Accumulation of endogenous inhibitors for nitric oxide synthesis and decreased content of L-arginine in regenerated endothelial cells

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1 We examined regeneration of endothelial cells (ECs), neointima formation, decreased endothelium-dependent relaxation (EDR) and changes in the contents of L-arginine, N^G-monomethyl-L-arginine (L-NMMA), asymmetrical N^G, N^G-dimethylarginine (ADMA) and symmetrical N^G,N^G-dimethylarginine (SDMA) in the regenerated ECs, 6 weeks after balloon denudation of the rabbit carotid artery.

2 Regeneration of ECs was completed in 6 weeks and a significant neointima formation accompanied by the decreased EDR was observed.

3 L-NMMA and ADMA contents in the regenerated ECs (23.5 ± 4.3 and 21.2 ± 2.0 pmol mg⁻¹ DNA, respectively) were significantly ($P < 0.05$ and $P < 0.01$) higher than those in the control ECs (8.8 ± 3.0 and 7.4 ± 1.9 pmol mg⁻¹ DNA, respectively), whereas L-arginine was significantly ($P < 0.005$) decreased in the regenerated ECs ($31,470 \pm 1,050$ pmol mg⁻¹ DNA) as compared to that in the control ECs ($47,870 \pm 1,890$ pmol mg⁻¹ DNA). SDMA content was below the assay limits.

4 L-NMMA and ADMA, but not SDMA, inhibited the EDR induced by acetylcholine in a concentration-dependent manner. The inhibition with L-NMMA and ADMA was prevented by an addition of L-arginine, but not by D-arginine.

5 These results suggest that the accumulation of endogenous inhibitors for nitric oxide synthesis and decreased L-arginine content are associated with decreased NO production/release from regenerated ECs and neointima formation.

Keywords: Neointima formation; endogenous inhibitors for NO synthesis; balloon denudation; decreased endothelium-dependent relaxation; EDRF/NO

Introduction

As proposed by Ross & Glomset (1976a,b) and Ross (1986), denudation and nondenudation intimal injuries may play an important role in the development of atherosclerotic vascular disease (Ip *et al.*, 1990). We have reported that restoration of the endothelium after balloon denudation of the rabbit carotid artery was completed in 6 weeks and that regenerated ECs were less capable of producing/releasing the endothelium-derived relaxing factor (EDRF/nitric oxide: NO) (Azuma *et al.*, 1990; 1992; Niimi *et al.*, 1994). EDRF/NO inhibits platelet adhesion and aggregation (Azuma *et al.*, 1986; Radomski *et al.*, 1987), which is considered as an initiating event for neointima formation (Ross, 1986; Schwartz *et al.*, 1981). It has also been demonstrated that NO inhibits smooth muscle cell proliferation (Garg & Hassid, 1989).

These findings when considered together suggest that the neointima formation after endothelial removal might be caused at least partly through impairment of the ability of ECs to produce/release EDRF/NO. However, the mechanism by which the production/release of EDRF/NO is reduced remains unclear. Recently, Vallance *et al.* (1992) have obtained evidence N^G-monomethyl-L-arginine (L-NMMA) and asymmetrical N^G,N^G-dimethyl arginine (ADMA) play a role as endogenous inhibitors of NO synthesis. The present experiments were, therefore, designed to determine the regeneration of ECs, the neointima formation, the decreased EDR and the changes in content of L-arginine, L-NMMA, ADMA and symmetrical N^G,N^G-dimethylarginine (SDMA) in the regenerated ECs after balloon denudation of the rabbit carotid artery.

Methods

Endothelial removal

Endothelial removal of the rabbit carotid artery was performed according to the method described previously (Azuma *et al.*, 1990). Briefly, the right common carotid artery of Japanese White male rabbit was exposed under anaesthesia with sodium pentobarbitone (25 mg kg⁻¹, i.v.) by aseptic surgery. An embolectomy catheter (12-040-3F/40 cm/3F, American Edwards Laboratories, Santa Ana, CA, U.S.A.) was then inserted into the proximal portion of the artery in retrograde fashion through a small nick made near the carotid bifurcation. The luminal surface of the artery from the origin to the balloon entrance was then gently rubbed back-and-forth with the balloon filled with 0.15 ml of air. This method of removal of ECs did not cause deep injury of the vascular wall (Azuma *et al.*, 1990; 1992; Niimi *et al.*, 1994). After the balloon was removed, the arterial incision was sutured with 10-0 nylon thread, keeping patency of the artery. The left carotid artery, underwent a sham operation and served as a control. At 6 weeks after the balloon denudation, animals were anaesthetized with sodium pentobarbitone and exsanguinated via the femoral arteries. This study complied with the Animal Welfare Regulations of Tokyo Medical and Dental University and the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society.

