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Balloon angioplasty and induction of non-endothelial nitric oxide synthase in rabbit carotid arteries [1]

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Abstract

The purpose of the study was to evaluate whether balloon angioplasty is associated with changes in nitric oxide synthase (NO synthase) activity. Normal rabbit carotid arteries were examined 10 min or 1, 2, 3 or 10 weeks after angioplasty with 2 or 2.5-mm balloons. Immunohistology was used to evaluate intimal thickening and endothelial cell regeneration. The NO synthase activity was studied functionally using isolated segments in organ chambers. Immunohistochemistry of the endothelial cell markers von Willebrand factor and platelet endothelial cell adhesion molecule-1 indicated that the regeneration of endothelial cells from patchy islands that remained after angioplasty was virtually complete within 2 weeks. However, the endothelium-dependent relaxations elicited by acetylcholine remained impaired up to 10 weeks after dilatation. Contractions elicited by 5-hydroxytryptamine (5-HT) were attenuated, but were significantly augmented by the NO synthase blocker, nitro-L-arginine. Furthermore, in contrast to normal arteries, the balloon-treated arteries developed marked contractions in response to nitro-L-arginine methyl ester (L-NAME), contractions which could be reversed by L-arginine. The latter contractions and relaxations were not influenced by endothelial removal. These results suggest that although the endothelium quickly regenerates after severe balloon injury, the endothelium-dependent release of nitric oxide remains disturbed. However, the functional data also suggest that angioplasty led to a significant induction of NO synthase in 'non-endothelial' cells of the artery.

Keywords: Balloon angioplasty; Neointima; Restenosis; Endothelium; Nitric oxide (NO); Nitric oxide (NO) synthase; Von Willebrand factor

1. Introduction

Percutaneous transluminal coronary balloon angioplasty is increasingly being used for the nonsurgical treatment of occlusive atherosclerotic coronary artery disease. Despite a primary success rate greater than 90%, the long-term efficacy continues to be compromised by the recurrence of stenotic lesions. Restenosis affects 30–40% of successfully dilatated patients (Popma et al., 1992; Topol et al., 1993). Necropsy and experimental studies suggest that restenosis is secondary to fibromuscular proliferation of vascular smooth muscle cells and extracellular matrix accumulation, resulting in intimal thickening (Liu et al., 1989; Ip et al., 1990; Haudenschild, 1994). In most experimental models endothelial denudation is accomplished by Fogarty balloon withdrawal, air drying or nylon filaments (Grotendorst et al., 1981; Bernstein et al., 1982; Chesebro et al., 1987; Fingerle et al., 1989; Weidinger et al., 1990; Lindner et al., 1993; Douglas et al., 1994). This superficial intimal damage evokes adhesion of platelets to denuded areas, followed by degranulation. These events not only trigger proliferation and migration of medial smooth muscle cells into the intima, where they maintain a high proliferation rate, but also initiate the migration and division of remaining endothelial cells to close the wound (Lindner et al., 1990; Casscells, 1992; Lee et al., 1993; Shirotani et al., 1993).

Endothelial cell regeneration and intimal thickening are regulated by many factors derived from endothelial cells, smooth muscle cells, platelets, macrophages or leukocytes. One of these factors could be nitric oxide (NO), a potent

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vasoactive autacoid released by vascular endothelium (for review see Moncada and Higgs, 1993). Nitric oxide is important in regulating vascular tone, inhibits platelet aggregation and is supposed to limit platelet–endothelial interactions. Finally, nitric oxide could play a role in maintaining the normal mitogenic state of vascular smooth muscle by inhibiting proliferation (Graf, 1993; Scott-Burden and Vanhoutte, 1993). The nitric oxide synthase (NO synthase) exists in at least two isoforms: a constitutive Ca^{2+} calmodulin-dependent NO synthase is present in endothelial cells and neuronal cells, and a Ca^{2+}-independent inducible form comes to expression in cytokine-activated macrophages and smooth muscle cells (Moncada and Higgs, 1993).

The aim of our study, using a model of severe balloon dilatation injury, was to evaluate a possible relation between the extent of vascular wall injury, endothelial cell regeneration and vascular NO synthase activity. The force development of isolated segments in organ chambers was used to study NO synthase activity functionally. A more detailed description of the morphological changes is beyond the scope of this text and is given separately (Bosmans et al., 1996).

2. Materials and methods

Eighty-two male New Zealand White rabbits weighing between 2.5 and 3 kg, and fed on a normal diet which was not supplemented with additional cholesterol, were studied. The right carotid artery was chosen for angioplasty, using a 2.0-mm or a 2.5-mm balloon, to allow use of the contralateral carotid artery as an uninjured control vessel. Segments of all arteries were examined histologically and functionally in organ chambers.

2.1. Angioplasty

Rabbits were anaesthetized with sodium pentobarbital (30 mg/kg body weight, i.v.), posed in a supine position and the ventral neck region was clipped and infiltrated with xylocaine for local anaesthesia. Both carotid arteries were exposed and dissected from the surrounding tissues. For angioplasty, direct arteriotomy was performed in the right common carotid artery, just proximal to the bifurcation. A standard 2.0-mm or 2.5-mm sized PTCA balloon dilatation catheter (Advanced Cardiovascular Systems, San Diego, USA) was introduced over a 0.014-inch floppy guide wire and advanced towards the aorta till about 5 cm from the incision. The balloon was inflated 3 times (6 atmospheres), for periods of 2 min each. Between the subsequent inflations the balloon was left deflated in the artery for 1 min. This insufflation protocol is a copy of the one commonly used in the human heart catheterisation laboratory, during coronary balloon angioplasty. However, in view of the possible effects of heparin on endothelial cell regrowth (Azizkhan et al., 1980; Maciag et al., 1981; Thornton et al., 1983), anticoagulant therapies were omitted. After removal of the catheter, the small distal incision was closed surgically, with restoration of arterial blood flow.

In 5 rabbits a sham operation was performed: after intravenous anaesthesia and dissection of both carotid arteries, direct arteriotomy was performed in the right common carotid artery. Eight minutes later, the incision was closed again, without insertion of a balloon catheter.

At the appropriate predetermined time points, the rabbits were anticoagulated with heparin (150 units/kg i.v.) and anaesthetized with sodium pentobarbital (30 mg/kg body weight i.v.). Both left and right carotid arteries were removed and placed in physiological salt solution. Afterwards, the animals were killed by an overdose of sodium pentobarbital.

