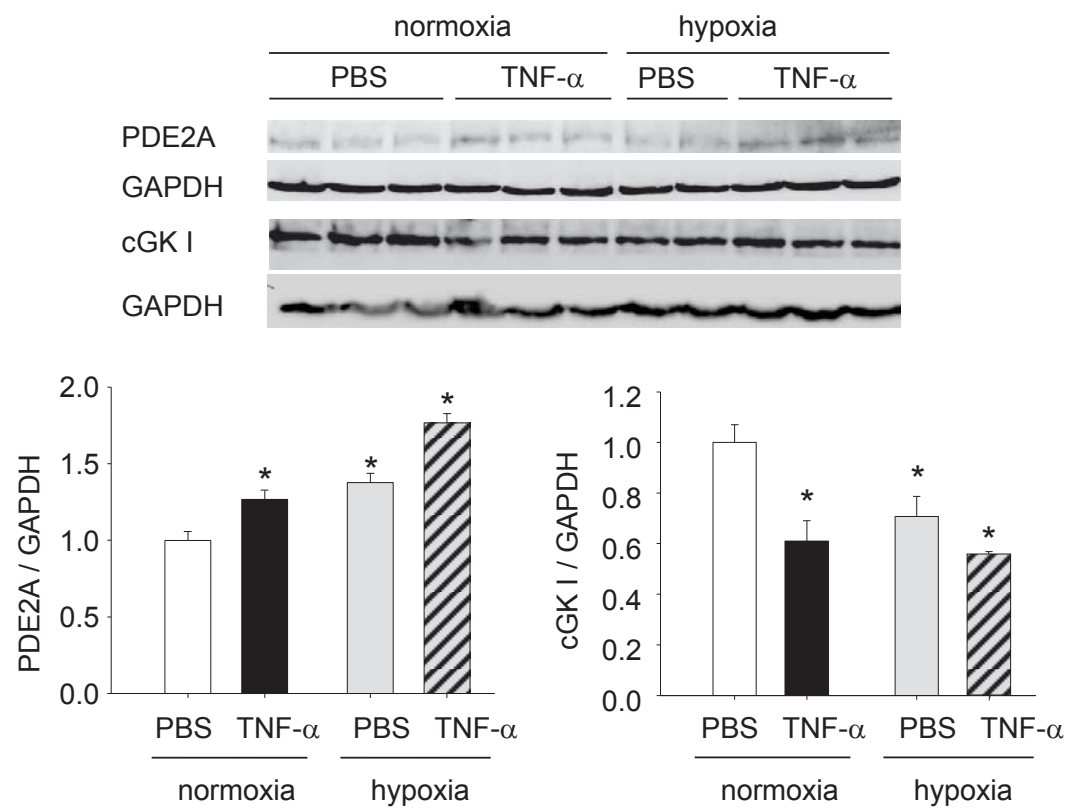
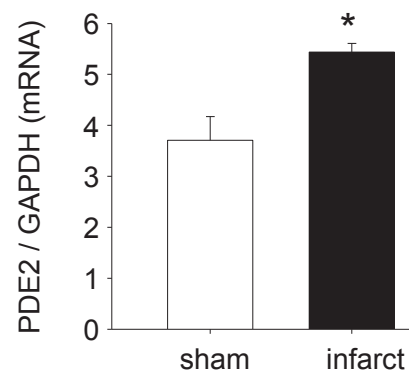
