$VEGF_{165}$ transfection decreases postischemic NF-κB-dependent myocardial reperfusion injury in vivo: role of eNOS phosphorylation

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ABSTRACT

Endothelial nitric oxide synthase (eNOS) phosphorylation increases nitric oxide formation, for example, after VEGF stimulation. We investigated whether nitric oxide formed after overexpression of VEGF or of phosphomimetic eNOS (S1177D) affects PMN-induced myocardial detriment after ischemia and reperfusion. Pigs (n=8 per group) were subjected to percutaneous liposome-based gene transfer by retroinfusion of the anterior interventricular vein 48 h before LAD occlusion (60 min) and reperfusion (24 h). Thereafter, regional myocardial function was assessed as subendocardial segment shortening (SES), and infarct size was determined. Tissue from the infarct region, the noninfarcted area at risk, and a control region was analyzed for NF-κB activation (EMSA), tumor necrosis factor (TNF)-α, and E-selectin mRNA and infiltration of polymorphonuclear neutrophils (PMN). L-NAME was applied in one group of VEGF-transfected animals. NF-κB activition, PMN infiltration in the infarct region, and AAR were reduced after transfection of VEGF or eNOS S1177D, but not after VEGF+L-NAME coapplication. Infarct size decreased, whereas SES improved after either VEGF or eNOS S1177D transfection, an effect inhibited by L-NAME coapplication. Retroinfusion of liposomal VEGF cDNA reduces NF-κB-dependent postischemic inflammation and subsequent myocardial reperfusion injury, an effect mediated at least in part by enhanced eNOS phosphorylation.

Rapid establishment of reperfusion after coronary occlusion is the main therapeutic target of current cardiologic therapy. However, postischemic reperfusion may induce organ dysfunction, which may be detectable as arrhythmias, myocardial stunning, or microcirculatory flow disturbances such as low or no reflow. These phenomena have been experimentally correlated with postischemic inflammation, in particular recruitment and activation of PMN, a major source of reactive oxygen species released during early reperfusion (1).

Antiinflammatory therapy is a longstanding approach to the ischemic and reperfused myocardium, interfering with rapid and delayed mechanisms of leukocyte recruitment and activation. Interestingly, corticosteroids, which are known to reduce transcription of proinflammatory proteins, have recently been found to exert cardiovascular protection from reperfusion injury through a nonnuclear activation of the endothelial isoform of NO synthase (eNOS) (2). In fact, this study confirmed the observations of other reports (including eNOS null mice) illustrating the critical role of NO in protecting myocardium from postischemic polymorphonuclear leukocyte (PMN)-mediated reperfusion injury (3–7). These studies suggest that the NO-mediated antiadhesive mechanisms involved are likely to be multiple. Rapid, nontranscriptional effects have been reported as those obtained with NO donors (3, 4), eNOS agonists (5), or L-arginine administration (8). We and others have also documented transcriptional effects of NO on NF-κB activation (9–11), a hallmark of subacute endothelial activation and delayed PMN recruitment (12).

Formation of NO has recently been found to occur after activation of PI3-kinase and Akt (protein kinase B), which phosphorylates eNOS independently of a maintained increase in $[Ca^{2+}]i$ (13). Accordingly, a permanent activation of eNOS can be achieved by mutating the Akt-phosphorylated serine residue in an aspartate (S1177D; human sequence), whereas the mutation of the serine in alanine will result in a nonphosphorylatable mutant (S1177A) (13, 14). VEGF has been demonstrated to utilize the PI-3-Kinase-AKT pathway to phosphorylate eNOS at this serine residue for increased formation of NO (13), initiating early angiogenic events such as migration and sprouting of endothelial cells (15). Consistently, VEGF as well as eNOS cDNA transfer into ischemic tissue induced therapeutic angiogenesis in animal models (16) as well as in patients with peripheral artery disease (17). VEGF application also is studied in the growing number of patients with extensive coronary artery disease without other therapeutic options of revascularization (18, 19).

However, in patients with end-stage coronary artery disease, recurrent ischemia and reperfusion is likely to occur. The role of VEGF for postischemic myocardial reperfusion injury *in vivo* has not been assessed in great detail. Moreover, the impact of VEGF on postischemic inflammation, an important cause of myocardial reperfusion injury (20), has not been studied yet. With respect to leukocyte recruitment, VEGF-stimulation of cultured endothelial cells has induced a differential response: Whereas VEGF appears to selectively enhance adhesion and transmigration of monocytes (21) expressing VEGF receptors 1 and 2, PMN or lymphocytes lacking VEGF receptors display a reduced adherence on stimulated endothelium after VEGF pretreatment *in vitro* (22) and *in vivo* (23, 24).

Given the potential of VEGF to down-regulate cytokine-induced endothelial activation and subsequent PMN adhesion, we hypothesized that VEGF is capable of attenuating myocardial reperfusion injury via nitric oxide *in vivo*. To study the role of eNOS phosphorylation and nitric oxide formation as mediators of a VEGF effect, we modulated nitric oxide formation by application of the NOS inhibitor L-NAME concomittantly with VEGF or by transfection of the phosphomimetic eNOS construct eNOS S1177D. Recently, we have demonstrated selective transfection of oligonucleotide-containing liposomes into the ischemic myocardium (12) and successful reporter gene transfection (25), using retroinfusion of the coronary vein for gene delivery. With this novel approach, we transfected liposomes containing $VEGF_{165}$ or eNOS S1177D into the area at risk (AAR) of the individual hearts.

MATERIALS AND METHODS

All chemicals used in this study were purchased by Sigma (Taufkirchen, Germany) if not stated otherwise. Oligonucleotides (ODNs) containing the NF-κB consensus binding site (5' AGT TGA GGG GAC TTT CCC AGG C 3' and 5' GCC TGG GAA AGT CCC CTC AAC T 3'), or a nonbinding sequence (5' TTG CCG TAC CTG ACT TAG CC 3' and 5' GGC TAA GTC AGG TAC GGC AA 3') were manufactured by MWG (Ebersberg, Germany). The polyclonal inducible isoform (iNOS) antibody was from Dianova (Hamburg, Germany).

Cell culture experiments

Neonatal rat coronary endothelial cells were cultured as described previously (26). Subconfluent cells, plated on 12-well plates, were transfected with Effectene (Qiagen) containing a construct of firefly luciferase ligated to an NF-κB-sensitive promotor of the human ICAM-1 gene (p277-ICAM-Luc) (27), a control construct of renilla luciferase ligated to an NF-κB-insensitive tyrosin-kinase promotor (pβ-actin-renilla) and the interventional gene of interest, for example, pCMV-$VEGF_{165}$, pCMV-eNOS-S1177D, or pCMV-eNOS-S1177A (14). Twenty-four hours later, cells were stimulated with TNF-α (100 ng/ml) or PMN (250.000/well). NF-κB decoy ODN (2 μg/well) containing liposomes was added at stimulation. After stimulation with TNF-α, cells were incubated for 24 h before they were harvested and pellets were analyzed for luciferase activity after substrate supplementation according to the manufacturer's instructions (Promega, Mannheim, Germany). Results are given as firefly luciferase activity divided by renilla luciferase activity (%).

For PMN adhesion assays, subconfluent cells were transfected with the gene of interest ($VEGF_{165}$, eNOS S1177D, eNOS S1177A). After 24 h, PMN (250.000/well) were added to the medium and allowed to attach for 1 h. Thereafter, nonadherent cells were removed by gentle washing (3× with phosphate-buffered saline [PBS]), and adherent cells were pelleted for MPO assay.

