British Journal of Pharmacology (1998) 123, 906–910

Inhibition of glycosphingolipid synthesis by threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) and the modulation of IL-1β-stimulated expression of inducible nitric oxide synthase in rat aortic smooth muscle cells

[1]Artur-Aron Weber, Claudia Seul, Volker Harth, Yon Ko, Stefan Seewald, Hans Vetter & [2]Agapios Sachinidis

Medizinische Universitätspoliklinik Bonn, Germany

1 The composition of glycosphingolipids is altered in atherosclerotic tissue. In order to study the possible modulation of interleukin-1β (IL-1β)-induced expression of inducible nitric oxide synthase (iNOS) by endogenously synthesized glycosphingolipids, we investigated rat aortic vascular smooth muscle cells (VSMC) grown in the presence of the inhibitor of glycosphingolipid synthesis, threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP).

2 Depletion of glycosphingolipids by PDMP (20–30 μM) was demonstrated by thin-layer chromatography of D-[1-^{14}C]-galactose- or L-[-U^{14}C]-serine-labelled glycosphingolipids. Nitrite generation was measured by the diaminonaphthalene assay, nitric oxide was determined by the oxyhaemoglobin technique and iNOS protein was detected by immunocytochemistry.

3 In VSMC grown in the presence of PDMP, the glycosphingolipid content was reduced by 30–50%. In PDMP-treated VSMC, IL-1β (3 u ml^{-1})-stimulated release of nitrite (135 ± 4 nmol mg^{-1} protein 48 h^{-1}) was significantly increased as compared to IL-1β-stimulated control cells (40 ± 3 nmol mg^{-1} protein 48 h^{-1}; $n=6$, $P<0.001$). Similarly, IL-1β (3 u ml^{-1}, 36 h)-stimulated release of nitric oxide was higher in PDMP-treated VSMC (6.1 ± 0.5 nmol mg^{-1} protein h^{-1}) as compared to untreated cells (2.0 ± 0.6 nmol mg^{-1} protein h^{-1}; $n=3$, $P<0.01$). These findings were confirmed by the demonstration of increased expression of iNOS protein (14.9 ± 1.2% vs 6.4 ± 0.2%; $n=4$, $P<0.001$), as shown by immunocytochemistry.

4 Evidence is presented that endogenous glycosphingolipids are important modulators of cytokine-induced iNOS expression. In view of an altered glycosphingolipid profile in atherosclerotic arteries, these mechanisms might be of relevance for the pathogenesis of atherosclerosis and restenosis subsequent to vessel injury.

Keywords: Atherosclerosis; glycosphingolipids; vascular smooth muscle cells; nitrite; nitric oxide; iNOS

Introduction

Inflammatory cytokines such as interleukin-1β (IL-1β) can stimulate the expression of the inducible nitric oxide synthase (iNOS) resulting in an increased formation of nitric oxide (NO) by vascular smooth muscle cells (VSMC), as well as by many other cell types (Busse & Mülsch, 1990; Beasley *et al*., 1991; Schini *et al*., 1991; Busse & Fleming, 1996). Generation of NO in injured vessels may contribute to the restoration of normal vascular functions. NO may prevent local vasospasms (Schini *et al*., 1991), reduce excessive VSMC proliferation (Scott-Burden *et al*., 1992), and inhibit platelet function (Durante *et al*., 1991). Thus, NO is considered to be a major determinant in the pathogenesis of cardiovascular diseases such as atherosclerosis or restenosis subsequent to balloon angioplasty (Loscalzo & Welch, 1995; Busse & Fleming, 1996).

The induction of iNOS in VSMC by cytokines is controlled by growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor (Scott-Burden *et al*., 1992), insulin-like growth factor (Schini *et al*., 1994), and also by blood-derived products such as thrombin (Schini-Kerth *et al*., 1995). In contrast, very little is known about the modulation of iNOS expression by reorganization of endogenous cellular lipids that occurs in atherosclerotic vessels. Interestingly, cholesterol enrichment of arterial smooth muscle cells by cationized low density lipoprotein (LDL) has been shown to increase cytokine induced nitrite production (Pomerantz *et al*., 1993).