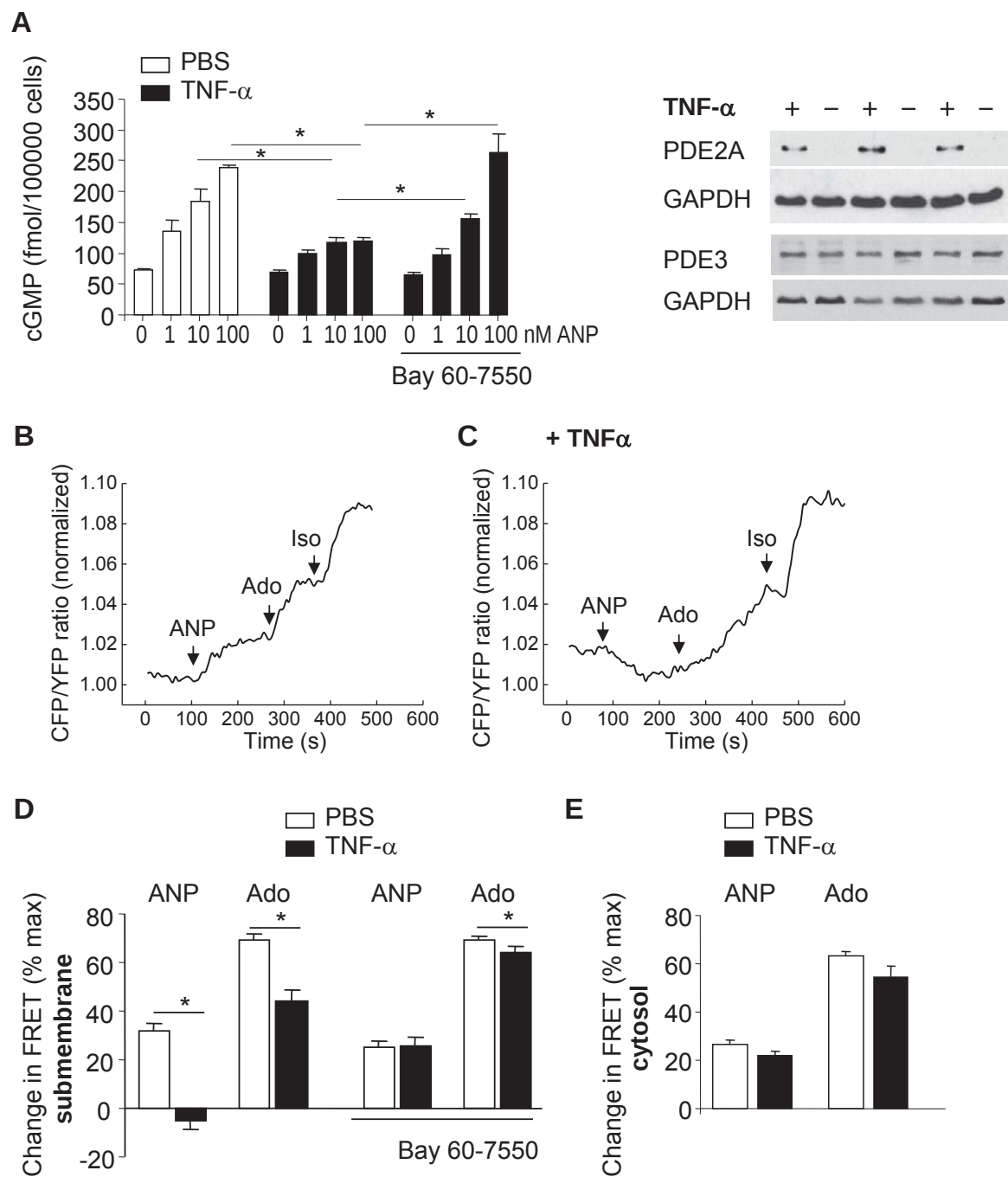
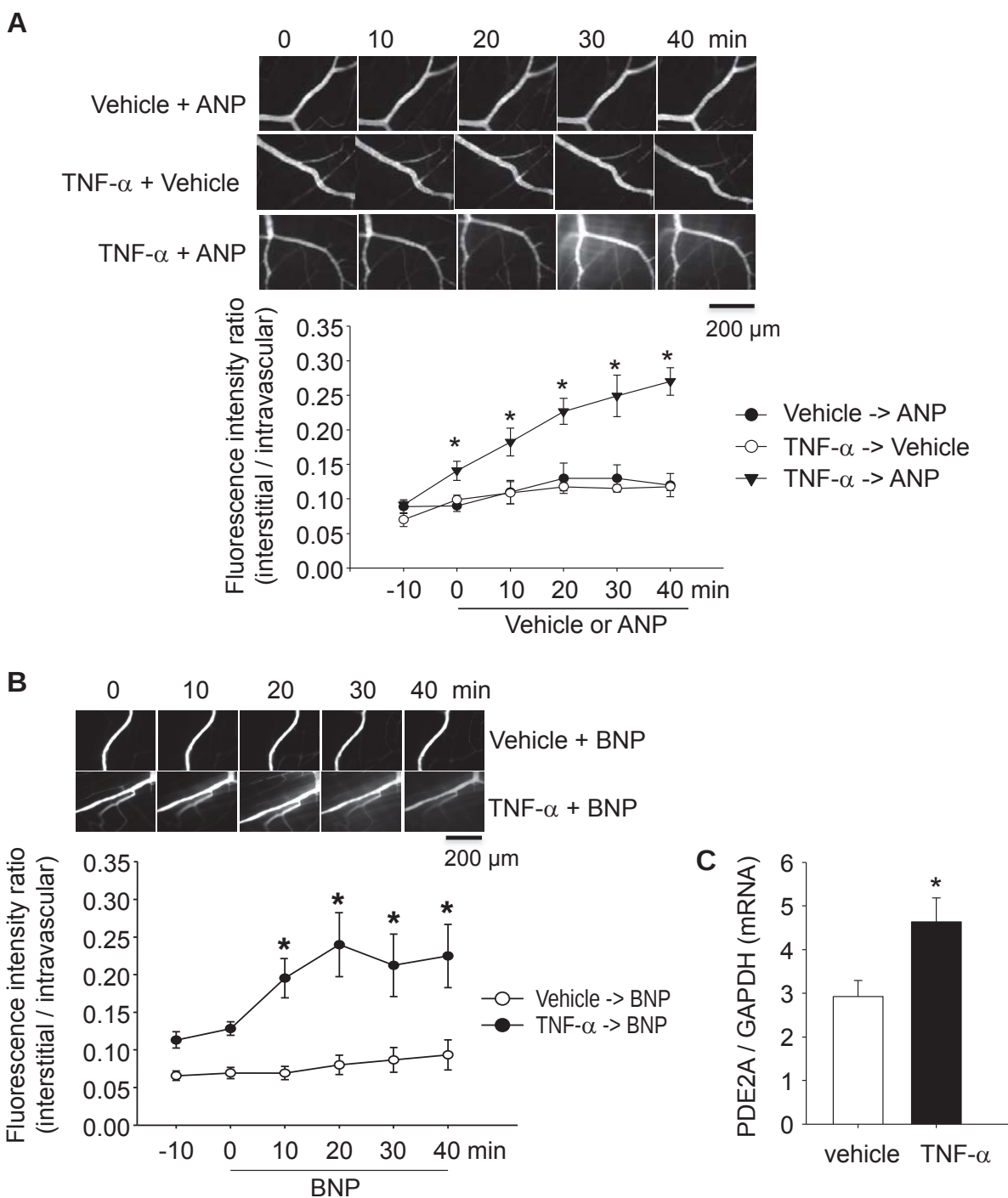