Morphological examinations

Scanning electron microscopic examinations were performed on the previously denuded and sham-operated carotid arteries. The method of preparing the specimens is described elsewhere (Azuma *et al.*, 1990). The density of normal and regenerated

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ECs was determined by scanning electron microscopy (ESM-3200, Elionics). To confirm the endothelial origin of the lining cells, vessels were stained with von Willebrand factor antibody (rabbit antihuman von Willebrand factor, Dako) using the labelled streptavidin-biotin staining technique (Dako LSAB kit) (Niimi *et al.*, 1994). Light microscopic observations were also made (Azuma *et al.*, 1990). To provide a semiquantitative parameter for assessing neointima formation, the intimal area and medial area were determined by means of an image analyzer (Quadra-950, Macintosh and Image scanner, Scanjet IIC, Hewlett Packard). The ratio (%) of the intimal area to the medial area was calculated.

Measurement of mechanical responses

Both common carotid arteries were isolated and immersed in cold modified Krebs solution. A transverse strip, 2 mm in width, was cut off with a razor blade and mounted vertically in an organ bath containing 5 ml modified Krebs solution continuously bubbled with 95% O₂ and 5% CO₂ at 37 ± 0.5°C. Special care was taken to avoid unintentional rubbing of the intraluminal surface. The mechanical responses were determined according to the method described elsewhere (Azuma *et al.*, 1990). To compare the EDR to acetylcholine (ACh) between the previously denuded right and sham-operated left carotid artery strips, E_{max} (% maximum response) and -log ED₅₀ (-log concentration producing 50% of the E_{max}) were obtained from the log concentration-relaxation curves to ACh. Relaxation induced by ACh was expressed as a percentage of the 10⁻⁶ M noradrenaline (NA)-induced contraction. Three different concentrations (3 × 10⁻⁵, 10⁻⁴ and 3 × 10⁻⁴ M) of authentic L-NMMA (Sigma) and ADMA, and 3 × 10⁻⁴ M SDMA (Sigma) were tested against ACh-induced EDR. Potency of these inhibitors was compared as E_{max} of the ACh-induced EDR and -log ED₅₀ values of ACh in the absence or presence of the inhibitors. ADMA was synthesized in our laboratory according to the method described by Kakimoto & Akazawa (1970).

Determination of L-arginine, L-NMMA, ADMA and SDMA

Contents of L-arginine, L-NMMA, ADMA and SDMA in the regenerated and control ECs were determined by means of automated high-performance liquid chromatography (h.p.l.c.) according to the method described by Villanueva *et al.* (1987) with minor modification. The automated analytical system was equipped with a solvent selection system (LV-980-03), 2 h.p.l.c. pumps (PU-980), an autosampler (AS-950), a column oven (CO-960), a fluorescence detector (FP-920) and an integrator (807-IT, all from Jasco, Tokyo). ECs were collected by gently rubbing the innermost surface of the previously denuded right and sham-operated left carotid arteries with a microspatula in 5 M ice-cold HEPES buffer (pH 7.4) and then sonicated at 20 W for 15 s (Sonifire 250, Branson), followed by an addition of trichloroacetic acid at a final concentration of 5%. Arterial strips from which endothelium had been deliberately removed were devoid of ECs on the intimal surface, and the intact subendothelial layer was identified by scanning electron microscopy. The homogenate was centrifuged at 1,300 g for 10 min. Supernatant was separated and 100 µl aliquot of the supernatant was applied for h.p.l.c. The composition of the eluting buffers and the elution time are shown in Table 1. The buffers were prepared from freshly distilled and deionized water, adjusted to the required pH and filtered through a Millipore filter (Type HA, 0.45 µm pore size; Millipore Waters). The flow-rates were 0.55 ml min⁻¹ for the buffers and 0.33 ml min⁻¹ for the orthophthalaldehyde fluorogenic reagent (Nakalai Chemicals Co., Kyoto, Japan). One column (Aapak Li, 6.0mmIDx100mm, Jasco) temperature, 60°C, was used during the analyses. Regeneration of the column was achieved in 15 min with a solution of 0.6 M lithium hydroxide and re-equilibration was completed in 60 min with the first buffer

