Vol. 191, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 26, 1993 Pages 103-109

CHOLESTEROL ENRICHMENT OF ARTERIAL SMOOTH MUSCLE CELLS UPREGULATES CYTOKINE-INDUCED NITRIC OXIDE SYNTHESIS

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Received December 29, 1992

Summary: Endothelium-derived relaxing factor/nitric oxide (EDRF/NO) is produced by the vascular wall and is a key modulator of vascular tone and blood pressure. NO is also produced by vascular smooth muscle (VSMC) where it can inhibit proliferation. Since cytokine-activated VSMC proliferation is a major event in the development of atherosclerosis, we investigated the influence of cholesterol (CE)-enrichment of VSMC on cytokine-induced NO synthesis. Treatment of VSMC with native LDL for one week did not promote CE-accretion or alter NO production following exposure to endotoxin (LPS). In contrast, CE-enrichment by cationized LDL augmented LPS-induction of NO synthesis 2-5-fold. While TNF-α promoted little NO synthesis in control VSMC, it was very potent after CE-enrichment. Similarly, CE-enrichment augmented IL-1α-induced NO synthesis. However, CE-enrichment did not affect the synergistic induction of NO synthesis by cytokines in combination with IFN-γ. Our findings suggest that CE-enrichment of VSMC upregulates signal transduction pathways which mediate cytokine and LPS induction of NO synthase activity.

Nitric oxide (NO) is a key cell-signalling molecule whose importance in vascular physiology and pathophysiology is becoming increasingly appreciated (1). NO is produced by NO synthases which are either constitutively expressed (cNOS), or inducible (iNOS), and arise from at least three distinct genes (2-5). NO is produced constitutively by cNOS in endothelial cells where it regulates vascular tone and blood pressure (6,7), and mediates responses to vasodilators (8,9). NO also inhibits VSMC proliferation (10), although it is unclear whether the quantities of endothelium-derived NO are sufficient to achieve this effect *in vivo*. However, high levels of NO are produced by iNOS, which is induced in VSMC and macrophages following exposure to bacterial lipopolysaccharide (LPS) or

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Abbreviations used in this paper: NO - nitric oxide; EDRF - endothelium-derived relaxant factor; iNOS - inducible nitric oxide synthase; cNOS - constitutive nitric oxide synthase; LPS - lipopolysacchride; TNF-α - tumor necrosis factor-α; IL-1α - interleukin-1α; IFN-γ - interferon-γ; LDL - low density lipoprotein; cLDL-cationized low density lipoprotein; CE - cholesteryl ester.

0006-291X/93 $4.00

cytokines (11-15). Thus, NO may regulate VSMC function in an autocrine and paracrine manner. Since NO inhibits VSMC proliferation, alterations in NO synthesis may be important in the pathophysiology of atherogenesis, which is characterized by VSMC hyperplasia and cholesteryl ester deposition. Indeed, atherosclerosis has been associated with either reduced (16), or elevated (17) NO synthetic capacity. However, the influence of CE-enrichment on NO generation by VSMC has not been explored. We report here that CE-enrichment augments cytokine-induction of iNOS activity.

MATERIALS AND METHODS

Materials: RPMI-1640, M-199, penicillin, streptomycin, and amphotericin B were from Gibco (Grand Island, NY). Fetal bovine serum (FBS) and newborn calf serum were purchased from Hyclone (Logan, UT). N,N-dimethyl-1,3-propanediamine was from Kodak Chemicals (Rochester, NY); 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide-HCl was purchased from Aldrich Chemicals (Milwaukee, WI). Human recombinant interleukin-1α (IL-1α), human recombinant tumor necrosis factor-α (TNF-α), and rat recombinant interferon-gamma (IFN-γ) were obtained from Hoffman La-Roche (Nutley, NJ). LPS (E.coli B0111:B4) was obtained from Sigma (St. Louis, MO). Other chemicals were obtained from Fisher Scientific (Springfield, NJ).

Preparation of plasma LDL and cationized LDL (cLDL): LDL (1.019 - 1.063 g/ml) was isolated by preparative ultracentrifugation of pooled donor human plasma (18). LDL was cationized as previously described (19). Lipoproteins were screened for the presence of thiobarbituric acid-reactive substances (TBARS), using malondialdehyde as standard (20). LDL and cLDL contained <2 nmol TBARS/mg protein. Lipoproteins were dialyzed against 0.154 M NaCl, 0.3 mM EDTA, 0.1 M $NaHPO_4$ (pH 7.4) for 48 h.