FIGURE 3



A**B**





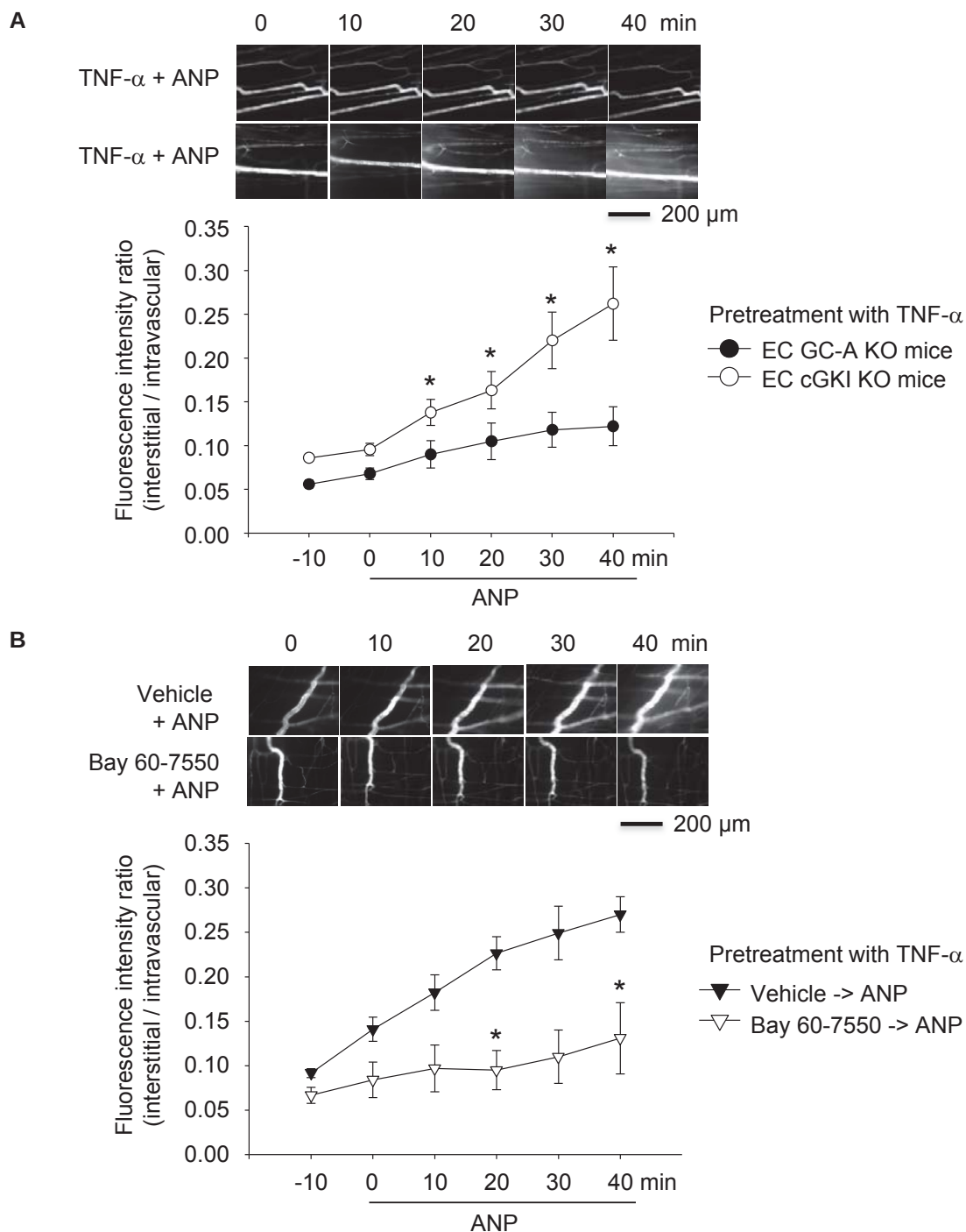
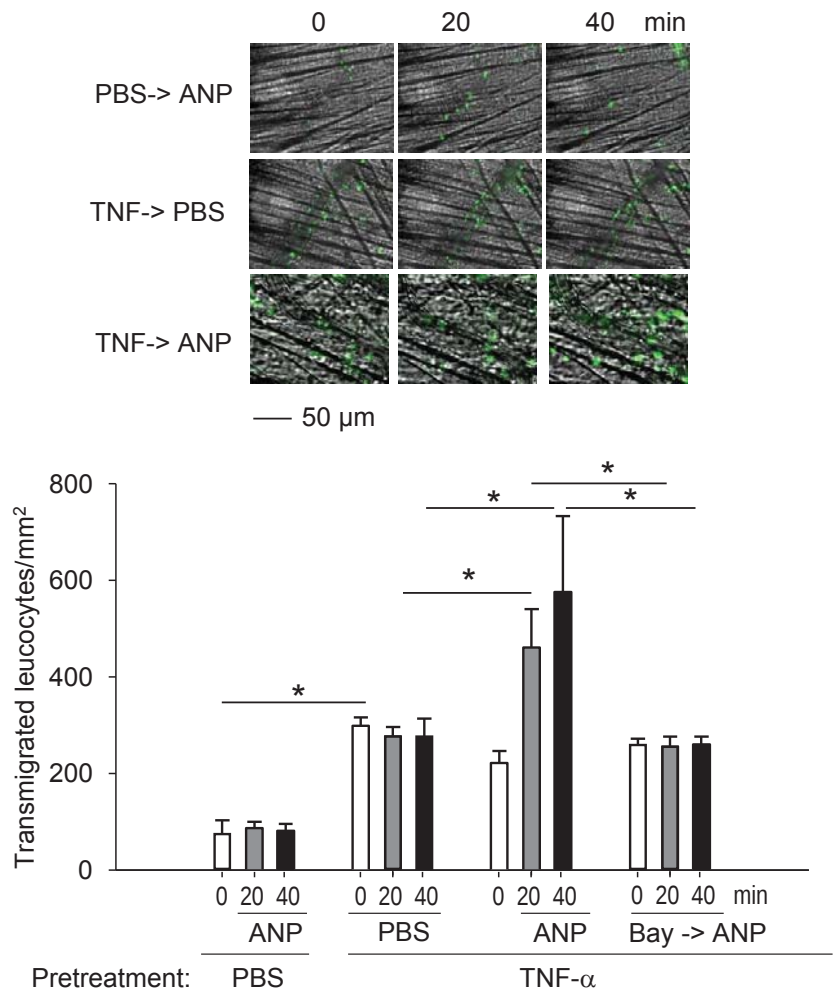