Animal instrumentation

All experimental procedures were approved by the Bavarian Animal Care and Use Committee (AZ 211–2531–07/01). All pig experiments were conducted at the Institute for Surgical Research of the University of Munich. German farm pigs were anesthesized and instrumented as described previously (12, 28). The external jugular vein and the carotid artery of the right side were canulated, and appropriate sheaths (8F) were placed.

Hemodynamic measurements

Before induction of ischemia and at the end of the experiment (24 h after ischemia), hemodynamic measurements were conducted. A Millar pressure tip catheter was placed in the left ventricle, providing parameters of global ventricular function such as left ventricular enddiastolic pressure (LVEDP); dP/dt_{max} as a parameter of systolic contractile function, which under pacing conditions is closely correlated with the contractile reserve of a heart; and dP/dt_{min} reflecting the diastolic relaxation, a decrease of which indicates diastolic dysfunction. All values

were acquired in repeated heart cycles, and for an individual heart, the value was expressed as the mean of five cardiac cycles/time point.

Retroinfusion

As described in detail previously (29), the retroinfusion catheter was advanced into the anterior interventricular vein (AIV), draining the parenchyma perfused by the LAD. After assessment of the individual systolic occlusion pressure of the venous system, retroinfusion pressure was set 20 mmHg above the latter. Continuous pressure regulated retroinfusion of NaCl 0.9% diluted liposomes containing either GFP (control group), VEGF, or eNOS S1177 D (1 mg cDNA each) was conducted for 2×10min at day 0 (during LAD occlusion). Then, venous and arterial sheaths were withdrawn, the vessels were ligated, and the muscle and skin were sutured. Animals were brought back to the animal facility where they were fed with water and nutrition ad libitum.

Ischemia and reperfusion

Ischemia and reperfusion of these animals were conducted at day 2, allowing for expression of the transfected gene product and avoiding effects of preconditioning. A balloon was placed in the LAD distal to the bifurcation of the first diagonal branch and was inflated. Correct localization of the coronary occlusion and patency of the first diagonal branch was ensured by injection of contrast agent under fluoroscopy. Ischemia was maintained for 60 min, whereafter the balloon in the LAD was deflated, initiating reperfusion of the LAD perfusion area.

Only retroinfusion of NF-κB decoy was performed at the day of myocardial ischemia, where indicated (for 5 min before reopening of the LAD) (12). Moreover, retroinfusion was used to apply L-NAME into the ischemic area over 15 min (500 ml, 500 μmol/l L-NAME) at the onset of reperfusion, inhibiting early nitric oxide synthase activity in the postischemic area. In addition, L-NAME-treated animals received water containing 500 μmol/l of the NOS inhibitor.

Regional and global myocardial function

After 24 h of reperfusion, animals were brought back to the OR, anesthetized, and instrumented. After hemodynamic measurements, sternotomy was performed and the pericardium was removed. For analysis of regional mycoardial function, subendocardial segment shortening (SES) was used. Therefore, sonomicrometry crystal pairs were placed 1 cm (AAR) and 3 cm distal to the balloon occlusion site into the anterior left ventricular wall. Each pair was inserted with its axis perpendicular to the LAD. A pair of control crystals was inserted in the Cx perfusion area. The distance between each crystal pair was followed continuously and evaluated for five heart cycles at each heart rate. Results of the infarcted area and the AAR were normalized to the subendocardial segmental shortening of the control region (Cx perfusion area), given in percent. Sonomicrometry measurements were performed under control conditions as well as with atrial pacing (100, 120, and 140/min for 1 min each) as previously described (29).

Infarct size determination

Before arrest of the heart, the LAD was reoccluded surgically and 20 ml of methylen blue was injected in the left ventricle for negative staining of the AAR. After heart excision, the infarct size was determined by pressure-controlled injection (80–100 mmHg) of 15 ml of 10%

tetrazolium red into the LAD distal to the occlusion. Thereafter, the left ventricle was cut into slices 5 mm thick, which were subjected to digital photography. Image processing of the digital pictures with trace measurements (SigmaScan) provided volume of infarct region, AAR, and total LV area for each slice.

EMSA, myeloperoxidase assay, immunoblot, and immunohistochemistry

The postischemic activation of NF-κB was assessed by electromobility shift assays (30), as described previously. Immunoblotting and immunoprecipitations were performed as described previously (31). Myeloperoxidase assay (32) was used to assess recruitment of leukocytes, preferentially PMN. Because macrophages are also capable of expressing myeloperoxidase, we refer to the results of this assay as inflammatory infiltrate. Immunohistochemistry was performed for detection of iNOS in frozen sections of the myocardium indicated, using iNOS antibody and a suitable secondary antibody labeled with horseradish peroxidase.

Statistical methods

The results are given as mean ±SE. Statistical analysis of results between experimental groups was performed with one-way ANOVA. Whenever a significant effect was obtained with ANOVA, we performed multiple comparison tests between the groups using Student-Newman-Keul's procedure. Only the comparison of pre- and postischemic LVEDP (Fig. 7) was performed with *t* test. All procedures were performed with an SPSS statistical program. Differences between groups were considered significant for $P<0.05$.

RESULTS

Effect of VEGF and eNOS S1177D transfection on NF-κB activation *in vitro*

As depicted in Figure 1, rat coronary endothelial cells were transfected 24 h before stimulation with an NF-κB-sensitive reportergene (p277ICAM-Luc) and a cDNA encoding $VEGF_{165}$, a growth factor known to phosphorylate eNOS at serine residue 1177. $VEGF_{165}$ transfection attenuated NF-κB activation after TNF-α stimulation (24 h) to a similar extent as specific inhibition of the transcription factor with a decoy oligonucleotide (NF-κB decoy ODN) or endothelial NOS S1177D transfection (Fig. 1B). The latter reduced TNF-α-dependent NF-κB activation to a larger extent than transfection of eNOS S1177A, a nonphosphorylable mutant of the wild-type enzyme ($P<0.05$, when tested separately from all other groups). Similarly, NF-κB activation upon exposure of purified PMN was blunted by $VEGF_{165}$ or eNOS S1177D transfection, but not eNOS S1177A transfection (Fig. 1C). Thus, *in vitro*, VEGF-activated or phosphomimetic eNOS is capable of silencing NF-κB activation upon exposure to TNF-α or adherent PMN, two well-characterized inflammatory stimuli occurring during postischemic reperfusion of the heart.

Concomittantly, the amount of adherent PMN on the endothelial cell layer decreased after VEGF or eNOS1177D transfection, an effect not detected after eNOS S1177A transfection (Fig. 1D). This effect occurred after 1 h of addition of PMN to the medium, relying on an acute, nontranscriptional effect. Thus, *in vitro* VEGF and eNOS S1177D decrease both acute

adhesiveness of coronary endothelium as well as transcriptional activation after inflammatory stimulation.