Although cholesterol is the major lipid accumulating in atherosclerotic lesions (Anitschkow & Chalatov, 1983), it is known that the composition of minor lipid components such as glycosphingolipids is also changed in atherosclerotic tissue (Breckenridge *et al*., 1975). Recently, a quantification of glycosphingolipid concentrations in human unlesioned and atherosclerotic aortae revealed a distinct distribution pattern of glycosphingolipids in atherosclerotic tissue as compared to unlesioned regions (Mukhin *et al*., 1995).

In recent years, the role of endogenous glycosphingolipids for cellular functions was sudied by the use of the glucosylceramide synthase inhibitor, threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) (Shayman *et al*., 1990; Barbour *et al*., 1992; Rani *et al*., 1995).

The present study was initiated to investigate the possible role of endogenous glycosphingolipids in IL-1β-induced expression of iNOS. Evidence is presented that inhibition of glycosphingolipid synthesis by PDMP results in a marked

[1]Present address: Institut für Pharmakologie, Heinrich-Heine-Universität Düsseldorf, Germany. [2]Author for correspondence at: Medizinische Universitätspoliklinik, Willhelmstr. 35–37, D-53111 Bonn, Germany.

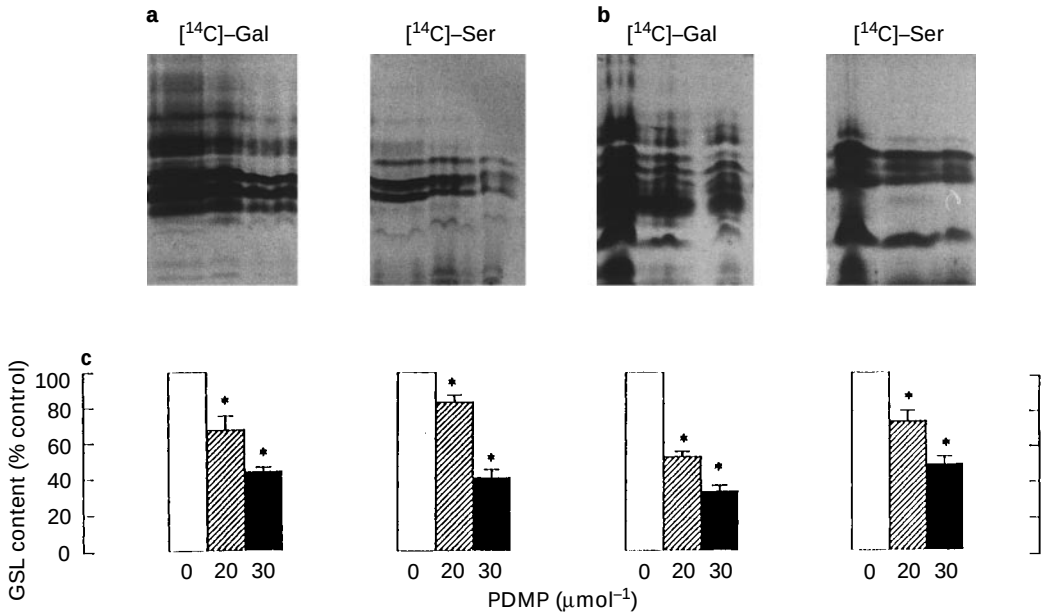

Figure 1 Thin layer chromatography of polar (a) and anionic (b) sphingolipids (GSL) from VSMC grown in the presence of PDMP (20–30 μM). Sphingolipids were labelled with D-[1^{14}C]-galactose ([^{14}C]-Gal) and L-[U^{14}C]-serine ([^{14}C]-Ser). After extraction and purification, aliquots corresponding to 0.5 mg protein were separated by thin-layer chromatography. Representative autoradiographs (a and b) and densitometric analyses (c) from $n=4$ experiments (mean $\pm$ s.e.mean) are shown. (* $P<0.05$ vs control cells).

increase in IL-1β-induced iNOS expression in VSMC. These mechanisms might be of relevance in atherosclerotic arteries.