Isolation and culture of aortic VSMC: VSMC were propagated from explants of rat thoracic aorta, and cultured as previously described (19). Cells were identified as VSMC by their hill-and-valley morphology and positive staining for α-actin. Cells were maintained in M-199/10% FBS (v/v) containing penicillin (80 μg/ml), streptomycin (80 μg/ml), and fungizone (2.0 μg/ml), and incubated at 37 °C in 5% CO_2 in air.

Preparation of foam cells: Cells were grown to confluence in 96-well cluster plates in M-199/10% FBS and treated with M-199/5% FBS alone, or containing LDL or cLDL (100 μg/ml) for seven days. These media were replaced on day 4. VSMC exposed to native LDL did not accumulate free or esterified cholesterol, while cells exposed to cLDL increased free and esterified cholesterol 2-fold and 10-30-fold, respectively (19).

Assay of NO: NO was measured as nitrite in the conditioned medium of normal and CE-enriched VSMC in 96-well cluster plates as previously described (12). Cells were exposed to stimulants in 200 μl of RPMI containing 10% newborn bovine calf serum, 2.5 mM glutamine, 25 mM HEPES and penicillin/streptomycin/fungizone. At appropriate time-points, 100 μl of medium were added to Greiss reagent (0.05% napthalenediamine and 0.5% sulfanilamide in 5% orthophosphoric acid,1:1) to form an azo dye adduct which was assayed in quadruplicate at OD_{550}, and quantified using nitrite as reference (0-100 μM). Background absorbance was determined using medium alone, and was subtracted from all values.

Miscellaneous assays: Cellular and lipoprotein protein content was measured by the method of Lowry et al (21), using bovine serum albumin as standard.

Statistical analysis: Data are expressed as mean ± SEM, and analyzed by Student's t test or analysis of variance, followed by Newman Keul's test. Differences with $p < 0.05$ were considered significant.

RESULTS

In initial experiments, the influence of cytokines and LPS on nitrite production was evaluated. As depicted in Table 1, LPS and IL-1α stimulated nitrite release

Table 1
LPS and IL-1α Induce Nitrite Production by Rat Aortic Smooth Muscle Cells

Treatment	Dose	Nitrite (fmoles)
Control		252 ± 21
LPS (μg/ml):	0.5	210 ± 21
	5.0	672 ± 21*
	50.0	3108 ± 252*
IL-1α (U/ml):	5.0	231 ± 42
	50.0	483 ± 42*
	500.0	3633 ± 252*
TNF-α (U/ml):	25.0	168 ± 21
	250.0	273 ± 42
	2500.0	357 ± 42

VSMC were exposed to media alone (control) and increasing concentrations of LPS, IL-1α, or TNF-α for 44 hours, followed by measurement of NO as nitrite (mean ± SEM , n=8, * = $p < 0.05$).

in a dose-dependent manner. In contrast, barely detectable levels of nitrite are attained even after exposure to high concentrations of TNF-α (2500 U/ml). Although basal release of nitrite-positive material is detected in 44-hr conditioned medium from control VSMC, it does not arise from NO since it is not reduced by treatment with a specific NOS inhibitor (1.0 mM N^{ω}-methyl-L-arginine, data not shown).

Next, experiments were performed to assess the influence of CE-enrichment of VSMC on the potency and efficacy of LPS as an inducer of NO synthesis (Figure 1). CE-enrichment augmented LPS-induced NO synthesis over 3-fold. Moreover, LPS induced NO synthesis in control cells only at concentrations exceeding 10 μg/ml, whereas LPS was active at concentrations 10-fold lower in eliciting nitrite production by CE-enriched cells. However, native LDL, which does not cause cholesterol enrichment (19), did not alter LPS-induced NO synthesis relative to control VSMC.

Since LPS induces the synthesis of numerous cytokines which mediate its bioactivity, we investigated the possibility that CE-enrichment could modulate induction of NO synthesis following exposure to TNF-α, IL-1α, and IFN-γ (Figure 2). In control VSMC, IL-1α, but not TNF-α and IFN-γ, induced NO synthesis. CE-enrichment significantly potentiated IL-1α-induced NO synthesis, and caused TNF-α to act as a potent inducer of NO generation. Clearly, the greatest release of nitric oxide by CE-enriched VSMC was observed with LPS, which stimulated NO production nearly 8-fold. In contrast, IFN-γ did not induce NO synthesis in either control or CE-enriched cells (Figure 2). These data suggest that CE-enrichment upregulates signal transduction pathways which mediate LPS- and cytokine-induced iNOS activity, and hence, NO production.

