Brief Definitive Report

Arterial Smooth Muscle Cells Express Nitric Oxide Synthase in Response to Endothelial Injury

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Summary

Endothelial cells regulate vascular tone by secreting paracrine mediators that control the contractility of arterial smooth muscle cells. Nitric oxide (NO) is an important vasodilating agent that is generated from L-arginine by the enzyme nitric oxide synthase (NOS), which is expressed constitutively by the endothelium. NO also inhibits platelet aggregation, contributing to the antithrombotic properties of the endothelial surface. It would therefore be expected that loss of the endothelium during arterial injury would lead to vasospasm and thrombosis but instead, the neointima formed after injury has a nonthrombogenic surface and a maintained vascular patency. We report here that arterial smooth muscle cells in the neointima formed after a deendothelializing balloon injury to the rat carotid artery express the cytokine-inducible isoform of NOS. Expression was detectable by reverse transcription-polymerase chain reaction from day 1–14 after injury and in situ hybridization showed expression of NOS mRNA by neointimal smooth muscle cells, particularly at the surface of the lesion. This was associated with systemically detectable NO production as revealed by electron paramagnetic resonance spectroscopic analysis of nitrosylated red cell hemoglobin. Local NO production by intimal smooth muscle cells after endothelial injury could represent an important mechanism for the maintenance of arterial patency and nonthrombogenicity in the injured artery.

Nitric oxide (NO) is a short-lived messenger molecule that is formed from L-arginine by the enzyme, NO synthase (NOS; EC 1.14.13.39) (1, 2). Three isoforms of NOS have been isolated, cloned, and expressed from cellular sources (3–11). NO formed by constitutively expressed endothelial NOS (eNOS) acts as a paracrine regulator of vascular tone by relaxing smooth muscle cells (SMC) (12, 13). NO is also an inhibitor of platelet aggregation (1, 2) and may contribute to the nonthrombogenic properties of the vascular surface. An inducible form of NOS (iNOS) is expressed in macrophages and several other cell types including vascular SMC after stimulation with the cytokines, IFN-γ, TNF, and IL-1, or with LPS (5–8, 14–16).

Deendothelializing vascular injury elicits a tissue response that is characterized by platelet adhesion and aggregation, leukocyte infiltration, smooth muscle migration and proliferation, and the formation of a neointima (17, 18). This may lead to arterial stenosis, which frequently occurs after human angioplasty (19). It is, however, remarkable that the denuded artery rapidly regains its nonthrombogenic properties and that the damaged arterial segment can be enlarged to compensate for the increased intimal thickness (20).

It has recently been shown that cytokine-stimulated SMC in vitro express high levels of iNOS and produce significant amounts of NO (14–16) and that deendothelialized arteries in vivo develop a relaxing capacity that is dependent on L-arginine and augmented by IL-1, compatible with an induction of iNOS in SMC (21). These observations suggest that SMC may be induced to express NOS in vivo after injury. We have tested this hypothesis by analyzing NOS mRNA and activity after deendothelializing injury to the carotid artery of the rat. Our results indicate that iNOS is rapidly induced in neointimal smooth muscle cells after arterial injury. This may represent a mechanism for maintaining patency and nonthrombogenicity in injured blood vessels.

Materials and Methods

Animals and Surgical Procedures. Arterial injury was inflicted in the common carotid artery of 5-mo-old Sprague Dawley rats (obtained from Alab, Stockholm, Sweden) with a Fogarty 2F balloon catheter (American Edwards Laboratory, Anasco, Puerto Rico) as described (22). In one group of rats, a balloon catheter was used to deendothelialize the thoracic aorta. Another group was subjected to a sham operation in which a skin incision and dissection of the carotid artery but no deendothelialization was performed. Rats (n = 4 per group) were killed by exsanguination under fluanisonium/fentanyl anesthesia at 24 h, 3, 7, and 14 d after surgery. All animal experiments were approved by the ethical committee of Gothenburg University.

