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SUPPRESSION OF CHOLESTERYL ESTER ACCUMULATION IN CULTURED HUMAN MONOCYTE-DERIVED MACROPHAGES BY LIPOXYGENASE INHIBITORS

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SUMMARY. Atherosclerotic lesions and xanthomas are characterized by the occurrence of cholesteryl ester (CE)-laden foam cells, which partly originate from macrophages. Little is known about the role of cyclo-oxygenase or lipoxygenase metabolites of arachidonic acid in the development of foam cells. In this study we investigated the influence of prostaglandins and inhibitors of the cyclo-oxygenase or the lipoxygenase pathway on CE accumulation in cultured human monocyte-derived macrophages. Accumulation of CE was achieved by incubation of the cells with acetylated low density lipoprotein (AcLDL). The stable prostacyclin analogue ZK 36 374 and prostaglandin E_2 showed no effect on cellular CE storage. Similarly, the cyclo-oxygenase inhibitor indomethacin failed to influence AcLDL-induced CE accumulation. By contrast, however, the inhibitors of lipoxygenase activity nordihydroguaiaretic acid (NDGA) and BW 755 C markedly suppressed the accumulation of CE in monocyte-derived macrophages. The inhibitory effect of NDGA was dose-dependent. Incubation of the cells with the anti-oxidant vitamin E gave no significant reduction of CE accumulation. Our results indicate that inhibition of the lipoxygenase pathway of arachidonic acid metabolism in cultured monocyte-derived macrophages effectively decreases the rate of experimentally-induced CE accumulation.

The role of cyclo-oxygenase products of arachidonic acid in the development of atherosclerosis has been the subject of some speculation (1). The opposite effects of thromboxane A_2 and prostacyclin (PGI_2) with regard to platelet aggregation and vascular tone are well documented (2,3). Furthermore, decreased levels of PGI_2 biosynthesis by aortic tissue (4,5) and by smooth muscle cells (6) derived from atherosclerotic lesions have been observed. There are also reports on the inhibition of PGI_2 synthesis by low-density lipoproteins (LDL) in aortic tissue and cultured endothelial cells, whereas high-density lipoproteins (HDL) have been shown to sti-

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ABBREVIATIONS: PGE_2, prostaglandin E_2; PGI_2, prostacyclin; LDL, low density lipoprotein; AcLDL, acetylated low density lipoprotein; HDL, high density lipoprotein; CE, cholesteryl ester; NDGA, nordihydroguaiaretic acid; ETYA, 5,8,11, 14-eicosatetraynoic acid; BSA, bovine serum albumin; M199, medium 199; PBS, Phosphate-buffered saline

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mulate PGI_2 production (7-9). These findings suggest a possible relationship between PGI_2 production in the arterial wall and atherogenesis, since raised levels of LDL are considered to be a positive risk factor for coronary heart disease, while HDL are assumed to represent a protective factor against this disease (10). As the development of atherosclerotic lesions is characterized by the generation of cholesteryl ester (CE)-laden foam cells within the arterial wall, the recently published observation of Hajjar and Weksler (11) that PGI_2 and prostaglandin E_2 (PGE_2) considerably affect the cholesteryl ester metabolism in cultured aortic smooth muscle cells, opens new aspects.

In the current study the effects of a stable prostacyclin analogue (ZK 36 374) and PGE_2 on CE accumulation in cultured human monocyte-derived macrophages have been investigated. At the same time, the influence of several agents, which are known to modify cyclo-oxygenase or lipoxygenase activity, has been studied in connection with cellular CE storage. Cultured monocyte-derived macrophages were used in our experiments because of their prominent role in foam cell formation during the development of early fatty streak lesions (12-14). Accumulation of CE was obtained by incubation of the cells with acetylated LDL (AcLDL), according to the experimental model of Goldstein and Brown (15). Our studies provided no evidence for a regulatory role of prostacyclin or PGE_2 on cellular CE storage. Inhibition of cyclo-oxygenase activity similarly failed to influence the rate of CE accumulation. By contrast, however, inhibition of the lipoxygenase pathway of arachidonic acid metabolism markedly suppressed AcLDL-induced CE accumulation in the cells.