Methods

Preparation of vascular smooth muscle cells

Rat vascular smooth muscle cells were isolated by enzymatic dispersion as previously described (Sachinidis *et al*., 1995) and were cultured in DMEM/10% foetal calf serum. In order to inhibit glycosphingolipid synthesis, threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) (20–30 μM), was added 4 h after plating of the cells. Cell viability was determined by means of trypan-blue exclusion test and was found to be unaffected by PDMP.

Analysis of glycosphingolipids

VSMC were cultured in medium containing either D-[1-^{14}C]-galactose (5 μCi ml^{-1}) or L-[-U^{14}C]-serine (5 μCi ml^{-1}). Glycosphingolipids were extracted and partitioned with chloroform-methanol-water (4:8:3, by vol) according to Schnaar (1994). The polar phase was purified by means of reversed-phase chromatography by use of Sep-Pak C_{18} cartriges (Millipore Corp., Milford, MA). After evaporation, polar and anionic glycolipids were separated with thin-layer chromatography plates (Merck, Darmstadt, Germany) in chloroform/methanol/2.5 M ammonia (60/40/9, by vol). Bands were detected by autoradiography and quantified densitometrically (Scan PacK, Biometra, Göttingen, Germany).

Nitrite analysis

Nitrite was determined according to the method of Misko *et al*., (1993). For nitrite analysis, VSMC were grown until subconfluency and the medium was then replaced by serum-free medium for 24 h. Subsequently, iNOS was stimulated by IL-1β (3 μ ml^{-1}) in the presence or absence of N^{G}-nitro-L-arginine-methyl-ester (L-NAME, 300 μM). Two hundred microlitres of the cell-free supernatant were diluted 1:5 with PBS and were allowed to react with 100 μl of 0.3 μM 2,3-diaminonaphthalene dissolved in 0.62 M HCl to form the fluorescence product 1-(H)-naphthotriazole. After 10 min, the reaction was terminated with 50 μl of 2.8 M NaOH and fluorescence was measured with excitation and emission

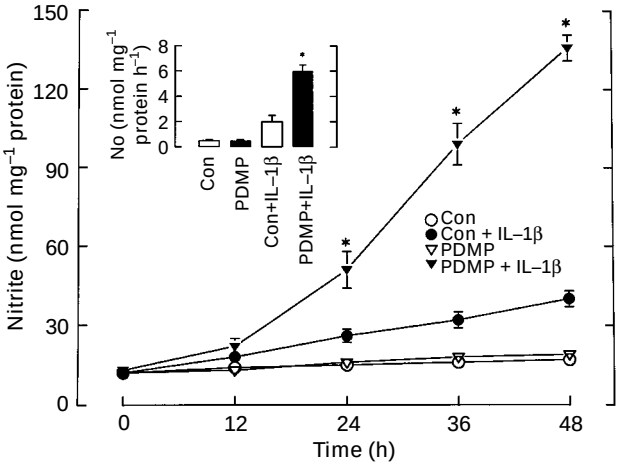

Figure 2 Effects of PDMP (20 μM) on release of nitrite and nitric oxide (insert, measurement 36 h after IL-1β-stimulation) in VSMC. Con = control cells, PDMP = PDMP treated cells, Con + IL-1β = control cells stimulated with IL-1β (3 u ml^{-1}), PDMP + IL-1β = PDMP treated cells stimulated with IL-1β (3 u ml^{-1}). Data are mean of $n=3-6$ experiments; vertical lines show s.e.mean. (* $P<0.001$ vs Con + IL-1β).

wavelengths of 365 nm and 450 nm, respectively (Perkin Elmer LS50, Überlingen, Germany). Nitrite concentrations were determined by use of sodium nitrite standards dissolved in culture medium/PBS mixture (1 : 5). Results are expressed as nmol nitrite mg^{-1} protein.