Cell Culture. Smooth muscle cells were isolated from the aorta

of 6-wk-old Sprague Dawley rats and propagated in culture as described (23). Cells of the fourth to sixth passage were used for experiments.

RNA Isolation. The common carotid artery was rapidly chilled, dissected free from adventitia, and frozen in liquid nitrogen. The tissue was homogenized with a Polytron tissue homogenizer (InterMed, Roskilde, Denmark) and total RNA isolated using the method of Chomczynski and Sacchi (24).

Reverse Transcription (RT) and PCR Amplification. 200 ng RNA was incubated with 2.5 mM random hexamers, 1 mM of each dNTP, 2.5 U/ml Moloney murine leukemia virus RT (Boehringer Mannheim, Mannheim, Germany), 1 U/ml RNasin (Boehringer Mannheim), and 5 mM $MgCl_2$ in 20 μl of RT buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3) at 42°C for 20 min. After denaturation at 99°C for 5 min, the synthesized cDNA was subjected to a 35-cycle PCR. The 5′ antisense primer corresponded to nucleotides 2698–2720 of the murine macrophage iNOS cDNA sequence, (5–7), and the 3′ sense primer to nucleotides 2941–2963. For comparison, β-actin transcripts were PCR-amplified from the same tissue RNA using a commercial primer set (Clontech, Palo Alto, CA). The PCR mix contained 20 pmol of each primer, 200 mM of each dNTP, and 2.5 U Taq polymerase (AmpliTaq; Perkin-Elmer Cetus, Norwalk, CT) in 100 μl of RT buffer with 2 mM $MgCl_2$.

Southern Blot. PCR products were transferred from agarose gels to nitrocellulose membranes (Hybond C extra; Amersham International, Little Chalfort, UK) and hybridized with a ^{32}P-labeled antisense oligonucleotide probe corresponding to nucleotides 2749–2778 of the murine iNOS (5–7) in 6× SSC, 5× Denhardt's solution, and 0.1% SDS at 42°C. The membranes were washed at 55°C in 0.5× SSC, 0.1% SDS.

Construction of Hybridization Probe. Cultured rat aortic SMC were treated with recombinant IFN-γ and TNF-α, both at 500 U/ml for 24 h. RNA was isolated and a NOS cDNA sequence reverse-transcribed and amplified by PCR using the primers described above. The plasmids, pYM3 and pYM4, containing the rat iNOS cDNA fragment (266 bp) were made by subcloning the PCR product in antisense (pYM3) and sense (pYM4) directions into the EcoRV site under the T7 promoter of the pT7 blue-T vector (Novagen, Inc., Madison, WI). The inserts were sequenced using the dideoxy chain termination method (*Taq* DyeDeoxy Termination Kit; Applied Biosystems, Inc., Foster City, CA) and a DNA sequencer (model 373A, Applied Biosystems, Inc.). RNA probes were synthesized from the linearized plasmids using T7 RNA polymerase and tagged with digoxygenin-labeled UTP using a DIG RNA labeling kit (Boehringer Mannheim).

In Situ Hybridization and Immunohistochemistry. 10-μm cryostat sections of the carotid arteries were adhered to slides coated with 3-aminopropyltriethoxysilane (Sigma Chemical Co., St. Louis, MO), and fixed and incubated in prehybridization solution as described (25). They were then incubated with the probe at 25 ng/section for 16 h at 45°C under coverslips. After hybridization, the coverslips were removed and the sections treated with RNase A followed by rinsing with 1× SSC and 0.1× SSC at 45°C (25). Sections were then incubated with a monoclonal antibody to digoxygenin (Boehringer Mannheim) followed by rabbit anti–mouse immunoglobulin, an alkaline phosphatase–antialkaline phosphatase complex (Dakopatts, Glostrup, Denmark) and a nitroblue tetrazolium substrate solution (25). Immunohistochemical staining for α-smooth muscle actin was carried out on 10-μm cryostat sections using a monoclonal antibody (23) followed by avidin-biotin-alkaline phosphatase detection (BioGenex, San Ramon, CA).