2.2. Histology

The study of vascular reactivity precluded perfusion fixation. Isolated segments were placed in methacarn fixative (60% methanol, 30% 1,1,1-trichloroethane, and 10% glacial acetic acid). Afterwards segments were cut into lengths of 4 mm. At least 4 segments were paraffin-embedded per artery. Transverse sections were stained with haematoxylin and eosin. After selection of the sirius haematoxylin-stained material, immunohistochemistry was carried out. The reactions were carried out by the indirect peroxidase antibody conjugate technique. The following monoclonal antibodies were used: α-smooth muscle cell actin, von Willebrand factor (1/100 dilution) and platelet endothelial cell adhesion molecule-1 (PECAM-1, CD 31 1/10 dilution). The monoclonal antibodies were diluted in phosphate-buffered saline. After 3 washes with phosphate-buffered saline, the sections were incubated with rabbit anti-mouse peroxidase for 45 min. For the demonstration of the complex, 3-amino-9-ethylcarbazole was used as a chromogen. The specificity of the primary antibodies was tested in previous studies (Kockx et al., 1992, 1993).

Luminal lining cells, which were immunoreactive for von Willebrand factor and platelet endothelial cell adhesion molecule-1 and negative for α-smooth muscle cell actin, were considered to be endothelial cells. Their presence was considered to be focal when separate patchy islands of endothelial cells were present, or continuous, when a continuous monolayer of endothelium was seen. The α-smooth muscle cell actin-stained sections were used to count the number of smooth muscle cell layers between the internal elastic lamina and the endothelium, and in the media from each balloon-treated and control segment.

In 14% (12/82) of the arteries, an occlusive thrombus was observed (1 week: $n = 2$; 2 weeks: $n = 3$; 3 weeks: $n = 7$, only in the 2.5-mm balloon-treated group). These arteries were excluded from further histological and functional evaluation.

An injury score was calculated, defined as the sum of the number (0–4) of quadrants in which either endothelium was absent, or the internal elastic membrane was ruptured or the media was torn (minimum 0, maximum 12).

2.3. Vascular reactivity

Rings, 3 mm in length, were cut from the central region of the balloon-treated and control arteries, carefully cleaned of loose connective tissue, and suspended in organ chambers, filled with 25 ml physiological salt solution, which was maintained at 37°C and continuously gassed with 95% O_2 and 5% CO_2. Tension was measured isometrically with a Statham UC2 force transducer (Gould, Cleveland, OH). After an equilibration period of 15 min, the preparations were gradually stretched to a tension of 8 g, which had been determined in preliminary experiments to bring both control and balloon-treated segments to the optimal point of their length–tension relationship (data not shown). The segments were then allowed to equilibrate for 45 min. Indomethacin (3×10^{-6} M) was always added to the organ chambers, 30 min before the administration of the agonists, to prevent interference due to the synthesis of vasoactive prostanoids.

In the first experiment vascular reactivity was evaluated 10 min ($n = 7$), 1 week ($n = 7$), 2 weeks ($n = 8$), 3 weeks ($n = 6$) and 10 weeks ($n = 4$) after angioplasty with a 2.5-mm balloon. Five supplementary rabbits were 'sham'-operated, to evaluate the surgical methodology without angioplasty after 2 weeks. Cumulative concentration–contraction curves were made for 5-HT (3×10^{-10} to 3×10^{-6} M) and phenylephrine (10^{-8} to 10^{-5} M). After contraction with phenylephrine in the concentration which produces a half-maximum contraction (EC_{50}; 3.5×10^{-7} M), cumulative dose–response curves were made for acetylcholine (3×10^{-9} to 10^{-6} M) and nitroglycerin (3×10^{-11} to 10^{-6} M). Between agonists, the bath solution was exchanged 3 times and tissues were allowed to equilibrate for 30 min. The 5 sham-operated arteries and their controls were also subjected to this study protocol.

In the second experiment the arterial wall was exposed to different degrees of injury by the use of a 2.5-mm ($n = 25$; 'severe' injury) or a 2.0-mm ($n = 10$; 'moderate' injury) balloon. Four rabbits were dilatated with 2-mm balloons and evaluated immediately to quantify morphologically the initial degree of 'moderate' vascular wall injury. Three weeks later 2 duplicate rings of each balloon-treated and control artery were studied. A cumulative dose–response curve was made for 5-HT (3×10^{-10} to 3×10^{-6} M), immediately followed by a dose–response curve for acetylcholine (3×10^{-9} to 10^{-6} M). Afterwards, relaxations elicited by nitroglycerin (3×10^{-11} to 10^{-6} M) and 3-morpholinosydnonimine (SIN-1, 1.6×10^{-5} to 10^{-9} M) were evaluated, after constriction with phenylephrine (3.5×10^{-7} M). This part was performed to exclude significant segmental variation within the arteries. In the second part, the protocol was repeated, but one balloon-treated and one control segment of each pair were evaluated in the presence of 30 μM nitro-L-arginine. Nitro-L-arginine rather than N^G-nitro-L-arginine methylester hydrochloride (L-NAME) was used in view of the possible antimuscarinic activity of the latter.

In the third experiment vascular responses to L-NAME (10^{-5} to 10^{-4} M) and L-arginine (10^{-4} to 10^{-3} M) were assessed after constriction with phenylephrine (3.5×10^{-7} M), in control and balloon-treated arteries, isolated 3 weeks after angioplasty, in rings both with and without endothelium. Mechanical rubbing was not a reproducible and reliable way to remove endothelium due to the intimal thickening. Selective removal of the endothelium was performed by giving a luminal bolus injection of deoxycholic acid (0.075%; 5 ml), immediately followed by extensive flushing with physiological salt solution. Endothelium removal was first functionally confirmed by the absence of relaxation in response to acetylcholine (3×10^{-9} to 10^{-6} M) after contraction with phenylephrine (3.5×10^{-7} M).

2.4. Materials

The physiological salt solution contained (mM) NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, CaEDTA 0.025, and glucose 11.1. Acetylcholine, L-arginine, N^G-nitro-L-arginine, N^G-nitro-L-arginine methylester hydrochloride (L-NAME) and anti-α smooth muscle cell actin antibody were obtained from Sigma Chemical Co. (St. Louis, USA). All other monoclonal antibodies were from Dako (Glostrup, Denmark). Indomethacin sodium tetrahydrate was from Merck Sharp & Dohme (Munich, Germany); nitroglycerin (1% solution in ethanol) was from Merck (Darmstadt, Germany); phenylephrine chloride was from Winthrop (Brussels, Belgium); 5-hydroxytryptamine was from Janssen Chimica (Beerse, Belgium); sodium pentobarbital was from Psyphac; Brussels, Belgium; and heparin Leo and SIN-1 were from Therabel Pharma (Brussels, Belgium).