Measurement of NO release

Nitric oxide (NO) was determined by the oxyhaemoglobin technique as previously described (Weber *et al*., 1993). Oxyhaemoglobin was prepared from bovine haemoglobin by means of reduction with sodium dithionite and subsequent purification by passage through a Sephadex G-25 column. After induction of iNOS with IL-1β for 36 h, as described above, the cells were washed with PBS and subsequently incubated with PBS supplemented with oxyhaemoglobin (4 μM) for 2 h at 37°C. The extinction difference was measured at 402 and 411 nm (Shimadzu UV-160, Kyoto, Japan). The values were corrected for autoxidation of oxyhaemoglobin.

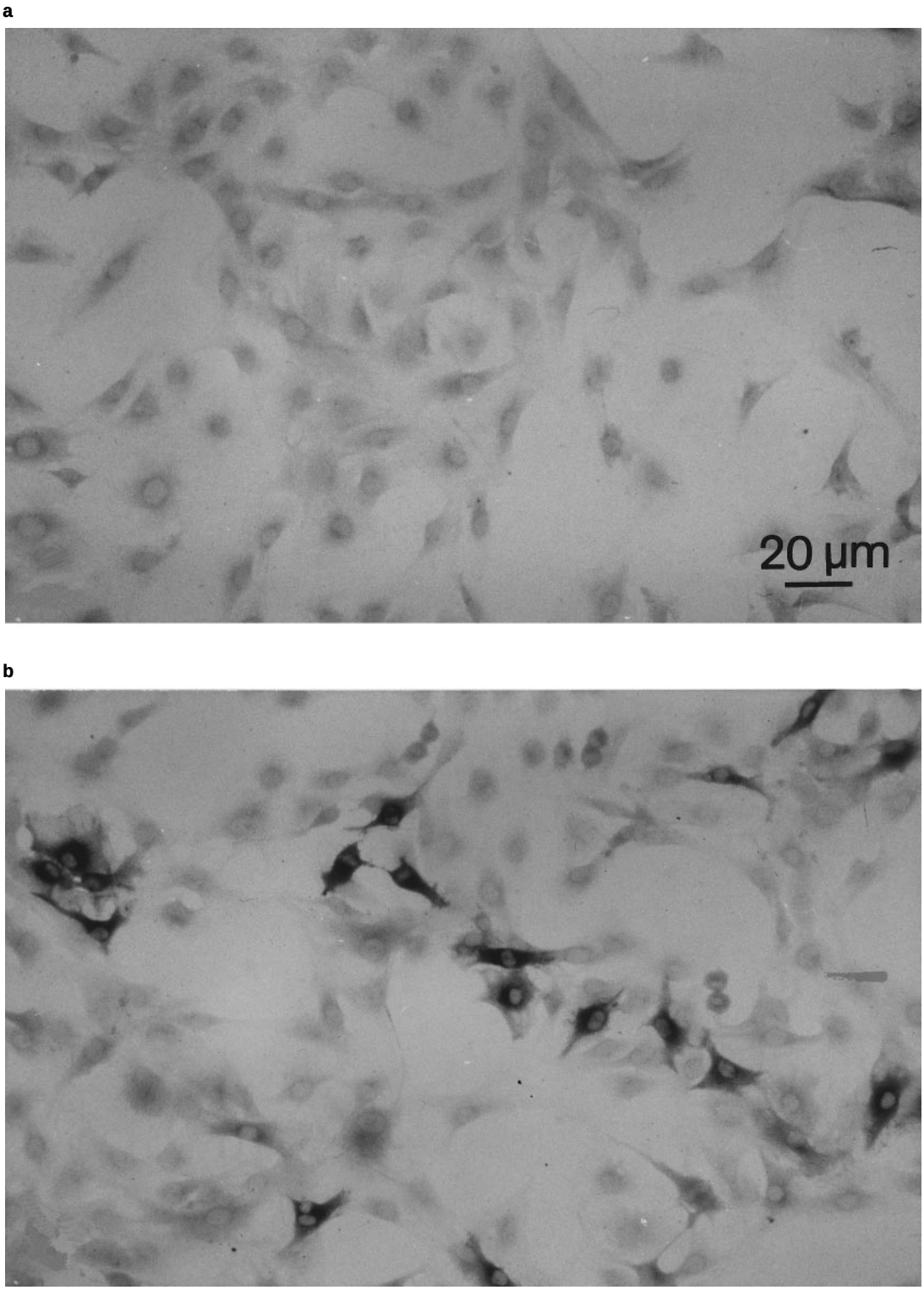

Figure 3 Immunostaining of basal (a) and IL-1β (3 u ml^{-1})-stimulated (b) iNOS expression in PDMP (20 μM)-treated VSMC

Immunocytochemistry

Sparse VSMC were grown on microscope slides and the medium was replaced by serum-free medium for 24 h. iNOS was then induced with IL-1β (3 u ml^{-1}) for 36 h. Subsequently, the cells were fixed with acetone and immunostaining was performed with an anti-iNOS antibody (1 : 500) and a secondary, biotinylated, anti-rabbit antibody (1 : 1,000) (both antibodies from Calbiochem Corp., San Diego, CA). After incubation with avidin-conjugated alkaline phosphatase, the slides were developed with naphthol AS phosphate/fast red violet LB. Different regions of the slides were photographed in a standardized way and a minimum of 300 cells was analysed in each experiment for quantification of iNOS-positive VSMC.

Materials

PDMP (ICN Biomedicals, Eschwege, Germany); DMEM, trypsin, nonessential amino acids, penicillin and streptomycin (Gibco Life Technologies, Eggenstein, Germany); hrIL-1β (Boehringer, Mannheim, Germany); D-[1-^{14}C]-galactose, L-[-U^{14}C]-serine, (Amersham-Buchler, Braunschweig, Germany). All other reagents were from Sigma, Deisenhofen, Germany.