MATERIALS AND METHODS

Isolation and culture of human monocytes. Mononuclear leukocytes were isolated from heparinized human blood by Ficoll-Isopaque density-gradient centrifugation (500 x g for 30 min). The cells at the interface were removed, washed three times with phosphate-buffered saline (PBS), and suspended in medium 199 (M199) containing 10% (v/v) pooled human serum. The cell suspension (10^7 cells/ml) was transfered to 10 cm^2 sterile plastic culture dishes and incubated for 2 h at 37°C in a humidified atmosphere with 5% CO_2. Thereafter, the adherent cells were washed three times with M199 and again incubated with M199 supplemented with 10% (v/v) pooled human serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). During cell culture, the medium was aspirated and replaced by fresh medium of the same composition every 48 h.

Lipoproteins. Low density lipoproteins (LDL, d = 1.03 - 1.05 g/ml) were prepared from fresh human serum by density gradient ultracentrifugation (16), followed by tube slicing. LDL was acetylated by repeated addition of acetic anhydride, as des-

cribed by Basu et al. (17). On agarose electrophoresis the AcLDL sample showed one single band with α-mobility.

Experimental procedures. After 10 days of culture, AcLDL (100 µg protein/ml) was added to the medium and the cells were further incubated for 70 h at 37°C. The following compounds were added to the medium together with AcLDL: arachidonic acid (Fluka, Buchs, Switzerland), the stable prostacyclin analogue ZK 36 374 (kindly provided by Dr.E.Schillinger, Schering A.G., Berlin), prostaglandin E_2 (Upjohn Diagnostics, Kalamazoo, MI, USA), indomethacin (Sigma Chemical Co., St.Louis, MO, USA), nordihydroguaiaretic acid (Sigma), 5,8,11,14-eicosatetraynoic acid (a gift from Dr.D.H.Nugteren, Unilever Research Lab., Vlaardingen, The Netherlands), compound BW 755 C (through courtesy of Dr.S.Moncada, Wellcome Research Lab. Beckenham, U.K.) and vitamin E (Sigma).

All these compounds were dissolved in absolute ethanol and added to the culture medium at a final concentration of 10^{-5} M. Furthermore, nordihydroguaiaretic acid (NDGA) was added to the medium at final concentration of 5×10^{-5} M. The maximal concentration of ethanol in the culture medium never exceeded 0.1% (v/v). Control dishes were incubated with medium (M199 plus 10% (v/v) pooled human serum) or with medium plus AcLDL (100 µg protein/ml) for 70 h at 37°C. Ethanol was included in the medium of control experiments as well, at a final concentration of 0.1% (v/v). Viability of the cells at the end of the incubation period was assessed by measuring the amount of lactate dehydrogenase released into the medium (18). After 70 hours, the cells were washed with M199. The adherent cells were scraped with a rubber policeman into 0.75 ml of BSA/Tris/NaCl buffer and washed with PBS. Subsequently, the cells were resuspended in 0.5 ml PBS. Aliquots (0.1 ml) were taken for measurement of protein content (19).

Lipids were extracted from the cell suspension with methanol : chloroform (1 : 2), according to Bligh and Dyer (20), after addition of cholesterol acetate (2 µg) as an internal standard. The lipids were separated by thin-layer chromatography on silica plates (HPTLC, Marchery & Nägel) after heat activation (130°C for 1 h) and a prerun with 10% 1 N NaOH in methanol. The thin-layer chromatography plates were developed in a Camag HPTLC apparatus (No.28520) by a solvent mixture of 87% hexane, 10% diethylether and 3% 2-propanol. The lipid bands were stained by incubation for 20 seconds in 50% (volume) methanol containing $MnCl_2$-$4H_2O$ (5 g/l) and H_2SO_4 (0.6 mol/l), followed by incubation for 30 minutes at 110°C. Thereafter, the lipid bands were quantified densitometrically with a Shimadzu CS 910 densitometer, using the reflection mode at 380 nm. The densitometric responses were integrated automatically using the Shimadzu data processor chromatopac C-RIB, programmed for the respective lipid densitometric response factors.