Venular transfection of $VEGF_{165}$ and eNOS S1177D by selective pressure-regulated retroinfusion

To test the hypothesis that eNOS phosphorylation is a crucial signaling event inhibiting postischemic myocardial inflammation *in vivo*, we transfected pigs with cDNA encoding $VEGF_{165}$ or eNOS S1177D via selective pressure-regulated retroinfusion 48 h before ischemia. With this approach, regional reportergene transfection in the AAR has been shown to be significiantly higher than using intracoronary application (25). To ensure comparability, control hearts received liposomes containing GFP. As depicted in Figure 2A, GFP expression was found in small vessels in the AAR. Further assessment of expression of the functional genes used in the study was pursued: for eNOS S1177D, the attached myc-tag was detected by immunoblotting in the ischemic area (Fig. 2B). To assess the absolute amounts of eNOS S1177D proteins expressed, we also performed immunoprecipitation, using c-myc antibody. Immunoblotting with eNOS thereafter revealed a 2- to 2.5-fold higher level of transgene expression in the AAR and infarct region (Fig. 2C) than in the control region. Moreover, after VEGF transfection, which increased VEGF protein expression, enhanced eNOS phosphorylation was found (Fig. 2D).

Postischemic NF-κB activation is reduced by VEGF and eNOS S1177D

A distinct activation of NF-κB, an essential element of postischemic inflammation, has been detected in tissue from the ischemic area in mock-transfected hearts (Fig. 3A). This NF-κB activation was substantially reduced in eNOS S1177D or VEGF-transfected hearts, except for L-NAME treatment during reperfusion (Fig. 3B, 3C). The expression of iNOS, in part, a NF-κB-regulated gene, in the vessel wall of AAR and infarcted was blunted after transfection of VEGF, except for the group concomittantly treated with L-NAME (Fig. 3D, 3E).

Postischemic inflammatory infiltrate after VEGF or eNOS S1177D transfection

Figure 4 demonstrates inflammatory cell influx into the ischemic area. The increase of leukocytes in the infarcted area was substantially higher than that in the noninfarcted AAR. The infarcted area and the AAR both displayed a reduction of leukocyte influx after VEGF or eNOS 1177D transfection, an effect abolished in the VEGF+L-NAME group. Interestingly, inhibition of NF-κB activation by NF-κB decoy ODN transfection exerted a similar decrease of inflammatory cell recruitment in the infarcted area, whereas in the AAR NF-κB decoy ODN, unlike VEGF or eNOS S1177D, did not significantly reduce leukocyte recruitment as compared with the mock-transfected group.

Effect of VEGF or eNOS S1177D transfection on infarct size

We next studied whether the inhibition of NF-κB-dependent inflammation affects myocardial infarct size. As depicted in Figure 5, 60 min of LAD occlusion resulted in an infarct area of 67±6% of the AAR at 24 h of reperfusion. Prior transfection with either VEGF or eNOS S1177D significantly decreased infarct size (41±4 and 39±5%, respectively), similar to the reduction

achieved by NF-κB inhibition with decoy ODN (38±5%). Additional treatment with L-NAME abolished the reduction of infarct size achieved by VEGF transfection.

Effect of VEGF or eNOS S1177D transfection on myocardial function

For the assessment of regional myocardial function in the ischemic area, SES was measured in the infarct region, the AAR, and normalized to the values of the control area. As depicted in Figure 6A, SES of the infarcted area in mock-transfected animals was compromised to 18±6% under resting conditions and further declined with increased pacing rates. However, preischemic transfection with VEGF or eNOS S1177D resulted in a significant higher preservation of SES under resting conditions and higher pacing rates (≥120/min), unless L-NAME was coadministered in the VEGF-treated group. In contrast, in the noninfarcted AAR, regional myocardial function under resting conditions was similar in control and transfected animals. In this region, a significant lack of contractile reserve was unmasked only after L-NAME application in VEGF-treated animals.

LVEDP, a parameter of global myocardial function, was found increased in mock-treated hearts after 24 h of reperfusion (Fig. 7A). This increase was absent in both VEGF and eNOS S1177D treatment. Coapplication of L-NAME in the VEGF-transfected hearts abolished the effect of VEGF.

Systolic contractility, assessed as dP/dt_{max}, tended to improve after VEGF transfection, in particular under pacing conditions (P=0.058), and in eNOS-transfected hearts (P=0.069), without reaching statistical significance. Diastolic relaxation, as determined by dP/dt_{min}, was improved after VEGF and eNOS S1177D transfection under pacing conditions, but not after VEGF+L-NAME application.

DISCUSSION

Defining the role of VEGF in the setting of subacute myocardial ischemia and reperfusion presents certain obstacles, because continuous VEGF protein application into the ischemic myocardium is difficult to conduct and poses the problem of severe hypotension (33). In the present study, we used a pig model of retrograde gene transfection into the LAD perfusion area 48 h before ischemia (1 h) and reperfusion (24 h) to investigate the nonangiogenic influence of VEGF *in vivo*. Using a novel mode of percutaneous transluminal retrograde delivery of liposomes carrying functional genes (34), we found an increased expression of VEGF (concomittantly with enhanced phosphorylation of native eNOS) or eNOS S1177D in the ischemic area. Both $VEGF_{165}$ and eNOS S1177D transfection reduced postischemic NF-κB activation and subsequent inflammatory infiltrate in the infarct region and the noninfarcted AAR, except for concomittant inhibition of NO formation by L-NAME in the VEGF-transfected hearts. These experiments indicate for the first time that VEGF-induced eNOS phosphorylation is a relevant event in down-regulation of postischemic inflammation and its contribution to PMN-dependent myocardial reperfusion injury.

The ability of nitric oxide to rapidly reduce leukocyte adhesion at reperfused coronary endothelium cells has been observed before (3–7). Consistently, our study demonstrates that transfection of VEGF or a constitutively active eNOS mutant *in vivo* decreases PMN recruitment

and subsequent myocardial damage. Implicitly, the rate of NO release after VEGF or eNOS S1177D transfection did not surpass the treshold of myocyte toxicity, caused in particular by peroxynitrite, which forms after reaction of NO with a superoxide radical (35, 36). Of note, peroxynitrite infusion at concentrations of 1–30 μmol/l caused myocyte damage in saline-perfused isolated hearts but was cardioprotective when hearts were perfused with blood (36). However, eNOS null hearts displayed reduced reperfusion injury in a saline perfusion model while revealing increased reperfusion injury *in vivo* (7). Thus, *in vivo*, the treshold of NO/peroxynitrite toxicity appears later than in saline-perfused models, enabling moderate liposomal overexpression of eNOS (Fig. 2C and ref 37) to exert cardioprotection .

Beyond its ability to acutely prevent neutrophil adhesion *ex vivo* and *in vivo*, nitric oxide interferes with the subacute window of neutrophil recruitment, regulated by transcriptional activation. Endothelial cell culture experiments have demonstrated a decreased expression of proinflammatory proteins such as the chemokine MCP-1 (9) and the adhesion molecules ICAM-1 (10) concomittantly with a decrease of NF-κB activation after nitric oxide donor application. In our study, eNOS S1177D transfection reduced induction of an NF-κB-dependent transcription *in vitro* (Fig. 1B) and *in vivo* (Fig. 3), attenuating postischemic inflammatory infiltrate (Fig. 4) and myocardial detriment (Fig. 5–7). The mechanism of NO antagonizing NF-κB activation has not been elucidated. Apparently, NO interferes with the redox sensitivity of the NF-κB system (38), as suggested by a concentration-dependent effect: Whereas small to moderate amounts of a potent NO donor (e.g., 10^{-9}–10^{-8} M SNAP) silence postischemic NF-κB activation, increasing amounts (10^{-7}–10^{-6} M SNAP) enhance NF-κB activation in isolated hearts (data not shown and ref 39), the latter potentially through peroxynitrite (40).