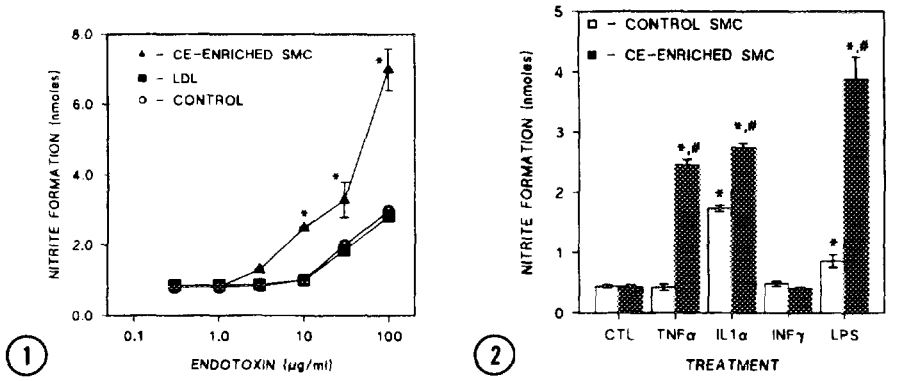

Figure 1. CE enrichment augments LPS induction of NO production by rat VSMC: cells were exposed to medium alone (open circles), medium containing native LDL (squares), or medium containing cLDL (triangles) for one week. Cells were then exposed to medium containing LPS (1-100 µg/ml) for 24 hours at 37 °C. Accumulation of NO was measured as NO_2^- (n=4, mean ± SEM, * = $p < 0.05$).

Figure 2. CE enrichment augments cytokine induction of NO synthesis: Normal and CE-enriched VSMC grown in 96-well cluster plates were exposed to serum-free M-199 alone (control), or to M-199 containing TNF-α (12.5 ng/ml), IL-1α (25 ng/ml), IFN-γ (50 ng/ml), or LPS (50 µg/ml) for 24 hours at 37 °C. NO was measured as described in Methods (n=4, mean ± SEM, * = $p < 0.05$ from cells not treated with cytokines or LPS, # = $p < 0.05$ normal vs CE-enriched VSMC).

We next tested the possibility that CE-enrichment would alter the synergistic induction of iNOS by INF-γ in combination with other cytokines (1). Normal and CE-enriched VSMC were exposed to TNF-α, IL-1α, or LPS in the presence and absence of IFN-γ. While IFN-γ did not induce NO synthesis when administered alone, it markedly potentiated induction of NO synthesis by other cytokines. Indeed, TNF-α, IL-1α or LPS in the presence of IFN-γ increased NO-release more than 6-fold despite the inability of TNF-α to induce NO production when administered alone (Figure 3, Panel A). CE-enrichment did not modify the synergistic effect of co-stimulation by INF-γ in the presence of TNF-α or LPS, but slightly reduced the synergistic stimulation of combined INF-γ/IL-1α treatment (Figure 3, Panel B).

DISCUSSION

Generation of NO within the blood vessel wall is important in the regulation of intrinsic tone and the maintenance of a non-thrombogenic surface. In this study, we show that CE-enrichment of VSMC augments LPS- and cytokine-induced iNOS activity. Although we did not directly measure iNOS mass or the steady state levels of iNOS mRNA, our data support the hypothesis that CE-enrichment can upregulate iNOS protein levels. Indeed, LPS (12,15) and IL-1α (22) induce NO production by VSMC by increasing transcription and translation of iNOS following a delay of 6-8 hr, most probably through upregulation of iNOS gene transcription (1). However, it is also conceivable that alterations in post-translational processing of iNOS by CE-enriched VSMC may mediate its enhanced activity. The