Statistical analysis

Data are mean $\pm$ s.e.mean of n experiments. Non-parametric statistical analysis was performed by the Mann-Whitney rank sum test. $P<0.05$ was considered to be significant.

Results

Effects of PDMP on glycosphingolipid composition in VSMC

In order to confirm the inhibition of glycosphingolipid synthesis by PDMP in rat aortic smooth muscle cells, the composition of global glycosphingolipids was analysed by thin-layer chromatography (t.l.c.). T.l.c. analysis showed that PDMP concentration-dependently reduced the content of polar and anionic [^{14}C]-galactose and [^{14}C]-serine labelled glycosphingolipids in VSMC. Representative autoradiographs and densitometric analysis from all experiments are shown in Figure 1.

Effects of PDMP on cytokine-induced iNOS expression in VSMC

IL-1β (3 u ml^{-1}) stimulated a time-dependent release of nitrite by VSMC. In cells grown in the presence of PDMP (20 μM), basal nitrite production was not changed. However, IL-1β-stimulated nitrite release was markedly increased in cells grown in the presence of PDMP. Nitrite release from both, untreated and PDMP-treated cells, was inhibited by L-NAME (300 μM), an inhibitor of NO synthesis, indicating that the nitrite measured in this system was due to NO synthase activity. For example, at 48 h incubation time, IL-1β-induced nitrite release was reduced by L-NAME from 136 ± 4 to 37 ± 3 nmol mg^{-1} protein in PDMP-treated cells ($P<0.001$, $n=4$), and from 32 ± 1 to 19 ± 1 nmol mg^{-1} protein in untreated cells ($P<0.001$, $n=4$). Similar results were obtained by direct measurement of NO by the oxyhaemoglobin technique showing significantly higher IL-1β-induced NO generation in PDMP-treated cells as compared to control cells. The data are summarized in Figure 2.