Moreover, reactive oxygen species such as hypochlorous acid and hydroxyl anion released from adherent neutrophils can contribute to NF-κB activation (Fig. 1 and ref 41). Thus, a positive feedback loop formed by initial endothelial activation, rapid neutrophil recruitment inducing further inflammatory protein expression via NF-κB appears to form during early reperfusion. Transfection of eNOS S1177D concomittantly targets two crucial elements of this feedback loop, leukocyte adhesion and leukocyte-dependent NF-κB activation. Transfection of microcirculatory venular endothelium by retroinfusion fittingly directed eNOS S1177D to the preferred site of either of both processes (5, 34).Of note, NF-κB activation after myocardial ischemia does not entirely rely on adherent PMN, because this process can also be detected in saline-perfused isolated hearts (30). Moreover, the fact that NF-κB decoy ODN transfection without enhanced nitric oxide formation reduced infarct size similarly to eNOS S1177D (Fig. 4A) indicates the relevance of transcriptional activation for the evolving myocardial reperfusion injury.

In addition, VEGF overexpression was able to reduce both postischemic inflammation and myocardial detriment. Of note, VEGF is up-regulated in the early course of reperfusion, even after limited ischemia (42), mainly released by myocytes, smooth muscle cells, and macrophages (43, 44). Under physiological circumstances, venular endothelium does not appear to be a major source of VEGF. Nevertheless, studies linking PMN adhesion and NF-κB activation to this vessel region render it a particularly attractive side for therapeutic and transient VEGF transfection for the purpose of NF-κB silencing (34, 45). Importantly, NF-κB-derived gene induction does not confound rapid PMN adhesion based on posttranslational changes of the endothelial phenotype, for example, P-selectin translocation (46). Notwithstanding, postischemic

inflammation is reduced by NF-κB antagonism by using, for example, decoy oligonucleotides (12) or VEGF transfection (Fig. 4–7).

Does VEGF transfection utilize the eNOS activation pathway to exert postischemic cardioprotection? The inhibitory effect of L-NAME indicates an involvement of nitric oxide synthases, which, however, could also be generated by the iNOS, an enzyme found to play a protective role after murine mycoardial ischemia (47). As depicted in Figure 3E, the percentage of iNOS-containing vessels in the postischemic regions was less elevated in VEGF- than in mock-transfected and VEGF+L-NAME-treated hearts at 24 h of reperfusion. This finding may reflect the fact that iNOS expression after myocardial ischemia is in part regulated by NF-κB (48) and down-regulated by eNOS-phosphorylating agents, for example, simvastatin (49).

In an earlier study, Griffioen et al. demonstrated a down-regulation of cytokine-induced leukocyte adhesion in the cremaster muscular microcirculation after VEGF pretreatment *in vivo* (23). In contrast, in brain microvascular endothelial cells (50) and in HUVECs (51), up-regulation of NF-κB and NF-κB-regulated genes such as ICAM-1 or IL-8, increasing PMN adhesion, has also been described after VEGF exposure. Besides the origin of the endothelial cells used in these studies, varying biological activities of transfected VEGF cDNA (our study) vs. VEGF protein application or the difference of VEGF affecting stimulated endothelial cells (our study) vs. unstimulated endothelial cells do not ensure sufficient comparability of the *in vitro* models. Importantly, the antagonistic effect of L-NAME after VEGF transfection in our study indicates the critical role of enhanced NO formation on transcriptional down-regulation of proinflammatory proteins (Fig. 1A, Fig. 3C, 3D), as previously described (9–11).

In summary, we have demonstrated for the first time that liposomal VEGF transfection via retroinfusion protects the postischemically reperfused heart by reducing NF-κB activation and subsequent PMN-dependent loss of regional myocardial function. Nitric oxide, generated by VEGF through eNOS S1177 phosphorylation, is an essential mediator of this cardioprotective effect of VEGF. These findings extend the therapeutic relevance of VEGF beyond treatment of chronic ischemia toward cardioprotection after acute ischemic events. It remains to be studied whether other growth factors, including insulin (52), insulin-like growth factor (53), and TGF-β (54), which is known to stimulate the eNOS-phosphorylation pathway, exert at least a part of their cardioprotective effect in the setting of ischemia and reperfusion via silencing of NF-κB. However, note that overexpression of VEGF in the coronary venular endothelium is an intervention with potential side effects, if released systemically, that has to be further characterized concerning the dose and time dependency of its antiinflammatory properties.