2.5. Data analysis

Forces were measured in g tension. Relaxations were expressed as a percentage of the initial tension. All data are given as the means ± S.E.M. The number of carotid arteries reported (n) equals the number of rabbits used. The negative logarithm (pD_2 value) of the concentration of the agonist that produces 50% of the maximum effect (E_{max}) was calculated for each ring after fitting the raw data with a logistic model (Nakashima et al., 1982). The E_{max} and pD_2 values were analysed by a factorial analysis of variance (Sokal and Rohlf, 1981). The number of neointimal smooth muscle cell layers in ballooned segments was compared with that of control segments, using the non-parametric Wilcoxon test (Sokal and Rohlf, 1981).

Table 1
E_{max} and pD_2 values of 5-hydroxytryptamine (5-HT) and phenylephrine, and maximum relaxation (%) elicited by acetylcholine and nitroglycerin in segments from sham-operated and non-manipulated control ($n = 5$)

Agonist	Sham		Control	
	E_{max}	pD_2	E_{max}	pD_2
5-HT (g)	7.5 ± 1.6	6.62 ± 0.12	8.3 ± 1.0	6.56 ± 0.13
Phenylephrine (g)	6.5 ± 1.8	6.18 ± 0.09	8.9 ± 0.9	5.97 ± 0.18
Acetylcholine % relaxation	77 ± 23	7.21 ± 0.07	75 ± 18	7.14 ± 0.17
Nitroglycerin % relaxation	80 ± 10	8.55 ± 0.16	97 ± 2	8.13 ± 0.51

Means ± S.E.M. ($n = 5$). E_{max} of 5-HT and phenylephrine expressed in g, and E_{max} of acetylcholine and nitroglycerin as percentage of the initial contration. Differences were statistically not significant.

A 5% level of significance was selected. All data were analysed with the SPSS (release 5) software (SPSS Inc, Chicago, Il, USA).

3. Results

3.1. Sham-operated arteries

Sham-operated animals ($n = 5$) were evaluated after 2 weeks. A monolayer of endothelial cells lined the intima. These cells were resting directly on the internal elastic membrane. Subendothelial smooth muscle cells were absent. Also in other aspects (absence of medial injury, intact elastic laminae, absence of thrombi), these sham-operated segments were indistinguishable from naive, non-manipulated rabbit carotid arteries.

Functional differences could not be detected between the sham-operated arteries and the contralateral, non-operated controls. Phenylephrine and 5-HT induced concentration-dependent contractions. Acetylcholine and nitroglycerin caused concentration-dependent incomplete or complete relaxations. The maximum and the pD_2 of the contractions elicited by 5-HT and phenylephrine, and of the relaxations elicited by acetylcholine and nitroglycerin were not significantly different (Table 1).

3.2. Balloon-treated arteries: histologic evaluation

3.2.1. Time course of 2.5-mm balloon-treated rabbits

Immediately after angioplasty ($n = 7$), marked segmental loss of smooth muscle cells and circular media tears were always observed. In 6/7 of the arteries the internal elastic membrane was fragmented. Yet, unfragmented sectors of the internal elastic membrane remained partially covered by sparse islands of endothelial cells (Fig. 1).

One week after angioplasty ($n = 5$), a thin rim of neointima was observed. Endothelial cells were still predominantly present in a focal way (4/5). The number of medial smooth muscle cell layers was slightly increased as compared to that of the contralateral control.

Two weeks after angioplasty ($n = 5$), the media still contained an elevated number of smooth muscle cell layers, but appeared to be nearly completely repaired. A prominent circular neointima was observed in all specimens. This neointima was always lined by a continuous layer of cuboidal endothelial cells that showed a dense flocculent immunoreactivity for von Willebrand factor. Beneath the endothelium, von Willebrand factor-immunoreactive material was found as well. The depth of this subendothelial accumulation varied.

Three weeks after angioplasty ($n = 24$), the prominent circular neointima was always lined by a continuous layer of endothelial cells (Fig. 1) which still showed a dense flocculent immunoreactivity for von Willebrand factor (Fig. 1). The number of medial smooth muscle cell layers was still elevated.

Ten weeks after dilatation ($n = 4$) a circular rim of neointima was observed in all sections, covered by a regenerated endothelial monolayer.

3.2.2. Effect of balloon size

The smaller balloon (2 mm, $n = 4$) caused less injury than the larger (2.5 mm, $n = 7$; mean injury scores respec-

Table 2
Summary of the morphology of carotid arteries, 3 weeks after balloon angioplasty

		Control ($n = 34$)	Balloon 2 mm ($n = 10$)	Balloon 2.5 mm ($n = 24$)
Intima				
Endothelium	Focal (%)	0	10	8
	Continuous (%)	100	90	92
Smooth muscle cell layers (n)		0	8.0 ± 1.5 [a]	14 ± 1.5 [ab]
Media				
Smooth muscle cell layers (n)		8.4 ± 0.1	10.5 ± 0.4 [a]	10.2 ± 0.4 [a]

Means ± S.E.M. of smooth muscle cell layers. Frequency of endothelial presence expressed as percentage of total number of arteries. [a] $P < 0.05$ compared to control; [b] $P < 0.05$ compared to 2-mm balloon.