FIGURE 8



Endothelial Actions of ANP Enhance Myocardial Inflammatory Infiltration in the Early Phase After Acute Infarction

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Supplemental Material

Materials and Methods

Genetic mouse models

Mice with conditional, endothelial (EC)-restricted inactivation of either GC-A (*Tie-Cre*^{+/0};GC-A^{flox/flox}; EC GC-A KO) or cGMP-dependent protein kinase I (*Tie-Cre*^{+/0};cGKI^{flox/flox}; EC cGKI KO) and their appropriate control littermates were generated by the Cre/loxP strategy as previously described (1). LysM-eGFP^{TG} mice with green fluorescent leucocytes have also been described before (2). Male and female mice, 2 months old, were studied. Cre-Transgenic and nontransgenic littermates were compared for both floxed mouse lines within all experiments. The experiments complied with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996) and were approved by the animal care committee of the Universities of Würzburg and Düsseldorf.

Infarct model

Experimental acute myocardial infarction (AMI) was induced as described previously (3). In brief, mice were anesthetized with 2.5% isoflurane, intubated, and put on a mechanical small-animal ventilator. Adequacy of anesthesia was monitored from the disappearance of the pedal withdrawal reflex. After left-sided thoracotomy, MI was induced by ligating the proximal portion of the left coronary artery. Buprenorphine (0.05 - 0.1 mg/kg BW) was used for postoperative analgesia. In parallel subgroups, a thoracotomy was performed to expose the heart, but no suture was made to the coronary artery (sham operation) (3).