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Fig. 1

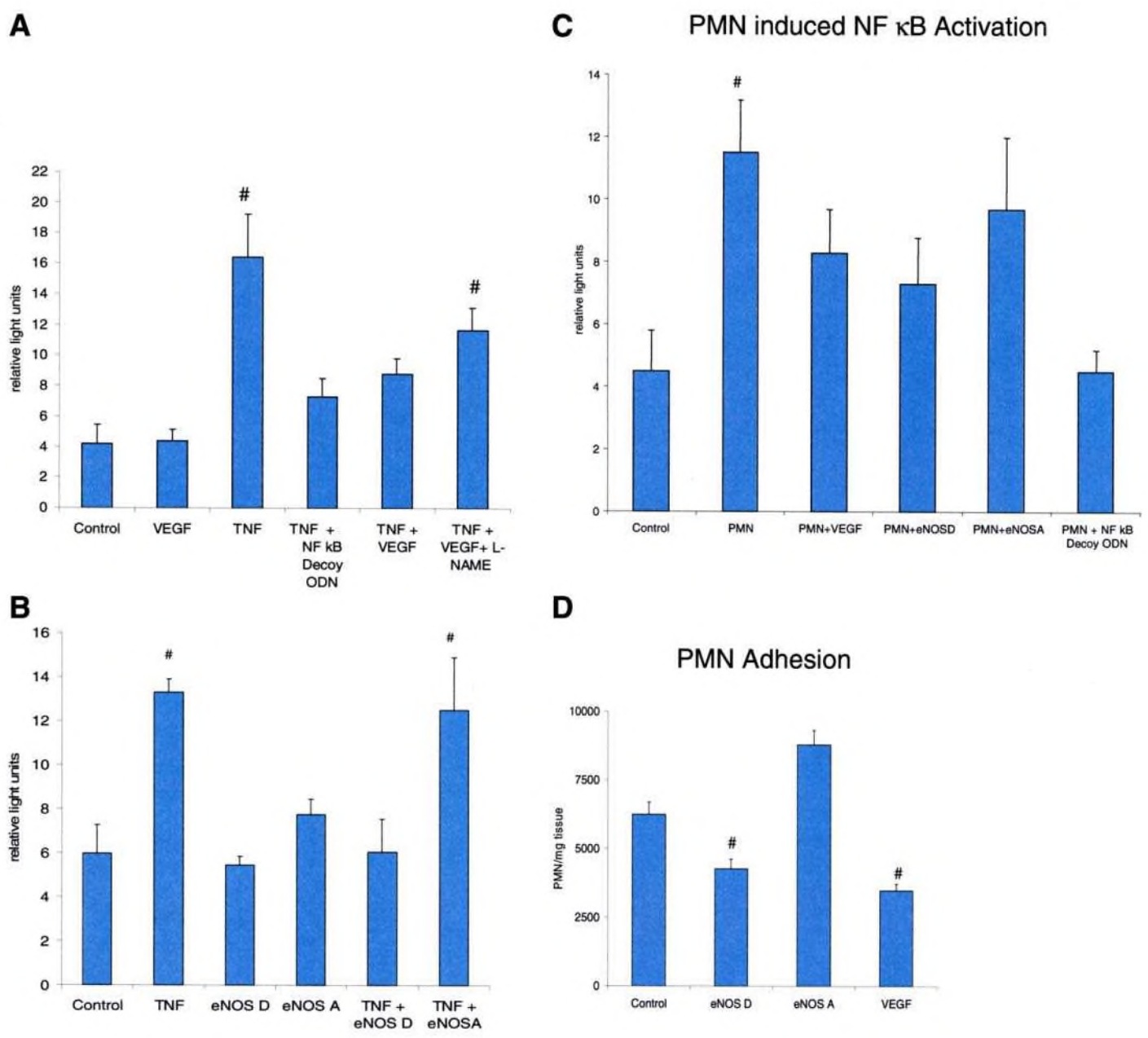

Figure 1. A) Rat coronary endothelial cells were transfected with a truncated NFκB-sensitive ICAM-1/promoter/luciferase/cDNA construct and a β-actin/promoter/renilla/luciferase construct and, where indicated, pCMV-VEGF. Twenty-four hours later, cells were stimulated with tumor necrosis factor (TNF)-α for 24 h and then analyzed for luciferase activity (percentage of renilla activity). NFκB decoy ODN was applied at the onset of stimulation (TNF-α). Compared with unstimulated cells, TNF-α increased the p277ICAM-Luc expression substantially, an effect largely attenuated by NFκB decoy ODN or VEGF transfection (n=4 independent experiments, $^{\#}P$<0.05 vs. control and VEGF-group). **B)** Similar inhibition of the TNF-α-induced induction of the NFκB-dependent pICAM277-Luc, when a pCMV-eNOS S1177D construct was coapplied, but not when a pCMV-eNOS S1177A was transfected before TNF-α stimulation (n=4, $^{\#}P$<0.05 vs. all other groups). **C)** After PMN stimulation (250.000/well, 24 h), an increased expression of pICAM277-Luc was detected, which was attenuated by prior VEGF- or eNOS-S1177D liposomal transfection, but not by eNOS S1177A transfection ($^{\#}P$<0.05 vs. control and PMN+NFκB decoy ODN group). **D)** VEGF and eNOS S1177 D transfection reduce PMN adhesion after 1 h of coincubation (n=4 independent experiments. $^{\#}P$<0.05 vs. unstimulated controls and eNOS A).

Fig. 2

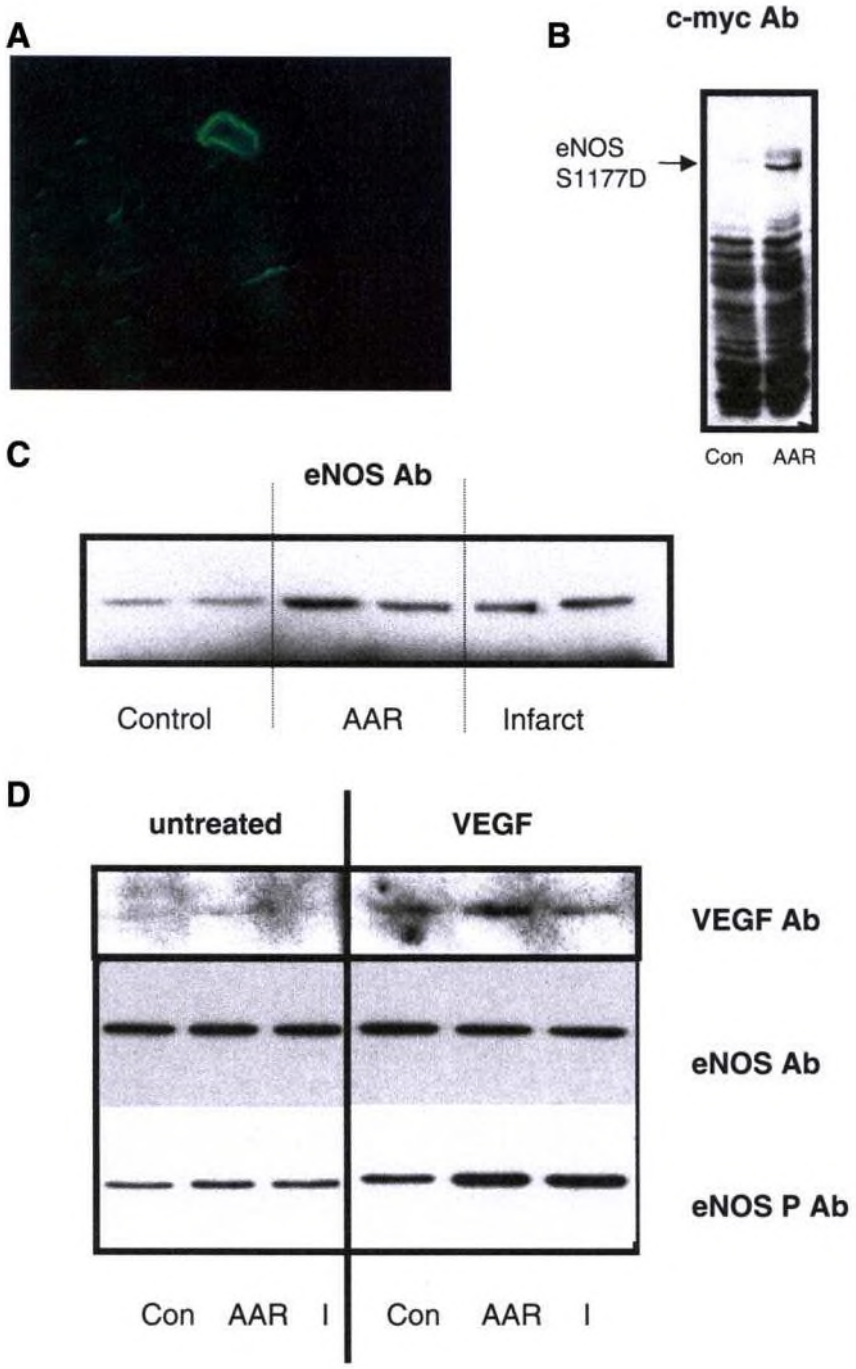

Figure 2. Detection of transfected cDNA in the indicated myocardial area 24 h after reperfusion. A) Liposomal transfection of a pCMV-eGFP construct revealed transfection of small vessels (15 μm diameter) and larger vessels (50–200 μm diameter). **B)** Detection of myc-tagged eNOS S1177D by immunoblotting with a c-myc Ab was successful in the ischemic area (right lane) but not in the control region of the same heart. **C)** eNOS S1177D transfection led to 2- to 2.5-fold higher levels of recombinant eNOS expression in the ischemic area than in the control area of the same heart, as depicted by eNOS immunoblotting after c-myc immunoprecipitation. **D)** In VEGF-transfected hearts, eNOS immunoblotting did not reveal expression differences between the untreated and transfected area, whereas an antibody detecting the phosphorylation of eNOS at S1177 revealed an increase in phosphorylated eNOS in the VEGF-transfected myocardium.