(0.1 N lithium citrate, pH 6.06). The column back-pressure generated during analyses was 28 kg cm⁻². The chromatograms and retention times of the authentic L-arginine, ADMA, L-NMMA, SDMA and paraphenylenediamine dihydrochloride (Wako Pure Chemicals) used as an internal standard are shown in Figure 1a. The equations of relationships between the concentrations (Y) of authentic compounds and the ratio (X: response factor) of the fluorescence peak area of the authentic compounds to that of the internal standard were determined as follows: Y = 59.38X - 0.515 (r = 0.9995) for L-arginine, Y = 71.26X - 0.437 (r = 0.9995) for ADMA, Y = 28.45X - 0.427 (r = 0.9996) for L-NMMA, and Y = 71.65X - 0.233 (r = 0.9998) for SDMA. The concentration of each compound in the ECs was determined by the equation described above after estimating the response factor (X) of the peak corresponding to the retention time of authentic compounds.

Determination of DNA

The microassay of DNA was performed according to the fluorometric method described by Kissane & Robins (1958). Protein concentration was measured with bovine serum albumin as a standard by the method of Lowry *et al.* (1951).

Table 1 Composition and conditions of eluting buffers

Buffer	Lithium citrate (N)	pH	Elution time (min)
1	0.1	6.06	8
2	0.6	6.85	8
3	1.1	7.00	25
4	1.4	7.05	70

Figure 1 Chromatographic analysis of a standard mixture of L-arginine and its methylated derivatives (a) and crude extract of regenerated ECs (b). Retention time (RT) is given as mean ± s.e. mean of 6 determinations on a mixture containing 200 pmol of each of the authentic compounds (a). Total of 6 determinations for the crude extract of the control (n = 3) and regenerated ECs (n = 3) were averaged (b). Paraphenylenediamine (11 nmol) was used as an internal standard (IS). The column and the elution programme employed gave a good overall resolution.

a	Peak	Compound	RT (min)	b	Peak	RT (min)
	1	IS	37.7 ± 0.12 (6)		1	37.7 ± 0.13 (6)
	2	L-Arginine	71.6 ± 0.05 (6)		2	71.6 ± 0.04 (6)
	3	L-NMMA	80.1 ± 0.04 (6)		3	80.1 ± 0.04 (6)
	4	ADMA	82.3 ± 0.04 (6)		4	82.3 ± 0.05 (6)
	5	SDMA	86.6 ± 0.03 (6)			

Statistical analysis

Values are expressed as mean $\pm$ s.e.mean. The statistical analysis was carried out by a two way analysis of variance (ANOVA). If the mean values were found to be different at $P < 0.05$ by means of ANOVA, either Scheffe or Student's *t* test was also applied.

Results

Morphological findings

In the sham-operated left carotid artery (under control conditions), scanning electron microscopy revealed that the luminal surface was completely covered with ECs, which were regularly arranged parallel to the direction of the blood stream. Six weeks after denudation, a full endothelial lining on the luminal surface was observed. However, the regenerated ECs were morphologically different from native ones; they were irregularly oriented. The density (cells mm^{-2}) of regenerated ECs was $9,540 \pm 317$ ($n = 5$), a value which was significantly ($P < 0.005$) different from $6,493 \pm 248$ ($n = 5$) in the control.

In the light microscopic observations of the cross sections of vascular wall, no noticeable changes could be detected in the control and the intima media⁻¹ ratio (%) was estimated to be 4.8 ± 0.6 ($n = 5$). Six weeks after denudation, a marked neointima formation was detectable. The intima media⁻¹ ratio was $38.7 \pm 8.2\%$ ($n = 5$), which was significantly ($P < 0.005$) different from the value in the control.