Evaluation of ischemic AAR and infarct size

Two days after AMI, the area at risk (AAR) and infarct area were evaluated (4). The thoracotomies were reopened, and 1 ml of 5% Evans blue (Sigma, Deisenhofen, Germany) was injected from the cardiac apex to delineate the nonischemic tissue. Thereafter the hearts were excised, washed with PBS, and cut into five transverse slices. The slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) solution to determine the infarct area, weighed, and photographed under a microscope (Olympus, Hamburg, Germany). Left ventricular (LV) area, AAR, and infarct were determined by computerized planimetry using a self-programmed automated-image analysis tool computing tissue volumes based on manual planimetry of digitalized images by the following formula: $[(A_1 \times W_1) + (A_2 \times W_2) + (A_3 \times W_3) + (A_4 \times W_4) + (A_5 \times W_5)]$, where *A* is the area of the slice denoted by the subscript and *W* is the weight of the respective section. Infarct area is expressed as a percentage of the AAR. Planimetry was performed independently by two observers blinded to the genotypes (4).

Immunohistochemical analysis of myocardial neutrophil infiltration

Left ventricular samples were fixed in 4% paraformaldehyde overnight and embedded in paraffin. 5- μ m sections were stained immunohistochemically for neutrophils (clone 7/4, Linaris, Wertheim, Germany, # 550274 (3)) and for myeloperoxidase (MPO) using a rabbit anti-human MPO antibody (dilution: 1:750; #A0398, Dako, Germany). Antibody binding was detected with Zytomed polymer POLHRP-100 (Zytomed Systems, Germany) and visualized with diaminobenzidine (DAB 530, Zytomed Systems, Germany). Quantitative assessment of neutrophil density was performed by counting the number of neutrophils and the MPO-positive cells in the ischemic area using the computer-assisted image analysis system Cell-D (Olympus, Hamburg Germany), with the investigator blinded to the genotypes.

Fluorescence-Activated Cell Sorting (FACS) Analysis of myocardial inflammatory infiltration

Cell suspensions from individual hearts were prepared by digestion with collagenase type 2 and protease type XIV (Sigma), as described in detail (3). Staining protocols were as described (3). The specific antibodies are depicted in Online Table I.

MPO activity assay

Myocardial MPO activity was measured as described by Pulli *et al.* (5). From each heart, the macroscopic infarct area and right ventricle (RV, as control) were dissected, washed with ice-cold PBS and then incubated on ice for 2 hours in extraction buffer (0.32 M sucrose, 1 mM CaCl₂, 10 U/ml Heparin in Hanks Balanced Salt Solution). Samples were homogenized by a mechanical homogenizer in 500 µl CTAB buffer (50 mM cetyltrimethylammonium bromide, Sigma, in 50 mM potassium phosphate buffer at pH 6.0), sonicated, and centrifuged at 15,000 g for 15 min at 4°C. Supernatants were used for measuring protein content and intracellular MPO activity. Protein content was analyzed by BCA protein assay. To specifically capture MPO, samples were incubated in MPO ELISA dilution buffer (Hycult, Plymouth Meeting, PA) on anti-MPO antibody coated 96-well plates (Hycult) for 1 hour at room temperature (duplicates of each sample). Plates were then washed 4 times with washing buffer (PBS with 0.05% Tween 20), and once with PBS only. MPO activity of antibody-captured MPO was assessed with ADHP (10-acetyl-3,7-dihydroxyphenoxazine, Sigma). PBS (49 µl), 1 µl 0.03% hydrogen peroxide (H₂O₂) and 50 µl of 200 µM ADHP solution were added to each well and MPO activity was analyzed immediately with a Wallac Victor2 microplate reader (PerkinElmer, USA) using an excitation wavelength of 535 nm and an emission wavelength of 590 nm. Enzyme activity was defined as relative fluorescent units (RFU) normalized to the sample protein concentration (5).

Detection of endothelial glycocalyx by fluorescent hyaluronic acid (HA) stainings

Fluorescent HA stainings were performed on cardiac cryosections (20 µm) as described (6). Endothelial cells of myocardial arterioles and capillaries were visualized by simultaneous CD31 stainings. Sections were embedded in ProLong Gold antifade reagent containing DAPI (Invitrogen, Karlsruhe, Germany). Imaging of the sections was performed using a Zeiss Axio Observer Z1 microscope and a 63x objective. The glycocalyx was analyzed employing ImageJ 1.42. Resulting integrated densities were normalized to the number of endothelial cells as apparent by CD31 and DAPI staining. From each heart two sections were analyzed and the glycocalyx of 3-6 vessels per section was measured. These analyses were performed independently by two persons.