Electron Paramagnetic Resonance (EPR) Analysis. Blood was drawn from the carotid artery during catheter surgery and from the heart at killing. The samples were frozen in liquid nitrogen and analyzed at 77 K on an EPR spectrometer (Varian Instruments Business, San Fernando, CA) (26). The EPR signals were recorded at a microwave frequency of 9.17 GHz and a microwave power of 8 mW and the spectra were scanned from 3,000 and 3,500 Gauss with a modulation amplitude of 8 Gauss and a scan rate of 25 Gauss/min.

Results and Discussion

NOS expression was first analyzed in cultured SMC using RT-PCR amplification of a 266 bp fragment from the coding sequence of NOS mRNA. No NOS expression was detectable under baseline conditions. It was, however, inducible by simultaneous stimulation of the cells with IFN-γ and TNF-α (Fig. 1). This PCR system also amplified constitutive NOS sequences from brain (data not shown). Direct sequencing was therefore performed on the PCR-amplified 266 bp cDNA fragment derived from SMC. It showed complete identity with the recently published rat smooth muscle iNOS sequence (8).

The RT-PCR system was used to analyze NOS expression in the rat carotid artery after deendothelializing injury. A balloon catheter was used to remove the endothelium and intima from a segment of the left common carotid artery, and arterial NOS expression during the response to injury was analyzed by RT-PCR. Fig. 2 *a* shows that NOS mRNA appeared in the injured artery already at 24 h post surgery. These transcripts persisted for 2 wk after surgery (Fig. 2 *a*). Southern hybridization of PCR products with an iNOS-

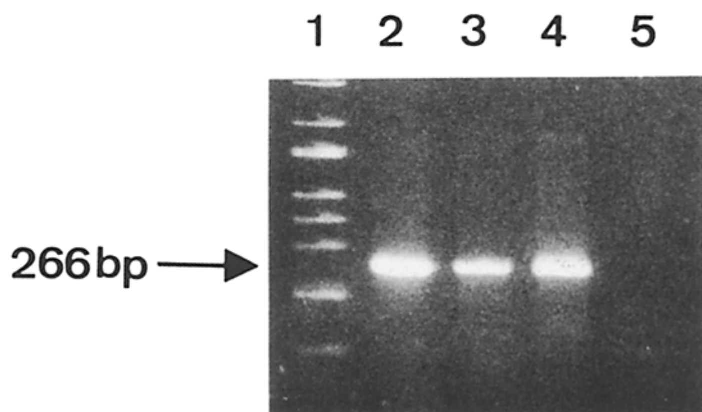


Figure 1. NOS mRNA expression is induced in cultured rat aortic SMC by stimulation with IFN-γ and TNF-α (200 U/ml of each for 16 h). RT-PCR, ethidium bromide-stained 2% NuSieve agarose (FMC Bioproducts, Rockland, ME) electrophoresis gel. Lane *1*, DNA ladder; lanes *2–4*, three different SMC cultures treated with IFN-γ + TNF-α; lane *5*, untreated control SMC.