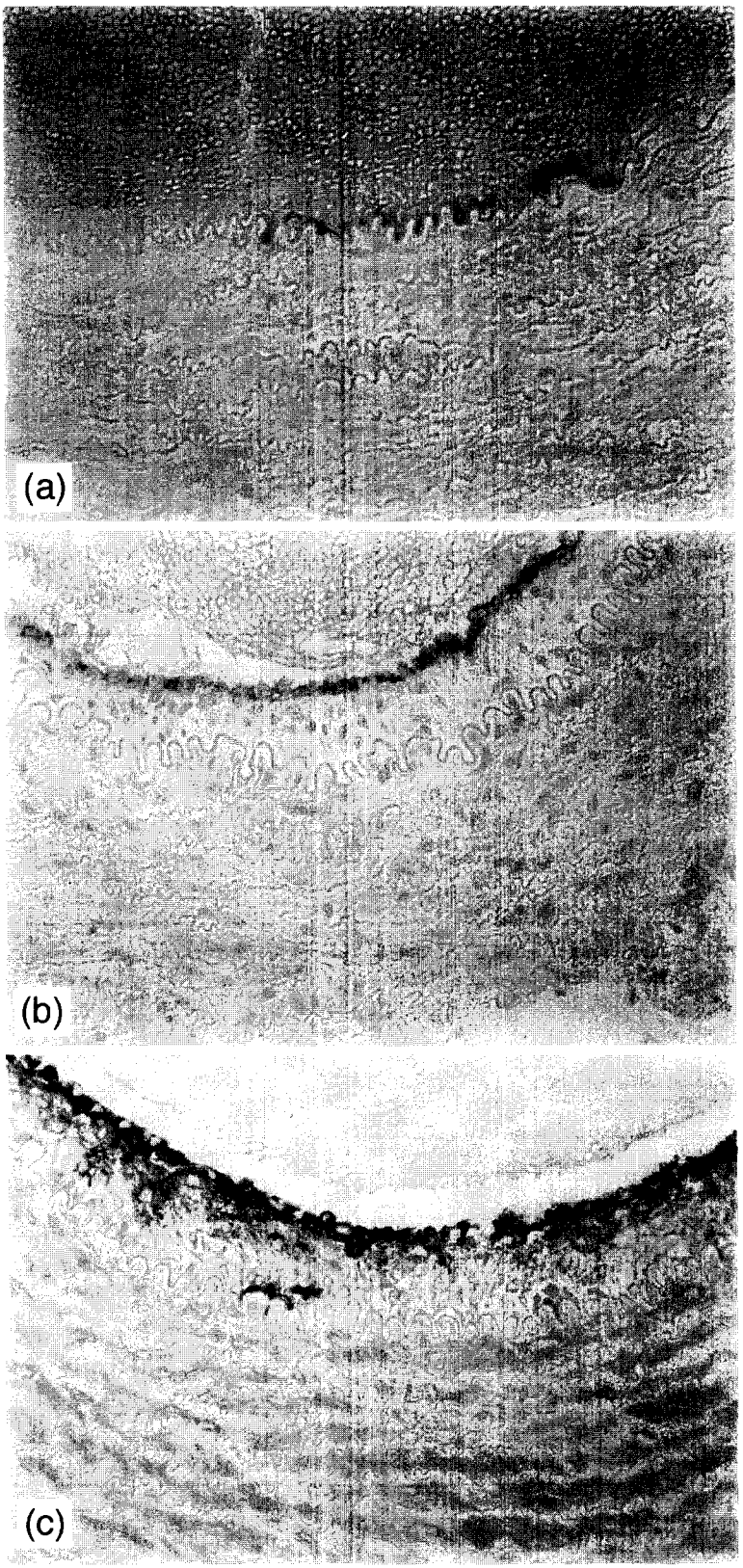

Fig. 1. (a) Rabbit carotid artery 10 min after balloon angioplasty. Immunohistochemical stain for platelet endothelial cell adhesion molecule-1 (CD 31). Small foci of immunoreactive endothelial cells are present along the luminal margin. (b) Rabbit carotid artery 3 weeks after balloon angioplasty. Immunohistochemical stain for platelet endothelial cell adhesion molecule-1 (CD 31). The cells along the luminal margin form a continuous immunoreactive cell layer indicating complete endothelial regeneration. (c) Rabbit carotid artery 3 weeks after balloon angioplasty. Immunohistochemical stain for von Willebrand factor. The endothelial cells demonstrate dense flocculent immunoreactivity. Deposition of von Willebrand factor is also present in the matrix of the neointima. All magnifications ×290.

Table 3
Time course of E_{max} and pD_2 values for 5-hydroxytryptamine (5-HT) and phenylephrine of control and 2.5-mm balloon-treated arteries

Agonist	Week (n)	Control		2.5-mm balloon	
		E_{max} (g)	pD_2	E_{max} (g)	pD_2
5-HT	0 (7)	5.9 ± 0.9	6.91 ± 0.07	1.5 ± 0.6 [a]	6.87 ± 0.12
	1 (5)	6.0 ± 1.1	6.72 ± 0.06	1.5 ± 0.5 [a]	6.80 ± 0.11
	2 (5)	8.3 ± 1.0	6.57 ± 0.13	1.5 ± 0.5 [a]	6.84 ± 0.24
	3 (4)	7.5 ± 0.7	6.81 ± 0.13	1.4 ± 0.7 [a]	7.06 ± 0.37
	10 (4)	4.5 ± 0.6	6.57 ± 0.12	3.1 ± 1.4	6.99 ± 0.12
Phenylephrine	0 (7)	7.9 ± 0.9	6.22 ± 0.15	1.8 ± 0.8 [a]	6.05 ± 0.24
	1 (5)	5.9 ± 0.7	6.16 ± 0.12	1.8 ± 0.8 [a]	6.09 ± 0.26
	2 (5)	8.9 ± 0.9	5.99 ± 0.17	2.2 ± 0.7 [a]	5.80 ± 0.29
	3 (4)	10.4 ± 0.4	6.05 ± 0.21	2.0 ± 0.9 [a]	6.37 ± 0.32
	10 (4)	6.4 ± 0.6	6.52 ± 0.20	4.1 ± 1.9	6.83 ± 0.15

Means ± S.E.M. Week 0: tested 10 min after angioplasty. [a] Different from contralateral control ($P < 0.05$ paired Student's t-test).

Table 4
Time course of maximum endothelium-dependent (acetylcholine) and -independent (nitroglycerin) relaxation in control arteries and arteries treated with a 2.5-mm balloon

Agonist	Week (n)	Control relaxation (%)	2.5-mm balloon relaxation (%)
Acetylcholine	0 (7)	85 ± 7	12 ± 3 [a]
	1 (5)	94 ± 3	17 ± 5 [a]
	2 (5)	94 ± 5	26 ± 12 [a]
	3 (4)	97 ± 1	35 ± 15 [a]
	10 (4)	77 ± 8	27 ± 3 [a]
Nitroglycerin	0 (7)	97 ± 1	89 ± 5
	1 (5)	80 ± 5	66 ± 12
	2 (5)	97 ± 2	68 ± 15
	3 (4)	93 ± 4	87 ± 4
	10 (4)	79 ± 13	87 ± 12

Means ± S.E.M. Week 0 = tested 10 min after angioplasty; relaxation expressed as percentage of the phenylephrine contraction. [a] Different from contralateral control ($P < 0.05$ paired Student's t-test).

Table 5
Effect of angioplasty and balloon size on sensitivity (pD_2 values) to SIN-1 and nitroglycerin

Agonist	Treatment			
	Control ($n = 9$)	2.0-mm balloon ($n = 9$)	Control ($n = 20$)	2.5-mm balloon ($n = 20$)
SIN-1	6.03 ± 0.08	6.14 ± 0.05	6.11 ± 0.05	6.16 ± 0.08
Nitroglycerin	7.43 ± 0.06	7.06 ± 0.06 [a]	7.53 ± 0.04	7.26 ± 0.07 [ab]

Means ± S.E.M. Two segments of each artery were studied, and the average of each pair calculated. A factorial analysis of variance pointed to significant effects of angioplasty ([a] $P < 0.001$) and balloon size ([b] $P = 0.004$) for nitroglycerin, but not for SIN-1. Statistically significant interactions were absent.