Culture of human umbilical vein endothelial cells (HUVECs) and cGMP determinations

HUVECs were isolated by collagen digestion and cultured in endothelial cell growth medium (PromoCell, Heidelberg, Germany). All experiments were performed with confluent HUVECs of passages 1 or 2 in mitogen-free, serum-reduced medium (0.5% fetal calf serum during 24 h prior to experimentation). In every experiment, HUVEC isolated from 3 different cords were combined. To study the effects of ANP on cyclic GMP content, cells seeded in 24-well plates were treated with human TNF-α (10 ng/ml; R&D Systems, Wiesbaden-Nordenstadt, Germany) or vehicle (PBS) during 24 h. Thereafter the cells were stimulated with ANP (Bachem, Bubendorf, Switzerland) for 10 min (with and without previous inhibition of PDE2A with 100 nM/L Bay 60-7550 (Alexis), added 15 minutes before ANP). The incubation medium was aspirated and intracellular cGMP was extracted with ice-cold 70% (v/v) ethanol. The samples were dried and reconstituted for determination of cGMP content by radioimmunoassay (1).

Western Blots with HUVECs

Cells were washed twice with PBS and then homogenized either in RIPA buffer (Figure 4A) or a buffer containing in mmol/L: 10 HEPES, 300 succrose, 150 NaCl, 1 EGTA, 2 CaCl₂, and 1 % Triton-X (Figure 5A). Proteins were quantified using BCA Protein Assay (Pierce). Samples were boiled at

95°C for 5 minutes, and 30 µg of total protein per lane were subjected to 10 % (Figure 4A) or 4-12 % (Bis-AA gradient gels, Criterion, Bio-Rad) SDS-PAGE (Figure 5A) and to immunoblot analysis using anti-PDE2A antibody (Fabgennix, 1:750) and a monoclonal GAPDH antibody (HyTest, both overnight). The blots in Figure 4 were developed using the ECL detection system (Amersham-Pharmacia, Freiburg, Germany) and results were quantitated by densitometry (Image Quant). For Blots in Figure 5 detection was performed with ECL Prime detection reagent (Amersham) and FujiFilm Super RX films developed with automated Konica SRX 101A developer.

RT-PCR analyses

Extraction of mRNA from heart and m. cremaster and reverse-transcription (RT) were performed as described (1). Expression levels of CXCL1, ICAM-1, VCAM-1, E-selectin, P-selectin and PDE2A mRNA were analyzed by real time RT-PCR using LightCycler Technology (LC-96; Roche, Mannheim, Germany). The following primers and probes were used: for CXCL1, sense: 5'AGAAGGGTGGTGTGCGAAAA 3'; antisense: 5'ACTGACATTCTTTTCAAGACATACAAA 3'; probe 75 (REF: 04688988001); for ICAM-1, sense: 5'CGAAGCTTCTTTTGCTCTGC 3'; antisense: 5'GTCCAGCCGAGGACCATA 3'; probe 10 (REF: 04685091001); for VCAM-1, sense: 5'TGGTGAAATGGAATCTGAACC 3'; antisense: 5'CCCAGATGGTGGTTTCCTT 3'; probe 34 (REF: 04687671001); E-selectin, sense: 5'TCCTCTGGAGAGTGGAGTGC 3'; antisense: 5'GGTGGGTCAAAGCTTCACAT 3'; probe 19 (REF: 04686926001); P-selectin, sense: 5'CCGAAAGACTGGATTGTTC 3'; antisense: 5'CCTGGACACTTGATGGCTTC 3'; probe 47 (REF: 04688074001); for PDE2A: sense: 5'AAAAGGTGAATGAAGCCCAAT 3'; antisense: 5'TTGGTGTATTCATCATCAGAGACC 3'; probe 83 (REF: 04689062001). Levels of GAPDH were used for normalization (GAPDH Gene Assay; REF: 05046211001, all Roche).

FRET to monitor subsarcolemmal and cytosolic cAMP dynamics in single living HUVECs

HUVECs were seeded onto 24 mm round glass coverslips, grown to 70-80% confluency and infected with pmEpac2-camps adenovirus or Epac2-camps adenovirus at multiplicity of infection (MOI) of 30-50. 40-48 h after infection, live-cell FRET measurements were performed exactly as described (7,8). Briefly, the cells were washed with FRET buffer (in mM: 144 NaCl, 5.4 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, pH 7.3), and 400 µL of the same buffer were added to the chamber. Measurements were performed using a Nikon Ti inverted fluorescent microscope equipped with 440 nm pE-1000 Cool LED, Dual View and ORCA 03-G camera. Images were acquired using MicroManager and analyzed using ImageJ software. 24 h prior to measurements cells were incubated with human TNF-α (10 ng/ml; R&D systems) or vehicle, and thereafter the acute responses to consecutive additions of ANP, adenosine and isoprenaline were studied.

Intravital microscopy of the mouse cremaster microcirculation

As a measure of microcirculatory endothelial permeability, leakage of fluorescein isothiocyanate-labeled (FITC)-Dextran (70 kDa; Sigma) from postcapillary venules within the mouse cremaster muscle was analyzed by intravital fluorescence microscopy as described (1). Mice were deeply anesthetized by an intraperitoneal injection of ketamine (100 mg/kg BW) and xylazine (10 mg/kg). Body temperature was maintained at 37°C. The cremaster muscle was exteriorized and continuously superfused with bicarbonate-buffered saline, at a rate of 1.2 ml/min. At the end of the experiment the mice were sacrificed via intravenous ketamine injection.