Fig. 3

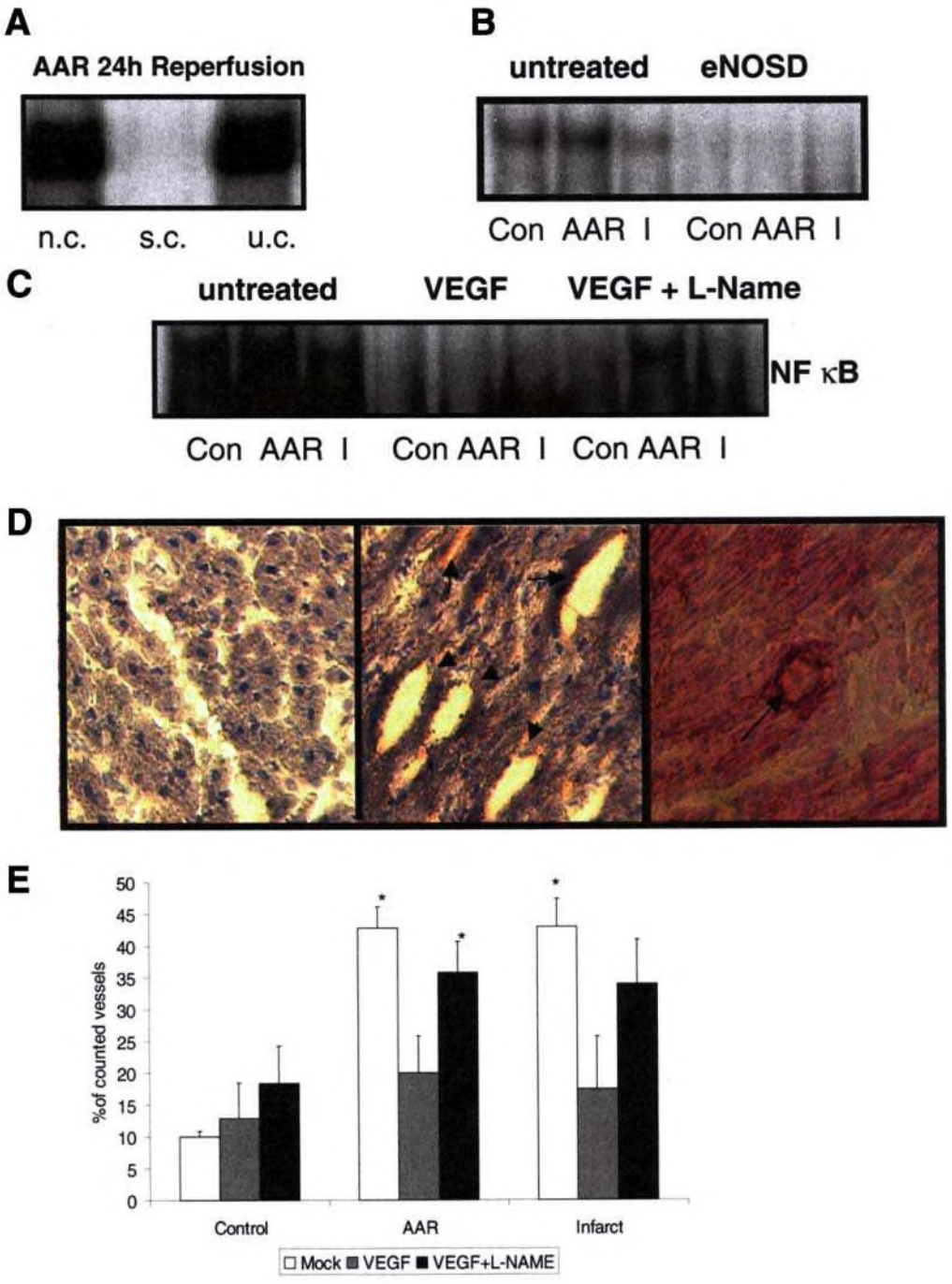

Figure 3. A) EMSA (See Materials and Methods) detected NFκB activation after 24 h of reperfusion in the area at risk (left lane), with a specific inhibition by in vitro addition of NFκB decoy ODN (s.c.) but not an unspecific ODN (u.c.). **B)** Compared with mock-transfected hearts, eNOS S1177D transfection blunted NFκB activation at 24 h of reperfusion. **C)** Similarly, VEGF-transfected hearts displayed a reduced NFκB activation in EMSA, with coapplication of L-NAME abolishing the effect of VEGF transfection. **D)** Immunohistochemical detection of iNOS in the vessel wall of infarcted myocardium (middle panel, hemalaun stain, 100×) and in greater magnification (Kernechtrot, 400×) of the same heart (right panel). Control staining with secondary antibody (left panel, 100×). **E)** Analysis of vessels staining positive for iNOS in the control area, AAR, and infarct (n=3, P<0.05 vs. the control area of the respective group).