Table 6
Effect of balloon size and nitro-L-arginine on sensitivity (pD_2 values) to 5-HT and acetylcholine

Agonist	Treatment			
	Control ($n = 9$)	2.0-mm balloon ($n = 9$)	Control ($n = 20$)	2.5-mm balloon ($n = 20$)
5-HT				
Saline	6.68 ± 0.04	6.62 ± 0.08	6.75 ± 0.05	6.36 ± 0.06 [b]
Nitro-L-arginine	6.76 ± 0.07	6.47 ± 0.08	6.82 ± 0.06	6.63 ± 0.04 [c]
Acetylcholine				
Saline	7.28 ± 0.09	7.10 ± 0.09	7.23 ± 0.05	7.14 ± 0.13
Nitro-L-arginine	6.82 ± 0.11 [a]	7.37 ± 0.18	6.96 ± 0.09 [a]	7.22 ± 0.14

Means ± S.E.M. [b] $P < 0.01$, balloon different from control; [a] $P < 0.05$, [c] $P < 0.01$, nitro-L-arginine different from saline. Interactions in ANOVA, followed by paired Student's t-test.

tively 1.5 and 6.5, $P < 0.05$, Wilcoxon). Three weeks later, the circular neointima was clearly less prominent in the 2-mm balloon-treated group (Table 2). It was completely covered by endothelial cells.

3.3. Balloon-treated arteries: vascular reactivity

3.3.1. Time course of the 2.5-mm balloon-treated rabbits

Immediately after angioplasty, the maximal contractions elicited by 5-HT and phenylephrine were significantly impaired, whereas the pD_2 values remained unchanged. The E_{max} remained depressed in the subsequent 3 weeks, but a partial recovery was seen 10 weeks after angioplasty. The pD_2 did not change and was never different from that of the control (Table 3). Immediately after angioplasty, the acetylcholine-induced relaxations were almost absent (Table 4). In the weeks after dilatation, the endothelium-dependent relaxations tended to be restored, but they still differed markedly from those of the control rings (Table 4). Balloon dilatation did not affect the maximal relaxations elicited by nitroglycerin (Table 4).

3.3.2. Effect of balloon size and nitro-L-arginine

From the 25 arteries dilatated with 2.5-mm balloons, 20 could be analysed, as well as 9 out of 10 arteries exposed to the 2.0-mm balloon. To exclude segmental differences within each artery, 2 rings of each vessel were first studied in the absence of nitro-L-arginine. The balloon treatment profoundly affected responses to acetylcholine and 5-HT (see below), but neither in control nor in balloon-treated arteries were there statistically significant differences among pairs of rings (results not shown). In spite of the reduced force development in response to the EC_{50} dose of phenylephrine, the subsequent relaxations in response to SIN-1 remained unchanged with respect to amplitude and sensitivity (Fig. 2; Table 5). Angioplasty with either balloon led to a small decrease in sensitivity to nitroglycerin (Fig. 3; Table 5). The amplitude of the relaxations remained unchanged after angioplasty with 2.5-mm balloons, but was slightly smaller in arteries with the smaller balloon (Fig. 3). Again, statistically significant differences were not noted among the duplicate segments taken from each artery with respect to the responses to SIN-1 and nitroglycerin.

The protocol was then repeated in the presence of nitro-L-arginine. A representative experimental trace is shown in Fig. 4. E_{max} (Figs. 4 and 5) and pD_2 (Table 6) for 5-HT were similar in both sets of control arteries and not influenced by nitro-L-arginine. The E_{max} for 5-HT was significantly attenuated after angioplasty. This effect was most pronounced in arteries treated with the larger balloon

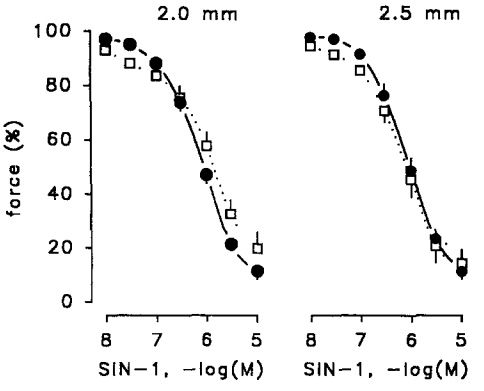

Fig. 2. Relaxations elicited by SIN-1 in rings of control (●, solid lines) or balloon-treated (□, dotted lines) carotid arteries 3 weeks after angioplasty with a 2.0-mm (left panel, $n = 9$) or a 2.5-mm (right panel, $n = 20$) balloon. Force expressed as a percentage of the contractions elicited by 0.35 μM phenylephrine, which were 6.6 ± 0.3 and 6.8 ± 0.4 g in control segments and 4.8 ± 0.6 and 3.4 ± 0.8 g in segments of arteries treated with 2.0- and 2.5-mm balloons, respectively. Means ± S.E.M. (vertical bars).

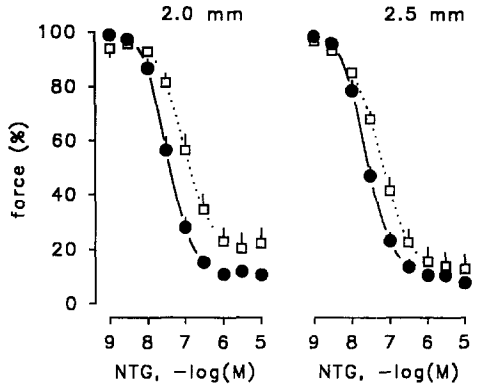

Fig. 3. Relaxations elicited by nitroglycerin (NTG) in rings of control (●, solid lines) or balloon-treated (□, dotted lines) carotid arteries 3 weeks after angioplasty with a 2.0-mm (left panel, $n = 9$) or a 2.5-mm (right panel, $n = 20$) balloon. Force expressed as a percentage of the contractions elicited by 0.35 μM phenylephrine, which were 5.3 ± 0.6 and 5.6 ± 0.6 g in control segments and 4.0 ± 0.8 and 3.2 ± 0.8 g in segments of arteries treated with 2.0- and 2.5-mm balloons, respectively. Means ± S.E.M. (vertical bars).