Endothelium-dependent relaxation (EDR) to ACh

ACh ($10^{-9} \sim 3 \times 10^{-6}$ M) produced a concentration-dependent relaxation during a contraction with 10^{-6} M NA. The ACh (10^{-6} M)-induced relaxation was abolished after the *in vitro* denudation of ECs and greatly inhibited by the pretreatment of

strips with N^G-nitro-L-arginine (10^{-4} M) and methylene blue (3×10^{-5} M), but was unaffected by indomethacin (10^{-5} M), indicating that the relaxation might be dependent on the endothelium and mediated by EDRF/NO, but not by prostacyclin. Comparison of the E_{max} and $-\log \text{ED}_{50}$ values between the control left and the previously denuded right carotid arteries showed that the EDR was greatly reduced in the latter. The E_{max} and $-\log \text{ED}_{50}$ were determined to be $72.4 \pm 7.8\%$ ($n = 5$) and 7.38 ± 0.07 ($n = 5$), respectively, in the control, and $18.9 \pm 2.6\%$ ($P < 0.005$, vs. control, $n = 5$) and 5.78 ± 0.09 ($P < 0.005$ vs. control, $n = 5$), respectively, in the previously denuded artery. The magnitude of precontraction with 10^{-6} M NA was not significantly different between the two groups [854 ± 169 mg in the control ($n = 5$) and 826 ± 172 mg in the previously denuded artery ($n = 5$)].

Contents of DNA, L-arginine, L-NMMA, ADMA and SDMA in ECs

The DNA content in the regenerated ECs (176.7 ± 4.7 μg mg^{-1} protein, $n = 3$) was significantly ($P < 0.005$) greater than that in the control (131.2 ± 4.8 μg mg^{-1} protein, $n = 3$). The changes in the L-arginine, L-NMMA, ADMA and SDMA contents are given in terms of pmol mg^{-1} DNA and shown in Table 2. L-NMMA and ADMA contents were significantly ($P < 0.05$ and $P < 0.01$) more increased in the regenerated ECs than in the control ECs. On the other hand, L-arginine contents showed a significantly ($P < 0.0005$) greater decrease in the regenerated ECs. The content of SDMA was below the assay limits.

Inhibitory activities of L-NMMA, ADMA and SDMA on the ACh-induced EDR

The inhibitory activities of L-NMMA, ADMA and SDMA are given in terms of E_{max} (%) and $-\log \text{ED}_{50}$ and shown in

Table 2 Comparison of L-arginine, N^G-monomethyl-L-arginine (L-NMMA), asymmetrical N^G,N^G-dimethylarginine (ADMA) and symmetrical N^G,N^G-dimethylarginine (SDMA) contents in the endothelial cells (ECs)

	n	L-Arginine	Tissue content (pmol mg^{-1} DNA)		
			L-NMMA	ADMA	SDMA
Control ECs	3	$47,870 \pm 1,890$	8.8 ± 3.0	7.0 ± 1.9	—
Regenerated ECs	3	$31,470 \pm 1,050^{***}$	$23.5 \pm 4.3^*$	$21.2 \pm 2.0^{**}$	—

Results are given as mean $\pm$ s.e.mean. Significant difference vs. control at $*P < 0.05$, $**P < 0.01$ and $***P < 0.005$. —: below the assay limitation by the h.p.l.c method.

Table 3 Inhibitory activities of N^G-monomethyl-L-arginine (L-NMMA), asymmetrical N^G,N^G-dimethylarginine (ADMA) and symmetrical N^G,N^G-dimethylarginine (SDMA) on the acetylcholine (ACh)-induced endothelium-dependent relaxation (EDR) in the control carotid artery strips

Treatment	Concentration (M)	n	ACh-induced EDR	
			E_{max} (%)	$-\log \text{ED}_{50}$
L-NMMA	—	5	76.9 ± 4.3	7.44 ± 0.13
	3×10^{-5}	5	$51.6 \pm 8.8^*$	$7.05 \pm 0.04^*$
	1×10^{-4}	5	$40.7 \pm 5.1^{***}$	$7.00 \pm 0.01^{**}$
	3×10^{-4}	5	$34.3 \pm 2.4^{***}$	$6.97 \pm 0.02^{**}$
ADMA	—	5	79.3 ± 3.7	7.45 ± 0.13
	3×10^{-5}	5	68.2 ± 5.1	7.22 ± 0.07
	1×10^{-4}	5	$58.9 \pm 7.3^*$	$7.12 \pm 0.06^*$
	3×10^{-4}	5	$48.0 \pm 7.2^{***}$	$6.98 \pm 0.01^{***}$
SDMA	—	5	77.6 ± 3.7	7.45 ± 0.19
	3×10^{-4}	5	77.9 ± 3.4	7.46 ± 0.21