After tail vein injection of FITC-Dextran, microscopy was performed using a fluorescence filter for FITC for epiillumination (Olympus). Topical application of ANP, BNP (both 100 nM/L; BNP as mouse sequence; American Peptide Co., Sunnyvale, CA), or vehicle was always started 30 min after i.v. administration of FITC-Dextran and continued for additional 40 min. Permeability responses to NPs were evaluated after intrascrotal injection of mouse TNF-α (200 ng (9); R&D Systems) or vehicle (200 µl PBS), in the absence or presence of the PDE2 inhibitor Bay 60-7550 (local superfusion of 100 nM/L, started 15 minutes before ANP). Quantitative off-line analysis of the microscopic images was

performed with the computer-assisted image analysis system Cell-D (Olympus) (1). The observer was blinded to the treatment and genotype. Changes in microvascular permeability to FITC-Dextran were measured using integrated optical intensity (IOI) as an index. Six interstitial areas, immediately adjacent to postcapillary venules, and corresponding intravascular equal areas, were randomly selected for IOI analysis. The same preselected areas were observed every 10 min, as indicated in the results section and corresponding Figures.

The experiments with LysM-eGFP mice (2) were performed with the same chemicals and experimental conditions. The number of extravascular fluorescent leucocytes was quantified in 3 fields of view ($160 \times 240 \mu\text{m}$) per animal at 3 time points: 0 (vehicle), 20 and 40 minutes (during superfusion of ANP, started at minute 1). The number of leukocytes per interstitial area (in square millimeters) was counted.

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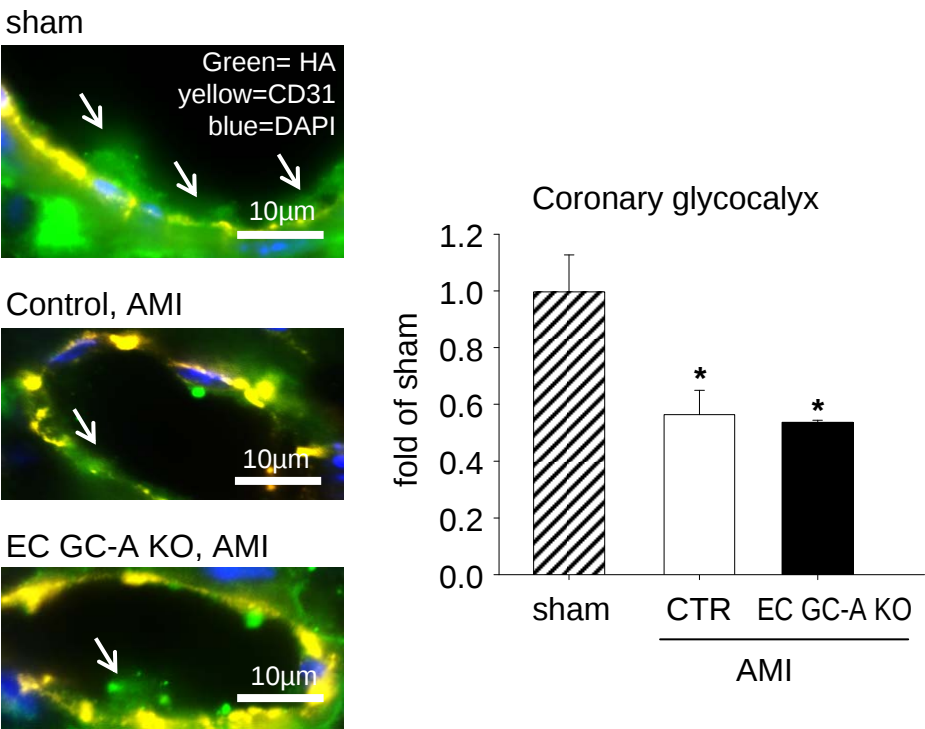
Legends for Online Figures I and II as well as for Online Table I

Online Figure I. Endothelial glycocalyx of myocardial arterioles and capillaries is reduced by experimental AMI. Control and EC GC-A KO mice were subjected to 2 days of coronary artery ligation or sham operation before analysis of the glycocalyx by fluorescent hyaluronic acid (HA) staining (green) by affinity histochemistry; arrows point toward the glycocalyx. Endothelial cells were identified by CD31 staining (orange) and nuclei were stained blue by DAPI. Representative images and quantitative analysis are presented. Fluorescent staining is expressed as integrated density per endothelial cell. (n=6-8 per group * $P < 0.05$ vs sham).