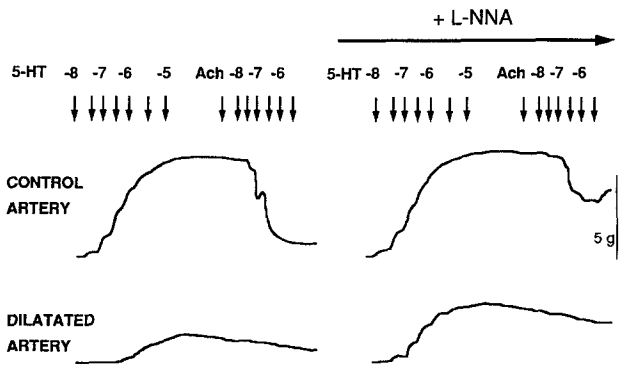

Fig. 4. Effect of the NO synthase inhibitor nitro-L-arginine on the responses to 5-hydroxytryptamine (5-HT) and acetylcholine (Ach). Representative tracings of a ring from a control artery (top panels) and a ring from the contralateral dilatated artery (bottom panels, 2.5-mm balloon) 3 weeks after angioplasty. The curves were obtained before (left panels) and after addition (right panels) of 30 μM nitro-L-arginine. 5-HT concentrations (10^{-8}–10^{-5} M) were given cumulatively and without changing the physiological salt solution; cumulative concentrations of acetylcholine (3×10^{-9}–3×10^{-6} M) were added to the organ bath.

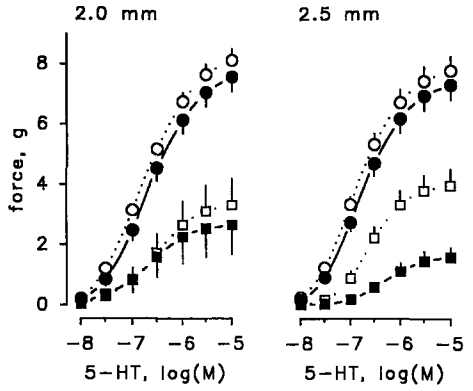

Fig. 5. Effect of 30 μM nitro-L-arginine (open symbols) on contractions elicited by 5-hydroxytryptamine (5-HT) in rings of control or balloon-treated carotid arteries 3 weeks after angioplasty with a 2.0-mm (left panel, $n = 9$) or a 2.5-mm (right panel, $n = 12$) balloon. ●, rings of control arteries without nitro-L-arginine; ○, rings of control arteries with nitro-L-arginine; ■, rings of balloon-treated arteries without nitro-L-arginine; □, rings of balloon-treated arteries with nitro-L-arginine. Means ± S.E.M. (vertical bars).

(Fig. 5). The latter arteries were also slightly less sensitive to 5-HT. Nitro-L-arginine normalized the pD_2 value, and significantly increased the E_{max} in arteries treated with the 2.5-mm balloon (Figs. 4 and 5, Table 6).

The relaxation elicited by acetylcholine was similar in the 2 sets of control curves, with respect to both maximum amplitude (Figs. 4 and 6) and pD_2 values (Table 6). In balloon-treated arteries the E_{max} became smaller, the effect being more pronounced in arteries treated with the larger balloon (Fig. 6). Moreover, in some segments the S-shape of the relaxation curves appeared to be lost. Neither balloon influenced the pD_2 values (Table 6). Nitro-L-arginine inhibited the amplitude of the acetylcholine relaxations to the same extent in both sets of control arteries, and in the

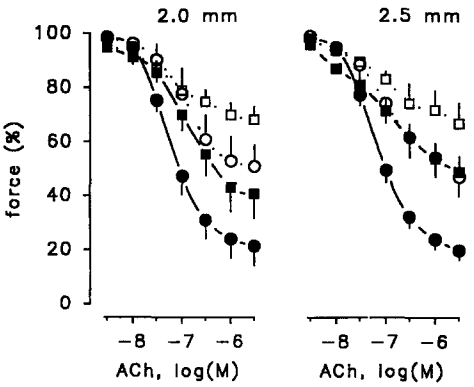

Fig. 6. Effect of 30 μM nitro-L-arginine (open symbols) on relaxations elicited by acetylcholine (ACh) in rings of control or balloon-treated carotid arteries 3 weeks after angioplasty with a 2.0-mm (left panel, $n = 9$) or a 2.5-mm (right panel, $n = 12$) balloon. ●, rings of control arteries without nitro-L-arginine; ○, rings of control arteries with nitro-L-arginine; ■, rings of balloon-treated arteries without nitro-L-arginine; □, rings of balloon-treated arteries with nitro-L-arginine. Force expressed as percentage of the contractions elicited by 10^{-5} M 5-HT, which are shown in Fig. 5. Means ± S.E.M. (vertical bars).

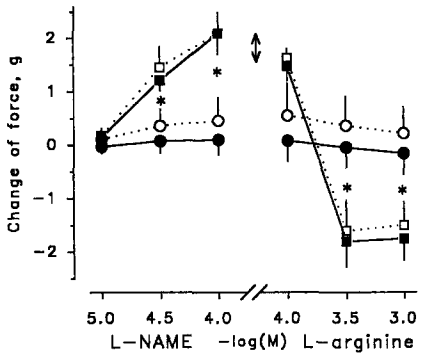

Fig. 7. L-NAME evokes contractions which are reversed by L-arginine in 0.35 μM phenylephrine-contracted rings of arteries obtained 3 weeks after angioplasty with a 2.5-mm balloon (■), but not in rings of contralateral control arteries (●). Removal of the endothelial cells (dotted lines) is without any effect. Rings of control arteries with (●) or without (○) endothelium; rings of balloon-treated arteries with (■) or without (□) endothelium. Means ± S.E.M. (vertical bars), $n = 6$. * $P < 0.05$, segments of balloon-treated arteries different from control arteries (ANOVA); ↕ $P < 0.05$, force after injection of 10^{-4} M L-arginine different from force evoked by 10^{-4} M L-NAME (paired Student's t-test).

arteries exposed to either large or small balloons. Nitro-L-arginine did not change the pD_2 values of acetylcholine in balloon-treated arteries, whereas a small decrease in sensitivity was seen in both groups of control arteries (Table 6).