Results are given as mean $\pm$ s.e.mean. As a result of ANOVA followed by Schaffe's test, $P < 0.01$ for control vs. 10^{-4} M L-NMMA and $P < 0.005$ for control vs. 3×10^{-4} M L-NMMA, $P < 0.05$ for control vs. 10^{-4} M ADMA and $P < 0.005$ for control vs. 3×10^{-4} M ADMA. Significant difference vs. corresponding control at $*P < 0.05$, $**P < 0.01$ and $***P < 0.005$, respectively (Student's *t* test). Inhibitory potency was expressed as E_{max} of the ACh-induced EDR and $-\log \text{ED}_{50}$ of ACh in the absence or presence of authentic L-NMMA, ADMA or SDMA. —: No treatment with authentic compound.

Table 3. The ACh-induced EDR was inhibited by L-NMMA and ADMA in a concentration-dependent manner, but not by SDMA at the highest concentration of 3×10^{-4} M. The activity in the reduction of $E_{\max}$ and ED_{50} values with L-NMMA was slightly more potent than that with ADMA. L-NMMA (3×10^{-4} M) and ADMA (3×10^{-4} M) did not produce inhibition of ACh-induced EDR in the presence of 3×10^{-3} M L-arginine. D-Arginine (3×10^{-3} M) was without effect on the inhibitory activities of L-NMMA and ADMA.

Discussion

Six weeks after removal of the endothelium from the rabbit carotid artery, the endothelial lining was re-established with significantly increased density of the regenerated ECs. Significant impairment of the ACh-induced EDR was accompanied by a marked neointimal formation. All these findings were similar to those in our previous papers (Azuma *et al.*, 1990; 1992; Niimi *et al.*, 1994). We have demonstrated that the impairment of the ACh-induced EDR is due to reduction in the production/release of EDRF/NO by the regenerated ECs and that the capacity to produce/release EDRF/NO per cell greatly reduces since the cell density had significantly increased over the normal (Azuma *et al.*, 1990; Niimi *et al.*, 1994). However, the mechanism by which impairment of the production/release of EDRF/NO is caused remains unclear. In the present experiments, we discovered that contents of L-NMMA and ADMA, but not of SDMA, were significantly higher in the regenerated ECs. L-NMMA and ADMA, but not SDMA, inhibited the ACh-induced EDR. The inhibition of ACh-induced EDR with the two methylarginine derivatives was prevented by addition of L-arginine, but not by D-arginine. These results combined with the evidence demonstrating that L-NMMA and ADMA play a role as endogenous inhibitors of NO synthesis (Vallance *et al.*, 1992) suggest that the reduced production/release of EDRF/NO in the regenerated ECs is at

least partly due to the accumulation of the endogenous inhibitors of NO synthesis. The content of L-arginine as a substrate for NO synthesis was significantly decreased in the regenerated ECs. This may partly account for the decreased production/release of NO in the regenerated ECs. It should be noted, however, that even in the regenerated ECs, the ratio of endogenous inhibitors to L-arginine was small. Therefore, it remains to be determined if these levels of L-NMMA and ADMA are able to inhibit NO synthesis in control cells and whether the degree of inhibition is enhanced in regenerated ECs in which accumulated levels of the inhibitors increased. Further studies on this aspect are in progress in our laboratories.

It is well established that EDRF/NO is an anti-aggregating substance (Azuma *et al.*, 1986; Radomski *et al.*, 1987), which is considered as an initiating event for neointima formation (Schwartz *et al.*, 1981; Ross, 1986) and it has been suggested by Garg & Hassid (1989) that endogenous EDRF/NO may act as an inhibitory modulator of vascular smooth muscle cell mitogenesis. In addition, the increase in plasma endothelin-1, a potent mitogen (Hirata *et al.*, 1989), is thought to reflect a rise in vascular production of the peptide in response to the impaired release of EDRF/NO from ECs (Boulanger & Lüscher, 1990). We have reported that the increased level of endothelin-1 in the vascular wall accompanied by a decreased production/release of EDRF/NO would be involved in neointima formation (Niimi *et al.*, 1994; Azuma *et al.*, 1994). If these findings are considered together, they suggest that the accumulation of endogenous inhibitors for NO synthesis is associated with the decreased NO production/release from regenerated ECs and neointima formation after endothelial removal in the rabbit carotid artery.