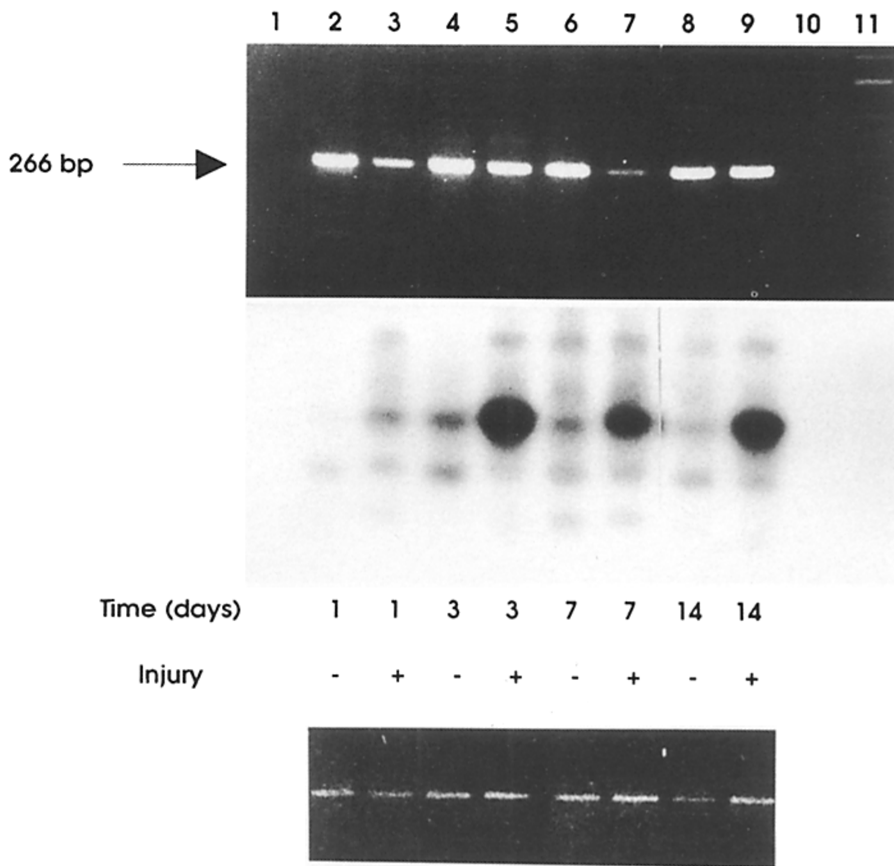


Figure 2. Expression of NOS mRNA isoforms in the arterial wall after injury. RT-PCR analysis and ethidium-bromide-stained agarose gel (*a*) and Southern hybridization with an iNOS-specific oligonucleotide (*b*) are shown. RT-PCR amplification of β-actin from the same samples is shown for comparison (*c*). Lane *1*, control without template; lane *2*, uninjured side, 24 h after balloon injury; lane *3*, injured side, 24 h after balloon injury; lane *4*, uninjured side, 3 d after balloon injury; lane *5*, injured side, 3 d after balloon injury; lane *6*, uninjured side, 7 d after balloon injury; lane 7, injured side, 7 d after balloon injury; lane *8*, uninjured side, 14 d after balloon injury; lane *9*, injured side, 14 d after balloon injury; lane *10*, control without template; lane *11*, DNA ladder.

specific antisense oligonucleotide revealed that NOS mRNA of injured arteries contained iNOS-homologous sequences (Fig. 2 *b*). This shows that the iNOS isoform was induced during the response to injury.

In the uninjured, contralateral artery, a NOS mRNA band was detectable at all time points after injury (Fig. 2 *a*). The RT-PCR material obtained 24 h after injury did not hybridize with the iNOS-specific oligonucleotide, implying that it represented constitutively expressed eNOS (Fig. 2 *b*). In contrast, bands amplified at subsequent time points contained iNOS-like material (Fig. 2 *b*). It is likely that these RT-PCR bands contained a mixture of eNOS and smooth muscle iNOS. The latter might have been induced by circulating cytokines during the systemic response to injury.

NOS expressing cells of the lesion were identified by in situ hybridization of injured carotid arteries using riboprobes transcribed from the pYM3 plasmid that contains the 266 bp NOS fragment in the antisense direction. Hybridization revealed NOS mRNA expression in the cells of the neointima (Fig. 3 *a*). Control hybridization with sense transcripts and irrelevant probes were consistently negative (Fig. 3 *b*). Staining of serial sections with antibodies to monocyte–macrophages (ED1) and T lymphocytes (OX19) excluded that any significant part of the cells were of hematogenous origin (data not shown). Since ~98% of the cells in the neointima are SMC, the abundant expression of NOS could be attributed to these cells. Staining with α-smooth muscle actin confirmed the SMC origin of some of the NOS expressing cells (Fig. 3 *d*). It should, however, be noticed that the majority of SMC in the neointima are dedifferentiated and do not express α-smooth muscle actin (27).