3.3.3. *Effect of endothelium, L-NAME and L-arginine*

Vascular responses to L-NAME and L-arginine were assessed after constriction with phenylephrine (3.5×10^{-7} M) in arteries isolated 3 weeks after angioplasty, both with and without endothelium (Fig. 7). In control segments, the removal of the endothelium did not influence the contraction elicited by phenylephrine. Addition of L-NAME or L-arginine to the precontracted control segments did not modify this contraction (Fig. 7). As in the previous experiments, the balloon-treated arteries developed less force in response to phenylephrine. Also in arteries after angioplasty, endothelium removal did not influence the contraction elicited by phenylephrine. However, balloon-treated arteries developed marked concentration-dependent contractions in response to L-NAME, which were concentration dependently reversed by L-arginine. Neither the contractions elicited by L-NAME nor the relaxations elicited by L-arginine were affected by removal of the endothelial cells (Fig. 7).

4. Discussion

Coronary artery restenosis, occurring in approximately 30% of patients, remains the major limitation of balloon dilatation and other percutaneous coronary revascularization techniques (Serruys et al., 1988). Most pharmacological treatments which appeared to be promising in models employing balloon denudation with only minimal medial

injury have not been found to be effective in the prevention of restenosis in clinical trials (Popma et al., 1992). Balloon dilatation in the clinical setting, however, frequently induces extensive injury of the vascular wall (Haudenschild, 1994). Hence, we developed a model of severe arterial balloon injury, creating deep vascular wall damage, and studied the time course of the changes in the morphology and vascular reactivity of isolated segments. The absence of atherosclerotic lesions in the normal rabbit carotid artery can be regarded as a drawback of the present model. However, since human atherosclerotic lesions are predominantly eccentric, clinical balloon angioplasty has its major impact on the relatively normal, uninvolved segment as well (Haudenschild, 1994), thereby supporting the experimental study of the normal vessel wall.

4.1. Endothelial cell regrowth and von Willebrand factor expression

The histology demonstrated that balloon dilatation always caused a marked segmental loss of smooth muscle cells and created circular media tears. The extent of vascular injury was dependent on the size of the balloon, and was significantly more pronounced with 2.5-mm balloons. In most arteries, the fragmented internal elastic membrane remained partially covered by sparse islands of endothelial cells. These were identified as endothelial cells by their shape and position, the presence of nuclei, and their immunoreactivity to von Willebrand factor and platelet endothelial cell adhesion molecule-1, and the absence of immunoreactivity to α-smooth muscle cell actin. In the weeks after angioplasty, circular intima thickening consistently occurred on the luminal side of the internal elastic membrane, and was most prominent in the 2.5-mm balloon-treated arteries. The neointima became rapidly lined by a continuous layer of regenerating cuboidal endothelial cells. The presence of platelet endothelial cell adhesion molecule-1 and von Willebrand factor and the absence of α-smooth muscle cell actin again suggested their endothelial nature. The re-endothelialisation appeared to be complete after 3 weeks, which is a major difference from models employing endothelial denudation of arteries with Fogarty balloons (Grotendorst et al., 1981; Bernstein et al., 1982; Chesebro et al., 1987; Fingerle et al., 1989; Lindner et al., 1993).

The increased and flocculent immunoreactivity to von Willebrand factor in the regenerating endothelial cells and in the subendothelial matrix after dilatation parallels the findings in collar-induced intimal thickening (Kockx et al., 1993). In normal arteries the endothelial cells secrete von Willebrand factor towards both plasma and the subendothelial space, where minimal deposits of this glycoadhesive protein are present in the basal lamina (Houdijk et al., 1986). Some authors attribute an important function to the basal lamina von Willebrand factor in endothelial cell adhesion (Wagner, 1990; Sporn et al., 1989). Cultured endothelial cells can indeed be stimulated to release additional large amounts of von Willebrand factor under conditions of pressure or shear stress (Sporn et al., 1989). The effect of the increased subendothelial von Willebrand factor deposition on neointima formation remains speculative, but might by itself contribute to smooth muscle cell migration and neointima formation (Kockx et al., 1993). Anyhow, the von Willebrand factor data clearly indicate that the endothelial cells remain functionally different even weeks after restoration of a continuous monolayer.

4.2. Contractile responses

In view of the injury of the media it was not surprising that angioplasty attenuated the maximum contractions elicited by phenylephrine and 5-HT. In addition, phenotypic shifts of the smooth muscle from a contractile to a biosynthetic state (Antonaccio et al., 1994), increased 'rigidity' of the vessel wall resulting from neointima formation and thrombus incorporation, or alterations in receptor function (Candipan et al., 1994) could have compromised force development in the weeks following the initial insult. Contractility improved 10 weeks after angioplasty, but remained deficient. Although light microscopy did not point to gross defects at that stage, incomplete recovery of the functional integrity of the media remains a possible explanation.

In general, balloon denudation, which is less damaging to the media compared to balloon angioplasty, has little impact on initial force development (Joly et al., 1992; Antonaccio et al., 1994; Douglas et al., 1994; Tarry and Makhoul, 1994), though it subsequently induces non-specific hyporeactivity to almost every spasmogen (Joly et al., 1992; Antonaccio et al., 1994; Douglas et al., 1994). Desensitisation to 5-HT and phenylephrine was not consistently seen in the study of the time course, but this is presumably due to the fact that a rather limited number of arteries, with a restricted contractile capacity and a large inherent variability, were evaluated. Indeed, desensitisation to 5-HT was clearly seen in the second experiment, when more arteries were investigated 3 weeks after angioplasty with the larger balloon. For α-adrenoceptors the desensitisation has been attributed to facilitated adrenergic neurotransmission after angioplasty (Candipan et al., 1994), but the non-specific nature of the hyporeactivity led other authors to suggest that induction of nitric oxide release was responsible (Joly et al., 1992; Douglas et al., 1994, vide infra).

4.3. Endothelium-dependent relaxations

Despite the rapid and apparently complete regrowth of the endothelium, the maximum endothelium-dependent relaxation elicited by acetylcholine remained impaired up to at least 10 weeks after balloon angioplasty. This defect was probably not caused by a decreased sensitivity of the

smooth muscle cells in the injured segment to nitric oxide, since the endothelium-independent relaxations elicited by the spontaneous nitric oxide donor SIN-1 were not affected at all by angioplasty. The latter finding also suggested that —in spite of the decreased contractile capacity of balloon-treated segments—the processes responsible for nitric-oxide-mediated relaxation remained undisturbed. In contrast to SIN-1, the sensitivity to nitroglycerin was slightly, but consistently, depressed after angioplasty. Further studies are required to test whether this is due to decreased biotransformation of organic nitrates in smooth muscle cells recovering from severe media injury or to decreased diffusion of nitric oxide derived from nitroglycerin in the endothelial cells to the underlying smooth muscle as a consequence of neointima formation. However, as regards SIN-1, the maximum amplitude of nitroglycerin-induced relaxation was generally not affected, in contrast to the endothelium-dependent relaxations.