These findings were further confirmed by the demonstration of increased expression of cytokine-induced iNOS protein in PDMP-treated cells (Figure 3). In untreated cells, $6.4\pm0.2\%$ of VSMC stained positive for iNOS after stimulation with IL-1β (3 u ml^{-1}). In contrast, in PDMP-treated cells, the proportion of iNOS-positive cells after stimulation with IL-1β was significantly increased to $14.9\pm1.2\%$ ($P<0.001$, $n=4$).

Discussion

The present study demonstrates that inhibition of glycosphingolipid synthesis by PDMP markedly increases IL-1β-induced iNOS expression in VSMC.

Accumulation of lipids in the intima is a key event of atherosclerosis (Anitschkow & Chalatov, 1983; Ross, 1990) and most studies have focused on the role of cholesterol in this process. However, the composition of glycosphingolipids is also changed in atherosclerotic tissue (Breckenridge *et al*., 1975; Mukhin *et al*., 1995). Although the majority of glycosphingolipids accumulates in the extracellular space of the intima, the glycosphingolipid content of cells isolated from hyperplastic layers of human aortic atherosclerotic plaques was also increased. The predominant glycosphingolipids that have been found to accumulate in these cells were glucosylceramide, lactosylceramide and ganglioside GM_3 (Mukhin *et al*., 1995). Several lines of evidence indicate that these glycosphingolipids may be involved in the pathogenesis of atherosclerosis (Prokazova & Bergelson, 1994). Intimal ganglioside GD_3, a major ganglioside of the intima of atherosclerotic aorta, has been shown to induce adhesion, spreading and aggregation of blood platelets (Mazurov *et al*., 1988); gangliosides stimulate aggregation of LDL (Mikhailenko *et al*., 1991), and increase the uptake of LDL by macrophages resulting in an increased accumulation of cholesteryl esters (Prokazova *et al*., 1991). However, no data on the role of endogenous glycosphingolipids in the regulation of VSMC functions that are relevant in the pathophysiology of atherosclerosis, such as cytokine-induced iNOS expression, are available so far.

In the present study, we demonstrated for the first time a modulatory function of endogenous glycosphingolipids on IL-1β-induced iNOS expression in VSMC.

In atherosclerosis, a reduced (Freiman *et al*., 1986) as well as an increased NO production has been found (Minor *et al*., 1990). On the basis of studies with L-arginine (Cooke *et al*., 1992) and NOS inhibitors (Cayette *et al*., 1994), NO was considered to be an anti-atherogenic molecule. iNOS-derived NO may inhibit local vasoconstriction (Schini *et al*., 1991), VSMC proliferation (Scott-Burden *et al*. 1992), as well as platelet function (Durante *et al*., 1991).

The importance of this finding for the situation *in vivo* is difficult to predict. However, our results clearly indicate a role of endogenous glycosphingolipids in the regulation of iNOS expression in VSMC. Thus, in view of the changes in glycosphingolipid composition in atherosclerotic arteries, one might speculate about an involvement of these lipids in the regulation of smoth muscle cell function in atherosclerotic vessels. However, further studies are needed to clarify this issue. In addition, the inhibition of glycosphingolipid synthesis by PDMP may provide a novel therapeutic approach to stimulate iNOS expression that would result in an enhanced generation of antiproliferative NO.

References

ANITSCHKOW, N. & CHATATOV, S. (1983). On experimental cholesterin steatosis and its significance in the origin of some pathological processes. *Arteriosclerosis,* **3,** 178 – 182.