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References

- AZUMA, H., FUNAYAMA, N., KUBOTA, T. & ISHIKAWA, M. (1990). Regeneration of endothelial cells after balloon denudation of the rabbit carotid artery and changes in responsiveness. *Jpn. J. Pharmacol.*, **52**, 541–552.
- AZUMA, H., HAMASAKI, H., NIIMI, Y., TERADA, T. & MATSUBARA, O. (1994). Role of endothelin-1 in neointima formation after endothelial removal in rabbit carotid arteries. *Am. J. Physiol.*, **267** (*Heart Circ. Physiol.*, **36**), H2259–H2267.
- AZUMA, H., ISHIKAWA, M. & SEKIZAKI, S. (1986). Endothelium-dependent inhibition of platelet aggregation. *Br. J. Pharmacol.*, **88**, 411–415.
- AZUMA, H., NIIMI, Y. & HAMASAKI, H. (1992). Prevention of intimal thickening after endothelial removal by a nonpeptide angiotensin II receptor antagonist, losartan. *Br. J. Pharmacol.*, **106**, 665–671.
- BOULANGER, C. & LÜSCHER, T.F. (1990). Release of endothelin from the porcine aorta, inhibition by endothelium-derived nitric oxide. *J. Clin. Invest.*, **85**, 587–590.
- GARG, U.C. & HASSID, A. (1989). Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J. Clin. Invest.*, **83**, 1774–1777.
- HIRATA, Y., TAKAGI, Y., FUKUDA, Y. & MARUMO, F. (1989). Endothelin is a potent mitogen for rat vascular smooth muscle cells. *Arteriosclerosis*, **78**, 225–228.
- IP, J.H., FUSTER, V., BADIMON, L., BADIMON, J., TABMAN, M.B. & CHESEBRO, J.H. (1990). Syndromes of accelerated atherosclerosis. *Am. J. Coll. Cardiol.*, **15**, 1667–1687.
- KAKIMOTO, Y. & AKAZAWA, S. (1970). Isolation and identification of N^G, N^G - and N^G, N^G -dimethylarginine, N^G -mono, di-, and trimethyllysine, and glucosylgalactosyl- and galactosyl- δ -hydroxylysine from human urine. *J. Biol. Chem.*, **245**, 5751–5758.
- KISSANE, J.M. & ROBINS, E. (1958). The fluorometric measurement of deoxyribonucleic acid in animal tissue with special reference to the central nervous system. *J. Biol. Chem.*, **233**, 184–188.
- LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.
- NIIMI, Y., AZUMA, H. & HIRAKAWA, K. (1994). Repeated endothelial removal augments intimal thickening and attenuates EDRF release. *Am. J. Physiol.*, **266** (*Heart Circ. Physiol.*, **35**), H1348–H1356.
- RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1987). The antiaggregating properties of vascular endothelium: interaction between prostacyclin and nitric oxide. *Br. J. Pharmacol.*, **92**, 639–646.
- ROSS, R. (1986). The pathogenesis of atherosclerosis - an update. *N. Engl. J. Med.*, **314**, 488–500.
- ROSS, R. & GLOMSET, J.A. (1976a). The pathogenesis of atherosclerosis (first of two parts). *N. Engl. J. Med.*, **295**, 369–377.
- ROSS, R. & GLOMSET, J.A. (1976b). The pathogenesis of atherosclerosis (second of two parts). *N. Engl. J. Med.*, **295**, 420–425.
- SCHWARTZ, S.M., GAJDUSEK, C.M. & SELDEN, S.C. (1981). Vascular wall growth control: the role of the endothelium. *Arteriosclerosis*, **1**, 107–171.
- VALLANCE, P., LEONE, A., CALVER, A., COLLIER, J. & MONCADA, S. (1992). Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet*, **339**, 572–575.
- VILLANUEVA, V.R., MARDON, M., LE GOFF, M.Th. & MONCELON, F. (1987). Improved high-performance liquid chromatographic analysis of methylated amino acids and related compounds and its application to the analysis of samples of polluted healthy and damaged *Picea* needles without extract purification. *J. Chromatogr.*, **393**, 107–113.

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