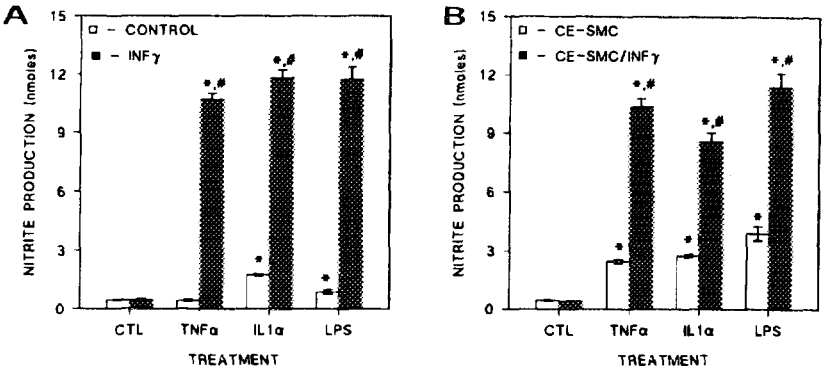

Figure 3. IFN-γ augments NO synthesis in response to cytokines. Normal (Panel A) or CE-enriched (Panel B) VSMC were exposed to TNF-α (12.5 ng/ml), IL-1α (25 ng/ml), or LPS (50 μg/ml) in the absence (open bars) and presence of IFN-γ (50 ng/ml) for 24 hours at 37° C (n=4, mean ± SEM, * = $p < 0.05$, control vs cytokine activation, # = $p < 0.05$, treatments alone vs treatments containing IFN-γ).

finding that CE-enrichment can modulate gene transcription is not without precedent. We have found that CE-enrichment upregulates the mRNA expression of the inducible form of cyclooxygenase (COX-2, unpublished observations), as well as the mRNA and protein for basic fibroblast growth factor (23) in VSMC. CE-enrichment could conceivably upregulate iNOS directly or secondarily through the generation of secondary cytokines which induce iNOS, such as IL-1 (24). Alternatively, CE-enrichment may decrease the synthesis of mediators which inhibit iNOS expression, such as TGF-β (25).

Although native LDL has been reported to inhibit endothelial-cell dependent relaxation of isolated rabbit thoracic aorta (26), we did not observe alterations in VSMC iNOS activity following treatment with LDL. Notably, native LDL does not cause CE-enrichment of VSMC (19). The effect of LDL on endothelial cell NO synthesis appears to be non-specific since VLDL and HDL inhibited NO release, possibly due to inhibition by lipid oxidation products, such as lysophosphatidylcholine (27). However, the effects of CE-enrichment on iNOS activity may well be cell-specific. In contrast to VSMC, oxidized LDL, but not acetylated LDL reduce NO synthetic capacity of macrophages despite similar increases in cellular cholesterol content after exposure to either LDL species (28). Thus, NO synthesis by macrophages may be inhibited by LDL-oxidation products, but not by LDL or LDL-derived cholesterol.

The synergistic effects of combined cytokine treatment on induction of NO synthesis have been previously reported for macrophages (29), endothelial cells (30), and VSMC (22). Although the mechanism of this synergy is unclear, IFN-γ also augments the induction of IL-1β mRNA in response to LPS or IL-1 in

endothelial cells (30), and IFN-γ and IFN-β stimulate the expression of LPS-inducible genes in macrophages (31). However, CE-enrichment did not modifying synergistic effect of co-stimulation by INF-γ in the presence of TNF-α or LPS., and slightly reduced the synergistic stimulation of combined INF-γ/IL-1α treatment. These observations suggest that CE-enrichment does not alter signal transduction pathways which mediate cytokine-induction of maximal NO synthesis. Collectively, our data indicate that CE-enrichment upregulates NO synthesis, an important modulator of blood vessel tone and proliferative state of VSMC. However, the influence of atherosclerosis on NO production and vascular responsiveness is not fully understood. Most studies have shown that atherosclerosis is associated with reduced NO synthesis, with concomitant hypersensitivity to exogenous NO (32). In contrast, atherosclerosis has also been associated with increased NO formation (presumably derived from VSMC), concomitant with decreased NO lifetime (17). Our findings provide evidence to support the latter *in vivo* findings. Increased NO synthesis by CE-enriched VSMC in the context of atherosclerosis may serve to attenuate the actions of mitogens which are overexpressed in developing lesions (PDGF, IL-1, TNF). Thus, VSMC-derived NO could serve a beneficial or protective role in atherogenesis by inhibiting unrestricted SMC proliferation.

ACKNOWLEDGMENTS: This work was funded by NIH P-01 HL46403, NIH R01 HL39701, and R01 HL34215.