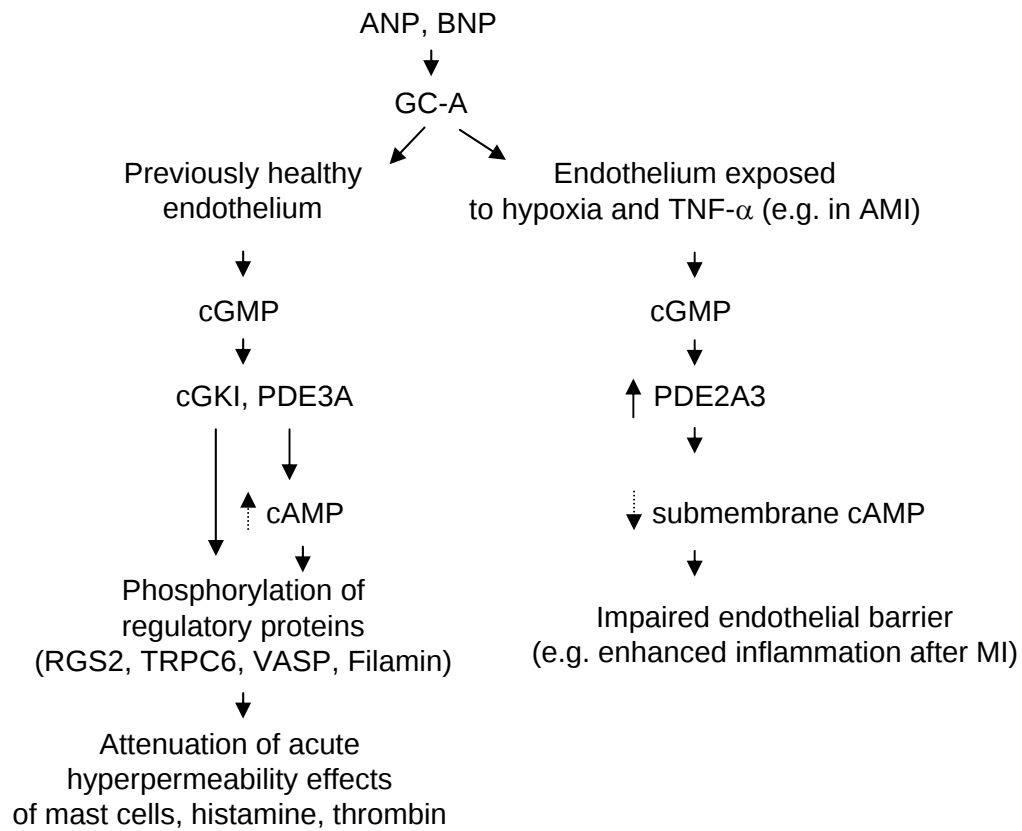
Online Figure II. Proposed model of the dual effects of ANP and BNP on endothelial barrier functions. In a previously healthy endothelium, NP/GC-A/cGMP signaling stimulates cGKI and inhibits PDE3, leading to phosphorylation of cytoskeleton- and calcium-regulating proteins (RGS2, VASP, TRPC6, filamin). This attenuates the hyperpermeability actions of mast cells and histamine (16) or thrombin (25). In AMI, hypoxia and TNF- α impair cGKI and induce PDE2A3 expression (25,28 and present study). In this situation, the NPs/GC-A/cGMP pathway can activate PDE2A3, leading to decreased submembrane cAMP levels and thereby contributing to the impairment of endothelial barrier properties.

Online Table I. Fluorescence-Activated Cell Sorting (FACS) Analyses of myocardial inflammatory infiltration in hearts enzymatically digested two days after coronary artery ligation. (A) Antibodies used for FACS Analyses. (B) Cell populations infiltrating the myocardium of control (n=6) and EC GC-A KO littermates (n=7) after AMI (in %). * $P < 0.05$ vs controls.

Online Figure I



Online Figure II



Online Table I

A. Antibodies	Specific clones	Company	Dilution
FITC rat anti-mouse CD45	Clone: 30-F11	BD Pharmingen	1:100
PE rat anti-mouse CD11b	Clone: M1/70	BD Pharmingen	1:100
Alexa Fluor 647 anti-mouse Ly6G	Clone: 1A8	BioLegend	1:250
Anti-mouse CD3 PE-Cy5	Clone: 145-2C11	BD Biosciences	1:100

B. Infiltrating inflammatory cell populations

	Control mice	EC GC-A KO mice
Neutrophils (CD45 ⁺ CD11b ⁺ Ly6G ⁺)/CD11b ⁺	52 ± 1.5 %	45.4 ± 2.4 %*
Monocytes/Macrophages (CD45 ⁺ CD11b ⁺ Ly6G ⁻)/CD11b ⁺	29 ± 2.4 %	26 ± 1.1 %
Myeloid cells (neutrophils, monocytes, macrophages)/CD45 ⁺	75 ± 3 %	69 ± 3 %
T cells (CD45 ⁺ CD3 ⁺)/CD45 ⁺	2.8 ± 0.3 %	3.7 ± 0.36 %