NOS expression was particularly frequent among the surface-forming, pseudoendothelial SMC (Fig. 3). In contrast, medial SMC were always negative with regard to in situ hybridization. In undamaged arterial segments, the endothelium exhibited a distinctive NOS hybridization signal but underlying, medial SMC were NOS negative (Fig. 3 *c*). The preferential localization of NOS mRNA to surface-forming, pseudoendothelial SMC could result in a high NO production at the luminal surface. Since NO inhibits platelet aggregation, this could explain the acquisition of nonthrombogenicity by the pseudoendothelial SMC.

The difference in NOS expression between medial and neointimal SMC could be explained by the phenotypic differences between the SMC of the two locations, by the presence of inducing factors such as cytokines only in the neointimal compartment, or by inhibitors of NOS expression in the media. Our previous detection of monocyte-derived macrophages and occasional T lymphocytes in the neointima (22, 28) supports a role for NOS-inducing cytokines. It is, however, also possible that the flow conditions at the vascular surface provide additional stimuli for NOS expression, that the more differentiated, contractile SMC of the media are less responsive to NOS-inducing stimuli, or that the local

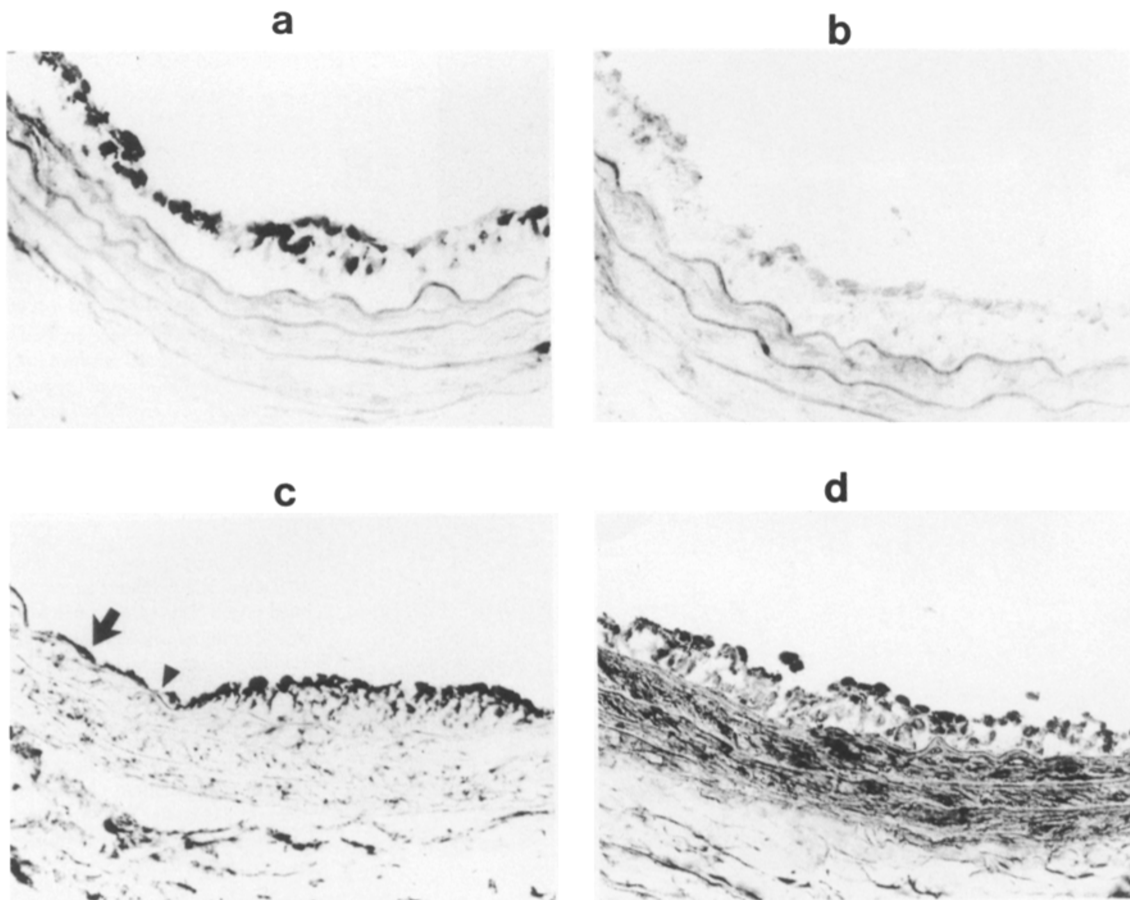


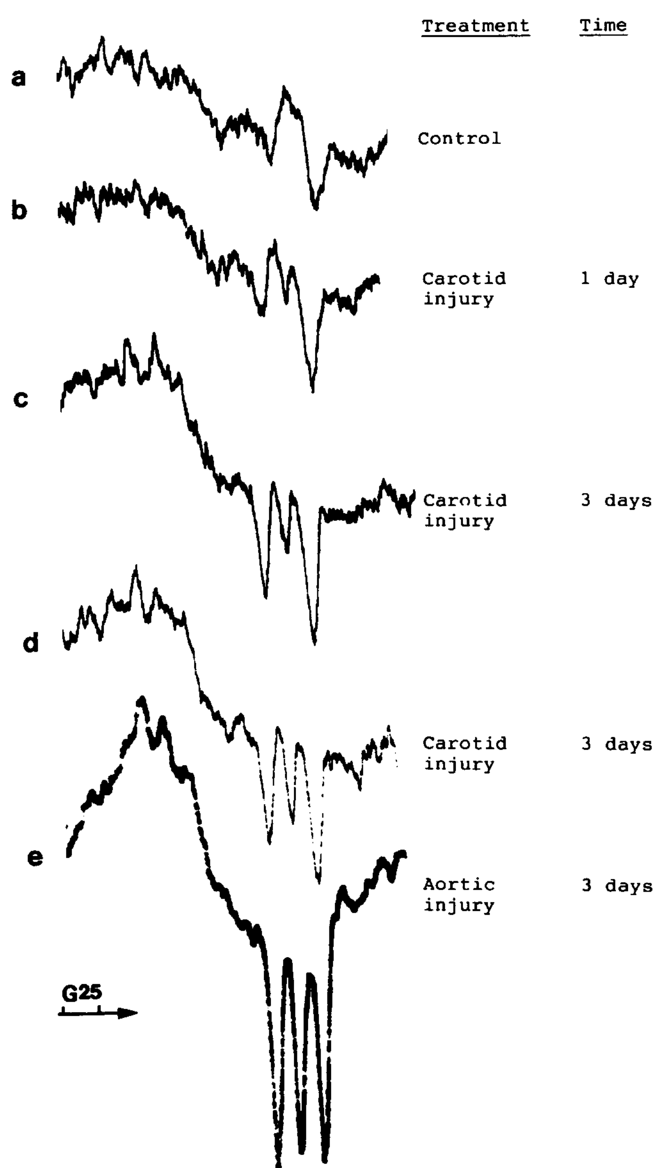
Figure 3. NOS mRNA expression in the postinjury neointima as revealed by in situ hybridization. Digoxygenin–antidigoxygenin-APAAP staining. (*a*) pYM3 antisense hybridization shows labeling of SMC in neointimal lesion, original magnification: 250×; (*b*) sense hybridization is negative, original magnification 250×; (*c*) antisense hybridization, border between lesion (*right*) and uninjured artery (*left*). The junction is indicated by an arrowhead. There is labeling of SMC in the neointima and of endothelium in the uninjured area (*arrow*), original magnification: 125×; (*d*) immunohistochemical staining for α-smooth muscle actin demonstrates SMC in the injured artery, original magnification: 250×.

environment of the media inhibits NOS expression. At any extent, SMC-derived NO may act as a paracrine rather than autocrine regulator of contractility, similarly to endothelium-derived NO.