Fig. 4

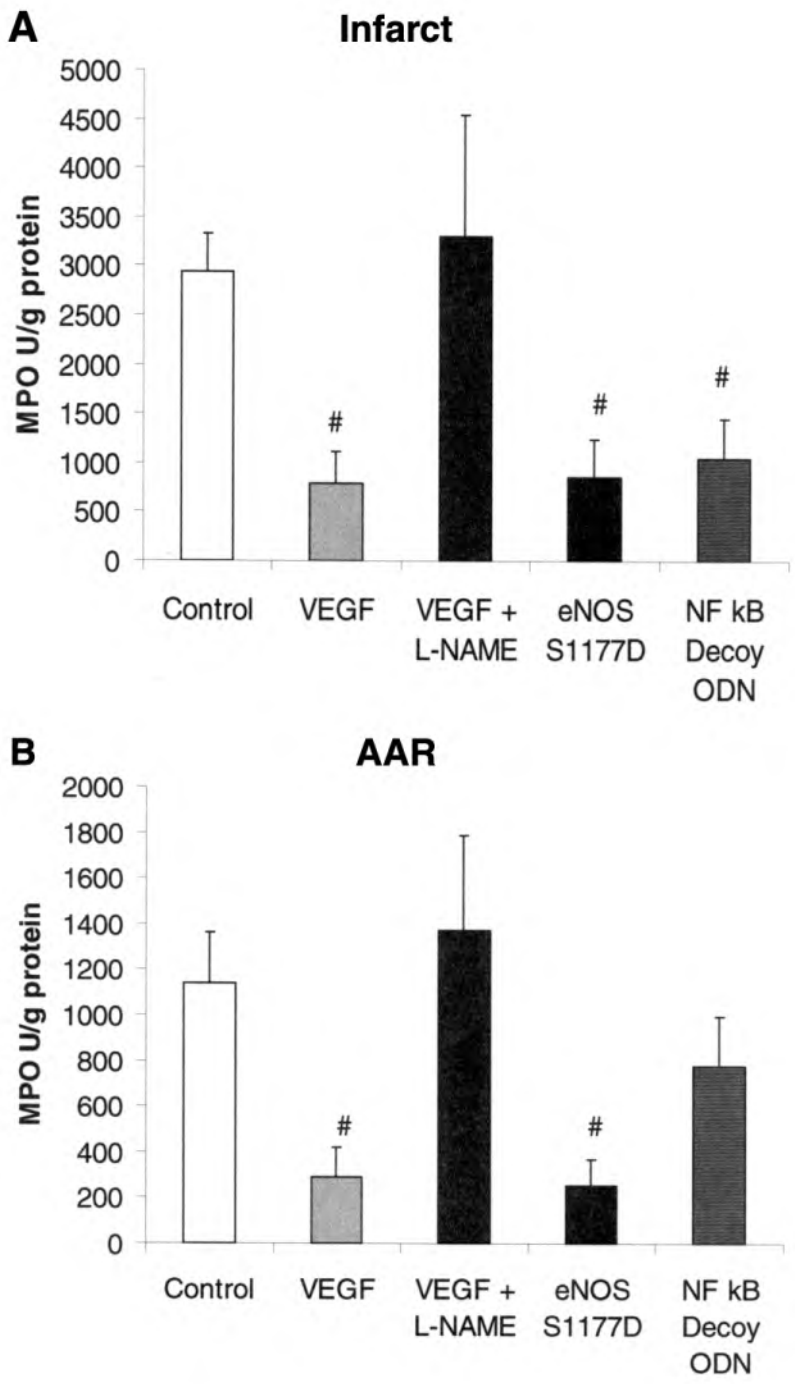

Figure 4. A) Inflammatory cell influx into the infarcted area of mock-transfected hearts (left column) was increased compared with VEGF- and eNOS-transfected hearts as well as NFκB decoy ODN-treated experiments. **B)** In the area at risk, VEGF and eNOS transfection also decreased PMN influx, compared with controls, VEGF+L-NAME and NFκB decoy ODN-treated heart. (n=8 per group, $^{\#}P$<0.05 vs. control group and VEGF+L-NAME group).

Fig. 5

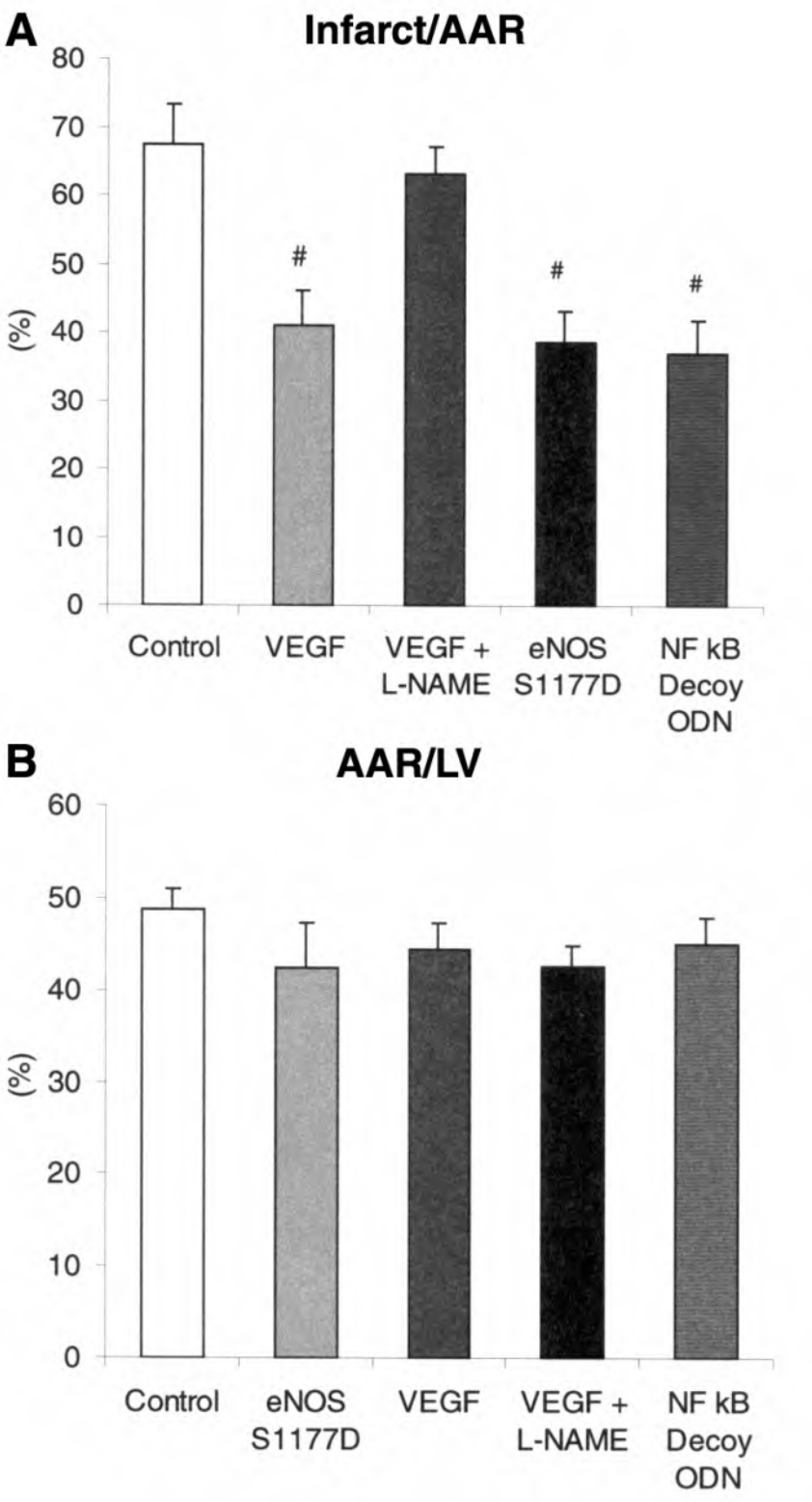

Figure 5. A) Infarct size was determined to be normalized to the area at risk AAR and is given in percent. Mock-transfected hearts displayed a larger relative infarct size than VEGF- or eNOS S1177D-treated hearts, similarly to VEGF+L-NAME-treated hearts. **B)** Area at risk normalized to the total LV revealed no difference in infarct size in all groups (n=8 per group, $^{\#}P<0.05$ vs. control group).