BARBOUR, S., EDIDIN, M., FELDING-HABERMANN, B., TAYLOR-NORTON, J., RADIN, N.S. & FENDERSON, B.A. (1992). Glycolipid depletion using a ceramide analogue PDMP alters growth, adhesion, and membrane lipid organization in human A431 cells. *J. Cell. Physiol.,* **150,** 610 – 619.

BEASLEY, D., SCHWARTZ, J.H. & BRENNER, M.J. (1991). Interleukin 1 induces prolonged L-arginine-dependent cyclic guanosine monophosphate and nitrite production in rat vascular smooth muscle cells. *J. Clin. Invest.,* **87,** 602 – 608.

BRECKENRIDGE, W.C., HALLORAN, J.L., KOVACS, K. & SILVER, M.D. (1975). Increase of gangliosides in atherosclerotic human aortas. *Lipids,* **10,** 256 – 259.

BUSSE, R. & FLEMING, I. (1996). Endothelial dysfunction in atherosclerosis. *J. Vasc. Res.,* **33,** 181 – 194.

BUSSE, R. & MÜLSCH, A. (1990). Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. *FEBS Lett.,* **275,** 87 – 90.

CAYETTE, A.J., PALACINO, J.J., HORTON, K. & COHEN, R.A. (1994). Chronic inhibition of nitric oxide production accelerates neointima formation and impairs endothelial function in hypercholesterolemic rabbits. *Arterioscler. Thromb.,* **14,** 753 – 759.

COOKE, J.P., SINGER, A.H., TSAO, P., ZERA, P., ROWAN, R.A. & BILLINGHAM, M.E. (1992). Antiatherogenic effects of L-arginine in the hypercholesterolemic rabbit. *J. Clin. Invest.,* **90,** 1168 – 1172.

DURANTE, W., SCHINI, V.B., SCOTT-BURDEN, T., JUNQUERO, D.C., KROLL, M.H., VANHOUTTE, P.M. & SCHAFER, A.I. (1991). Platelet inihbition by an L-arginine-derived substance released by IL-1β-treated vascular smooth muscle cells. *Am. J. Physiol.,* **30,** H2024 – H2030.

FREIMAN, P.C., MITCHELL, G.G., HEISTAD, D.D., ARMSTRONG, M.L. & HARRISON, D.G. (1986). Atherosclerosis impairs endothelium-dependent vascular relaxation to acetylcholine and thrombin in primates. *Circ. Res.,* **58,** 783 – 789.

LOSCALZO J. & WELCH, G. (1995). Nitric oxide and its role in the cardiovascular system. *Prog. Cardiovasc. Dis.,* **38,** 87 – 104.

MAZUROV, A.V, PROKAZOVA, N.V., MIKHAILENKO, I.A, MUKHIN, D.N., REPIN, V.S. & BERGELSON, L.D. (1988). Stimulation of platelet adhesion and activation by ganglioside GD3 adsorbed to plastic. *Biochim. Biophys. Acta,* **238,** 167 – 171.

MILHAILENKO, I.A., DUBROVSKAYA, S.A., KOREPANOVA, O.B., TIMOFEEVA, N.G., MOROZKIN, A.D., PROKAZOVA, N.V. & BERGELSON, L.D. (1991). Interaction of low-density lipoproteins with gangliosides. *Biochim. Biophys. Acta,* **1085,** 299 – 305.

MINOR, R.L., MYERS, P.R., GUERRA, R., BATES, J.N. & HARRISON, D.G. (1990). Diet-induced atherosclerosis increases the release of nitrogen oxides from rabbit aorta. *J. Clin. Invest.,* **86,** 2109 – 2116.

MISKO, T.P., SSHILLING, R.J., SALVEMINI, D., MOORE, W.M. & CURRIE, M.G. (1993). A fluorometric assay for the measurement of nitrite in biological samples. *Anal. Biochem.,* **241,** 11 – 16.