Statistical analysis. The results were analyzed by a two-way analysis of variance, followed by Scheffé's method for multiple comparisons (21).

RESULTS

Incubation of cultured human monocyte-derived macrophages with AcLDL induced a considerable increase of cellular CE content (Fig.1). Addition of arachidonic acid to the culture medium together with AcLDL for an incubation period of 70 h did not influence the rate of CE storage in the cells. Similarly, the stable prostacyclin analogue ZK 36 374, PGE_2, and the cyclo-oxygenase inhibitor indomethacin did not significantly affect AcLDL-mediated CE accumulation (Fig.1, $p > 0.05$).

In contrast, nordihydroguaiaretic acid (NDGA) and BW 755 C markedly suppressed the AcLDL-induced CE accumulation when added to the medium of cultured monocyte-

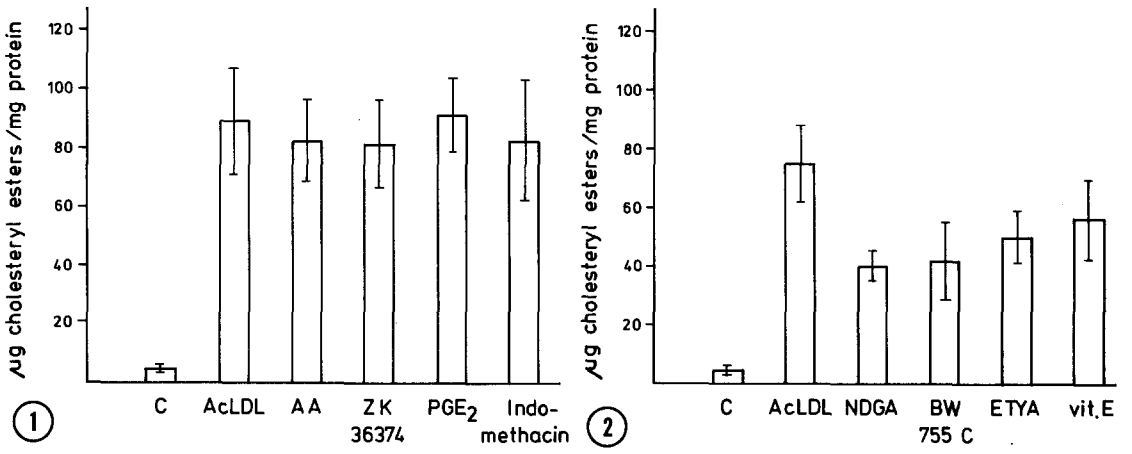

Fig. 1 AcLDL-induced cholesteryl ester (CE) accumulation in cultured human monocyte-derived macrophages is not influenced by arachidonic acid (AA), the stable prostacyclin analogue ZK 36 374, prostaglandin E_2 (PGE_2), or indomethacin ($p > 0.05$). The concentration in the culture medium was 10^{-5} M for all these compounds. The amount of CE measured in cells not incubated with AcLDL is shown as a control (C). The results shown in the figure represent the mean values (± SEM) of four experiments. Each experiment was performed in triplicate.

Fig. 2 Inhibition of AcLDL-induced cholesteryl ester (CE) accumulation in cultured human monocyte-derived macrophages by lipoxygenase inhibitors. Cells incubated with AcLDL plus nordihydroguaiaretic acid (NDGA) or BW 755 C showed a reduction of CE accumulation compared with cells incubated with AcLDL alone ($p < 0.05$). The amounts of CE stored in cells incubated with AcLDL plus 5,8,11,14-eicosatetraynoic acid (ETYA) or vitamin E was not significantly reduced ($p > 0.05$). The concentration in the medium was 10^{-5} M for all these compounds. The amount of CE measured in cells not incubated with AcLDL is shown as a control (C). The results shown in the figure represent the mean values (± SEM) of four experiments. Each experiment was performed in triplicate.

derived macrophages (Fig.2, $p < 0.05$). For NDGA this effect appeared to be dose-dependent (Fig.3). Although 5,8,11,14-eicosatetraynoic acid (ETYA) also suppressed the cellular CE accumulation, this effect was not statistically significant ($p > 0.05$ To evaluate whether the inhibition of CE storage was due to anti-oxidative activities of the lipoxygenase inhibitors, the cells were also incubated with vitamin E. As shown in Fig.2, vitamin E did not significantly inhibit AcLDL-induced CE accumulation ($p > 0.05$). None of the additions used resulted in increased release of lactate dehydrogenase into the culture medium.