Fig. 6

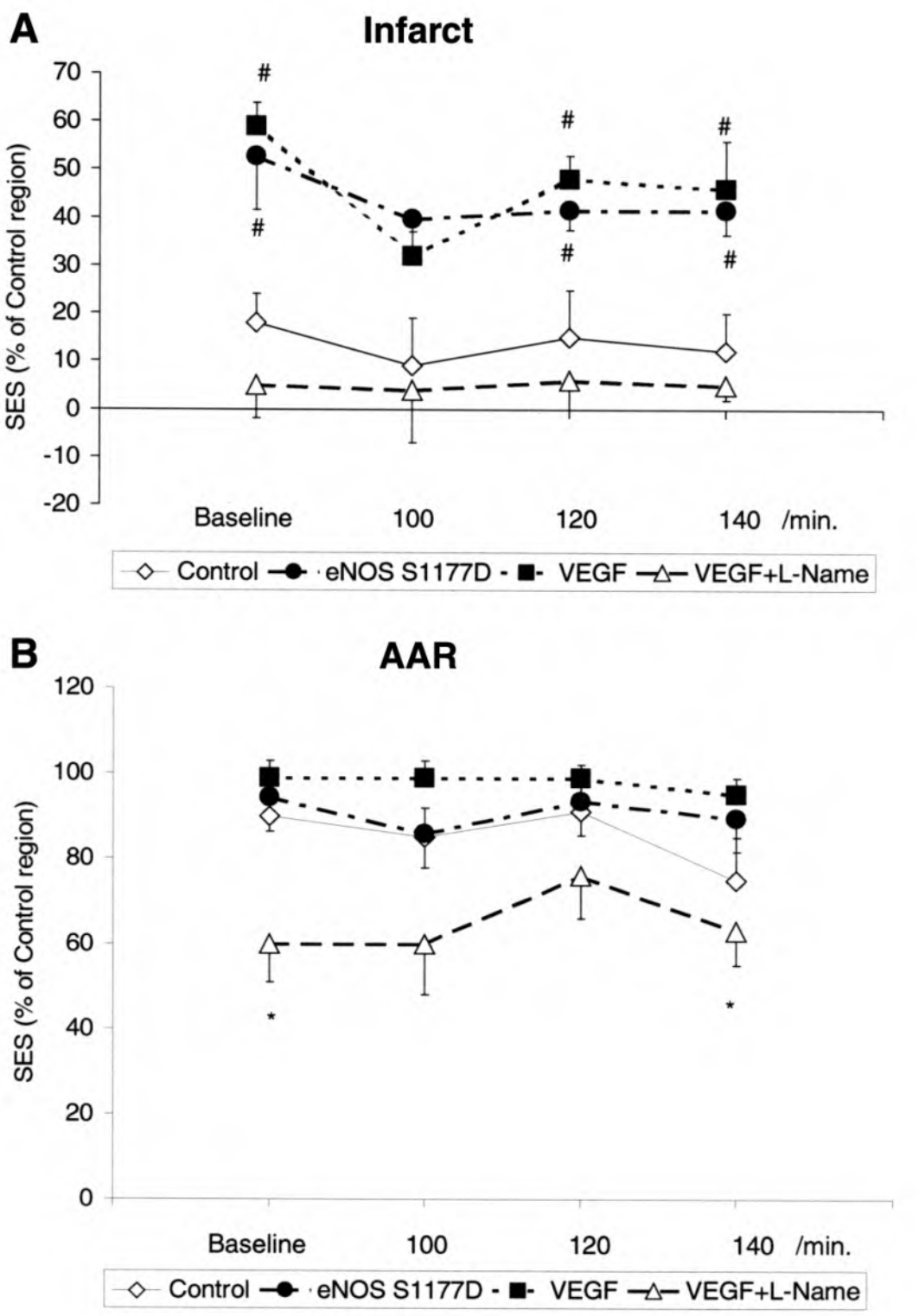

Figure 6. A) Subendocardial segment shortening (SES) of sonomicrometry crystals in the infarcted area was found to be below one-fifth of the RCx perfusion area at rest in the mock-transfected or VEGF+L-NAME-treated hearts, whereas VEGF- and eNOS-treated hearts displayed improved SES in the infarcted region. **B)** SES in the area at risk of mock-, VEGF-, or eNOS S1177D-transfected hearts did not reveal differences. However, in hearts that were subjected to VEGF transfection and L-NAME, a decrease in SES was present at native heart rate and pacing (140/minute) (n=8, $^{\#}P$<0.05 vs. control, $^{*}P$<0.05 vs. VEGF).

Fig. 7

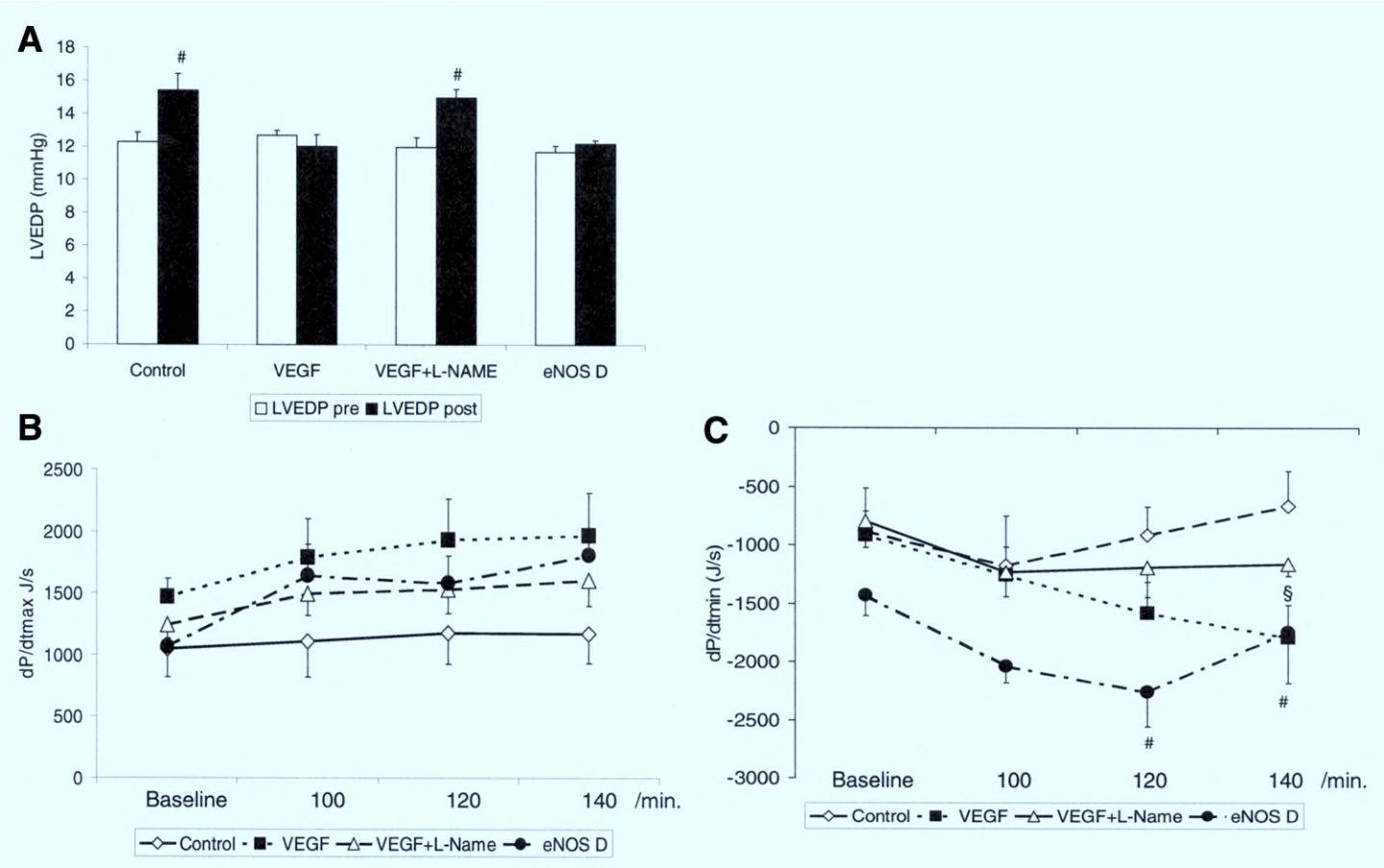

Figure 7. A) LVEDP comparison before (pre) and 24 h after the ischemic insult (post). In control hearts, a distinct increase in LVEDP was observed, which was not detected in the VEGF- and eNOS S1177D-treated animals. However, L-NAME treatment after VEGF transfection again induced an increase in postischemic LVEDP. **B)** dP/dt max tended to be increased in the VEGF- and eNOS S1177D transfected hearts, without reaching statistical significance. L-NAME coapplication abolished this effect in the VEGF-transfected group. **C)** Compared with control hearts, dP/dtmin increased in VEGF- and eNOS S1177D-transfected hearts, whereas L-NAME blunted the effect of VEGF (n=8 per group, $^{\#}P<0.05$ vs. control hearts).