MUKHIN, D.N., CHAO, F.-F. & KRUTH, H.S. (1995). Glycosphingolipid accumulation in the aortic wall is another feature of human atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.,* **15,** 1607 – 1615.

POMERANTZ, K.B., HAJJAR, D.P., LEVI, R. & GROSS, S.S. (1993). Cholesterol enrichment of arterial smooth muscle cells upregulates cytokine-induced nitric oxide synthesis. *Biochem. Biophys. Res. Commun.,* **191,** 103 – 109.

PROKAZOVA, N.V. & BERGELSON, L.D. (1994). Gangliosides and atherosclerosis. *Lipids,* **29,** 1 – 5.

PROKAZOVA, N.V., MIKHAILENKO, I.A. & BERGELSON, L.D. (1991). Ganglioside GM3 stimulates the uptake and processing of low density lipoproteins by macrophages. *Biochem. Biophys. Res. Commun.,* **177,** 582 – 587.

RANI, C.S.S., ABE, A., CHANG, Y., ROSENZWEIG, N., SALTIEL, A.R., RADIN, N.S. & SHAYMAN, J.A. (1995). Cell cycle arrest induced by an inhibitor of glucosylceramide synthase. *J. Biol. Chem.,* **270,** 2859 – 2867.

ROSS, R. (1990). The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature,* **362,** 801 – 809.

SACHINIDIS, A., FLESCH, M., KO, Y., SCHRÖR, K., BÖHM M., DÜSING, R. & VETTER, H. (1995). Thromboxane A_2 and vascular smooth muscle cell proliferation. *Hypertension,* **26,** 771 – 780.

SCHINI, V.B., CATOVSKY, S., SCHRAY-UTZ, B., BUSSE, R. & VANHOUTTE, P.M. (1994). Insulin-like growth factor I inhibits induction of nitric oxide synthase in vascular smooth muscle cells. *Circ. Res.,* **74,** 24 – 32.

SCHINI, V.B., JUNQUERO, D.C., SCOTT-BURDEN, T. & VANHOUTTE, P.M. (1991). Interleukin-1 β induces the production of L-arginine-derived relaxing factor from cultured smooth muscle cells from rat aorta. *Biochem. Biophys. Res. Commun.,* **176,** 114 – 121.

SCHINI-KERTH, V.B., FIBLTHALER, B., ANDERSON, T.T., FENTON, J.W., VANHOUTTE, P.M. & BUSSE, R. (1995). Thrombin prevents the expression of inducible nitric oxide synthase in vascular smooth muscle cells by a proteolytically-activated thrombin receptor. *Thromb. Haemost.,* **74,** 980 – 986.

SCHNAAR, R.L. (1994). Isolation of glycosphingolipids. *Methods Enzymol.,* **230,** 348 – 370.

SCOTT-BURDEN, T., SCHINI, V.B., ELIZONDO, E., JUNQUERO, D.C. & VANHOUTTE, P.M. (1992). Platelet-derived growth factor suppresses and fibroblast factor enhances cytokine-induced production of nitric oxide by cultured smooth muscle cells: effects on cell proliferation. *Circ. Res.,* **71,** 1088 – 1100.

SHAYMAN, J.A., MAHDIYOUN, S., DESHMUKH, G., BARCELON, F., INOKUCHI, J. & RADIN, N.S. (1990). Glucosphingolipid dependence of hormone-stimulated inositol triphosphate formation. *J. Biol. Chem.,* **265,** 12135 – 12138.

WEBER, A.-A., STROBACH, H. & SCHRÖR, K. (1993). Direct inhibition of platelet function by organic nitrates via nitric oxide formation. *Eur. J. Pharmacol.,* **247,** 29 – 37.

(Received April 28, 1997
Revised November 4, 1997
Accepted November 12, 1997)