Taken together, these functional studies suggest that relaxations mediated by endothelium-derived nitric oxide remain disturbed for weeks after balloon angioplasty, even after regeneration of the endothelial cells. This confirms the persistent attenuation of receptor (acetylcholine)-mediated and non-receptor (calcium ionophore A-23187)-mediated endothelium-dependent relaxations after arterial injury, in spite of rapid endothelial regeneration (Weidinger et al., 1990). In another model of intimal thickening, in which endothelial cell injury and proliferation are much less important (Kockx et al., 1993), the impaired endothelium-dependent relaxations (De Meyer et al., 1991; Arthur and Dusting, 1992) are presumably due to a defect of the endothelial muscarinic receptors (De Meyer et al., 1991). Also in the hypercholesterolaemic rabbit, endothelium-dependent relaxations became progressively inhibited as the degree of fatty streak formation increased (Verbeuren et al., 1986), partly due to dysfunctional endothelial muscarinic receptors (Bult et al., 1995). The present data do not allow further differentiation between alterations of endothelial receptors, decreased activity of the constitutive endothelial NO synthase or diminished diffusion of nitric oxide towards the smooth muscle through the thickening neointima. However, the diminished non-receptor-mediated endothelium-dependent relaxations after arterial injury reported by Weidinger et al. (1990) suggest that the activity of the endothelial NO synthase decreased or that the developing neointima represented a diffusion barrier.

In addition to regulating vasomotor tone, nitric oxide may inhibit or promote vascular smooth muscle cell proliferation (Graf, 1993; Scott-Burden and Vanhoutte, 1993). The dysfunctional endothelium, regenerating after angioplasty, may thus contribute to an imbalance between growth-promoting and growth-inhibiting factors, and thus favour 'restenosis', which is one of the major problems associated with this procedure. Angiographic data indeed demonstrated a relationship between impaired endothelium-dependent relaxations and the extent of restenosis after angioplasty of human coronary arteries (Bertrand et al., 1989).

4.4. Induction of non-endothelial NO synthase

Despite the dysfunctional responses of the endothelial NO synthase, the augmented sensitivity and force development in response to 5-HT by the NO synthase inhibitor nitro-L-arginine, and the marked contractions evoked by L-NAME, another NO synthase inhibitor, clearly suggest the induction of NO synthase (iNOS) in the arterial wall, although its activity was not measured directly. Isolated rabbit macrophages fail to express inducible NO synthase in response to stimuli which are operative in rat cells (Hey et al., 1995), but NO synthase activity has previously been documented after development of atherosclerotic lesions in the aorta of hypercholesterolaemic rabbits (Verbeuren et al., 1993). In contrast to the rat carotid artery, in which NO synthase is induced after superficial injury evoked by balloon denudation (Joly et al., 1992; Douglas et al., 1994; Hansson et al., 1994), NO synthase activity has not yet been reported in rabbit arteries exposed to either balloon denudation or after the more severe injury (type III) evoked by balloon dilatation. The more marked activity in arteries treated with 2.5-mm balloons as compared to 2-mm balloons indicated that the expression of the non-endothelial NO synthase was related to the severity of the initial insult. In addition, the activity persisted for weeks after angioplasty, whereas both mRNA for (Hansson et al., 1994) and the activity of inducible NO synthase (Douglas et al., 1994) disappear 2 weeks after denudation of the rat carotid. The spasmogenic activity of L-NAME in balloon-treated arteries was not only abolished by L-arginine, but even reversed to relaxations at higher concentrations of the NO synthase substrate, indicating that endogenous substrate availability is a limiting factor for maximum activity of the inducible enzyme in the isolated artery. Since none of these effects was observed in control arteries, a contribution of the constitutive NO synthase, resulting from endothelial cell activation by serotonin or phenylephrine, seems unlikely. Indeed, the effects of arginine and L-NAME were not influenced at all by removal of the endothelial cells from balloon-treated arteries. This further indicates that the activity of the inducible NO synthase was predominantly present in non-endothelial parts of the arterial wall. Denudation of the rat carotid artery induces immediate activity in the media (Joly et al., 1992), and subsequently NO synthase mRNA is transiently expressed in neointimal smooth muscle cells, particularly at the surface of the lesion (Hansson et al., 1994). Whether balloon angioplasty induced mRNA expression in macrophages or smooth muscle cells present in the neointima, the organizing mural thrombi or the media cannot be determined from the present study.

The induction of NO synthase and its stimulation by supplementation with exogenous substrate may partly ex-

plain the observation that administration of L-arginine reduces intimal hyperplasia following superficial injury by balloon denudation in rats (McNamara et al., 1993) and normolipidaemic rabbits (Tarry and Makhoul, 1994). Moreover, Von der Leyen et al. (1995) have shown that neointima formation in the rat carotid artery is attenuated by restoring endothelial cell NO synthase expression in the vessel wall by using a highly efficient Sendai virus/liposome gene transfer technique. Furthermore, L-arginine administration can suppress the development of fatty streaks and induces regression of pre-existing lesions (Candipan et al., 1996), whereas L-NAME augments atheroma development (Cayatte et al., 1994; Naruse et al., 1994) in the thoracic aorta of hypercholesterolaemic rabbits. Nitric oxide donors such as SPM-5185 and the SIN-1 precursor molsidomine have been found to inhibit intimal thickening in rat (Guo et al., 1994) and rabbit (De Meyer et al., 1995) following superficial injury. However, the observation that SIN-1 administration inhibits platelet adhesion (Groves et al., 1993) and smooth muscle cell replication (Groves et al., 1995) after deep vascular injury evoked by angioplasty of pig coronary arteries without influencing intimal thickening (Groves et al., 1995) indicates that the results obtained after superficial injury may not be representative of clinical benefit of molsidomine administration following angioplasty and thus await confirmation in a large scale, randomized, double-blinded trial. Since balloon dilatation is performed at sites of atherosclerosis where endothelial function (i.e., NO release) is already known to be attenuated, and smooth muscle cells and macrophages resident within the lesion could possibly express inducible nitric oxide synthase, effects of angioplasty remain uncertain in humans.

In conclusion, these results indicate that although the endothelium quickly regenerates after severe balloon injury, and demonstrates high immunoreactivity for von Willebrand factor, it remains functionally disturbed with respect to the endothelium-dependent release of nitric oxide. However, angioplasty led to a marked 'non-endothelial' induction of NO synthase. This may be a 'protective' mechanism for the maintenance of arterial patency (Cooke and Tsao, 1994), by inhibiting smooth muscle cell proliferation and by counteracting the effect of contractile agonists like platelet-delivered 5-HT.

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