Enzymatic activity of NOS was monitored by EPR analysis of nitrosylated hemoglobin (HbNO) in the circulating blood from operated rats. A triplet hyperfine signal characteristic for nitrosylation of heme groups was detectable after injury, culminating after 1 wk (Fig. 4). In contrast, no HbNO could be observed in unoperated control rats or in sham-operated rats subjected to arterial dissection but not deendothelialization. The HbNO signal was significantly larger when the thoracic aorta was deendothelialized compared with the common carotid artery, indicating that the amount of HbNO was proportional to the size of the lesion (Fig. 4). The EPR data therefore strongly suggest that at least a substantial part of the circulating HbNO was formed by NO released from NOS-expressing neointimal cells. Furthermore, they suggest that iNOS induction in the injured artery generates NO in sufficient amounts to nitrosylate proteins of circulating erythrocytes, whereas endothelial NOS of intact arteries do not generate systemically detectable NO (29). The difference in their capacity to induce HbNO fits with the difference in activity between eNOS and iNOS (1, 2).

The NO radical is extremely short-lived and reacts with other compounds such as proteins within seconds after its formation. It is therefore likely that all NO produced in the deeper layers of the neointima will react with intra- and extracellular molecules in the tissue. The most probable explanation of the significant amounts of HbNO complexes in circulating erythrocytes is that NO was produced in high amounts by the iNOS-expressing pseudoendothelium at the luminal surface. This would permit nitrosylation of hemoglobin present in erythrocytes rolling over the injured segment. The high degree of oxygenation of arterial blood does not exclude that nitrosylation could take place there since 1–2% of the hemoglobin is not oxygenated and the affinity of hemoglobin for NO is 10^6-fold higher than that for oxygen (30).

Several mechanisms could be involved in local iNOS induction in the neointima. At later time points, i.e., 7 and 14 d, it is probable that local expression of IL-1, IFN-γ, and



TNF could stimulate iNOS expression. The finding of iNOS expression already at 24 h post injury, i.e., at a time point when hardly any mononuclear cells are present (31, 32), was more surprising. One possible explanation is that tissue injury per se induces iNOS expression, by releasing preformed cytokines or other molecules that could activate the NFκB-responsive iNOS promoter (33). Endotoxemia after surgery could contribute to the early phase of iNOS expression but the finding of iNOS mRNA in the injured but not in the uninjured side at 24 h in most of the rats suggests that local inducing factors in the response to injury are more important than systemic ones.

In conclusion, the results of the present study indicate (*a*) that eNOS mRNA is lost upon deendothelialization of the artery; (*b*) that iNOS mRNA is induced in the injured arterial segment; (*c*) that iNOS mRNA expression is localized to SMC in the neointima and particularly to the pseudoendothelial SMC; and (*d*) that iNOS mRNA expression is associated with significant NO production, which is detectable in the circulation of operated animals. Together, the present results unveil a homeostatic mechanism that may be important for the maintenance of vascular tone and nonthrombogenicity, and that depends on the induction of NO production in vascular smooth muscle cells of the injured artery.

Figure 4. EPR detection of HbNO in peripheral blood erythrocytes after arterial injury. (*a*) Blood sample from sham-operated control rat contains no HbNO. (*b*) A triplet hyperfine signal representing HbNO is barely detectable 1 d after deendothelializing injury to the common carotid artery. (*c–d*) Significant triplet hyperfine HbNO signals are found 3 d after carotid injury. (*e*) An HbNO signal with a larger amplitude is detected 3 d after a larger, deendothelializing injury is inflicted in the thoracic aorta.

We thank Stina Nygren, Marianne Almqvist, and Ann-Sofi Petersson for skillful technical assistance.

This project was supported by grants from the Swedish Medical Research Council (project no. 6816, 7120, and 4341), the Swedish Heart-Lung Foundation, King Gustaf V 80th Anniversary Fund, the Swedish National Society against Rheumatism, and a grant from Astra-Hässle, Mölndal, Sweden.

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Received for publication 19 January 1994 and in revised form 20 April 1994.

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