REFERENCES

1. Nathan, C. (1992) FASEB J. 6, 3051-3064.
2. Bredt, D., Hwang, P., Glatt, C., Lowenstein, C., Reed, R., and Snyder, S. (1991) Nature 351, 714-718.
3. Xie, Q., Cho, H., Calaycay, J., Mumford, R., Swiderek, K., Lee, T., Ding, A., Troso, T., and Nathan, C. (1992) Science 256, 225-228.
4. Lyons, C., Orlof, G., and Cunningham, J. (1992) J. Biol. Chem. 267, 6370-6374.
5. Sessa, W., Harrison, J., Barber, C., Zeng, D., Durieux, M., Drew, D., D'Angelo, D., Lynch, K., and Peach, M. (1992) J. Biol. Chem. 267, 15274-15278.
6. Aisaka, K., Gross, S., Griffith, O., and Levi, R. (1989) Biochem. Biophys. Res. Comm. 160, 881-886.
7. Rees, D., Palmer, R., and Moncada, S. (1989) Proc. Natl. Acad. Sci. USA 86, 3375-3378.
8. Ignarro, L. (1990) Ann. Rev. Pharmacol. Toxicol. 30, 536-560.
9. Furchgott, R. and Vanhoutte, P. (1989) FASEB J. 3, 2007-2018.
10. Garg, U. and Hassid, A. (1989) J. Clin. Invest. 83, 1774-1777.
11. Schini, V., Junquero, D., Scott-Burden, T., and Vanhoutte, P. (1991) Biochem. Biophys. Res. Comm. 176, 114-121.
12. Gross, S., Madera, A., Griffith, O., and Levi, S. (1991) FASEB J. 5, A1728.
13. Beasley, D., Schwartz, H., and Brenner, B. (1991) J. Clin. Invest. 87, 602-608.
14. Neufeld, G. and Gospodarowicz, D. (1985) J. Biol. Chem. 260, 13860-13868.
15. Gross, S. and Levi, R. (1992) J. Biol. Chem. in press.

16. Osborne, J., Siegman, M., Sedar, A., Mooers, S., and Lefer, A. (1989) Am. J. Physiol. 256, C591-C597.
17. Minor, R., Myers, P., Guerra, R., Bates, J., and Harrison, D. (1990) J. Clin. Invest. 86, 2109-2116.
18. Havel, R., Eder, H., and Bradgon, J. (1955) J. Clin. Invest. 34, 1345-1353.
19. Pomerantz, K. and Hajjar, D. (1989) J. Lipid. Res. 30, 1219-1231.
20. Marshall, P., Warso, M., and Lands, W. (1985) Anal. Biochem. 145, 192-199.
21. Lowry, O., Rosebrogh, N., Farr, A., and Randall, J. (1951) J. Biol. Chem. 193, 265-275.
22. Kilbourn, R., Gross, S., Lodato, R., Adams, J., Levi, R., Miller, L., Lachman, L., and Griffith, O. (1992) J. Natl. Cancer Inst. 84, 1008-1016.
23. Kraemer, R., Pomerantz, K., Joseph-Silverstein, J., and Hajjar, D. (1993) J. Biol. Chem. (in press).
24. Joly, G., Schini, V., and Vanhoutte, P. (1992) Circ. Res. 71, 331-338.
25. Junquero, D., Schini, V., Scott-Burden, T., and Vanhoutte, P. (1992) Am. J. Physiol. 262, H1788-H1795.
26. Bernhardt, J., Tschudi, M., Dohi, Y., Gut, I., Urwyler, B., Buhler, F., and Luscher, T. (1991) Biochem. Biophys. Res. Comm. 180, 907-912.
27. Takahashi, M., Yui, Y., Yasumoto, H., Aoyama, T., Morishita, H., Hattori, R., and Kawai, C. (1990) Am. J. Physiol. 258, H1-H8.
28. Jorens, P., Rosseneu, M., Devreese, A., Bult, H., Marescau, B., and Herman, A. (1992) Europ. J. Pharmacol. 212, 113-115.
29. Ding, A., Nathan, C., and Stuehr, D. (1988) J. Immunol. 141, 2407-2412.
30. Suzuki, H., Shibano, K., Okane, M., Kono, I., Matsui, Y., Yamane, K., and Heihachiro, K. (1989) Amer. J. Pathol. 134, 35-43.
31. Hamilton, T., Bredon, N., Ohmori, Y., and Tannenbaum, C. (1989) J. Immunol. 142, 2325-2331.
32. Freiman, P., Mitchell, G., Heistad, D., Armstrong, M., and Harrison, D. (1986) Circ. Res. 58, 783-789.