DISCUSSION

The possibility of *in vitro* modulation of cellular cholesteryl ester (CE) storage by cyclo-oxygenase and lipoxygenase metabolites of arachidonic acid might elu-

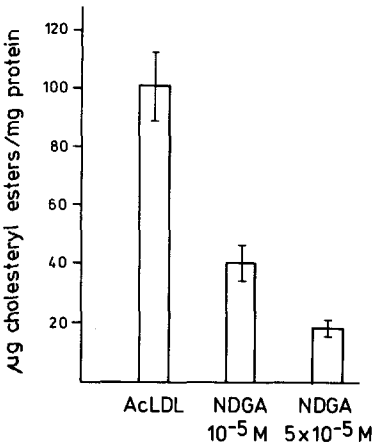

Fig. 3 Dose-responsive inhibition of AcLDL-induced cholesteryl ester (CE) accumulation in cultured human monocyte-derived macrophages by nordihydroguaiaretic acid (NDGA). The cells were incubated with AcLDL, of with AcLDL plus NDGA at two different concentrations (10^{-5} M or 5×10^{-5} M). The results shown in the figure represent the mean values (± SEM) of an experiment performed in triplicate.

cidate certain mechanisms which occur in the arterial wall during the development of atherosclerotic lesions. The model system for AcLDL-mediated CE accumulation in cultured macrophages, as devised by Brown and Goldstein (15), provides a useful method for investigations on foam cell formation. Using this model, we have found no measurable effects of the prostacyclin analogue ZK 36 374 (22) or prostaglandin E_2 (PGE_2) on AcLDL-induced CE accumulation. We had to use a stable analogue, since prostacyclin itself is very unstable and therefore unsuitable for prolonged incubation periods. PGE_2, on the other hand has been shown to be chemically stable in aquous solutions for up to 3 days (23). Addition of arachidonic acid to the culture medium did not influence CE accumulation either. Inhibition of cyclo-oxygenase activity by indomethacin also failed to affect CE storage in cultured monocyte-derived macrophages incubated with AcLDL. Our findings are in agreement with the results of Sing et al. (23), who investigated the effects of PGE_2 and 6-keto-$PGF_{1\alpha}$ on accumulation and removal of CE in 3T3 mouse fibroblasts loaded with CE. Since these prostaglandins did not affect the cellular CE metabolism, the same authors subsequently studied the role of endogenously synthesized prostaglandins in similar cell systems (24). Cholesteryl ester accumulation and removal were found to be unaffected by both stimulation and inhibition of cyclo-oxygenase activity in cultured 3T3 mouse fibroblasts and pig arterial smooth muscle cells (24).

Cultured human monocytes have not only been shown to produce prostaglandins and thromboxanes, but also to secrete lipoxygenase products (25). This is of great relevance to our finding that nordihydroguaiaretic acid (NDGA) and BW 755 C, both specific lipoxygenase inhibitors (26), markedly suppressed the amount of CE stored in monocyte-derived macrophages incubated with AcLDL (Fig.2). This effect appeared to be dose-dependent for NDGA (Fig.3). To discriminate whether the influence of lipoxygenase inhibitors could be based on a non-specific anti-oxydative mechanism, the cells were exposed to vitamin E in similar experiments. Vitamin E failed to reduce cellular CE accumulation significantly. Therefore, we assume that inhibition of the lipoxygenase pathway of arachidonic acid metabolism in cultured human monocyte-derived macrophages effectively decreases the rate of experimentally induced CE accumulation. To our knowledge, such a relationship between the lipoxygenase pathway and CE metabolism in macrophages has not been reported before. The nature of this relationship has yet to be revealed.

Nevertheless, our findings may have consequences for pharmacological intervention in the processes of atherosclerosis and xanthomatosis.

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REFERENCES

1. Moncada, S. (1982) Br.J.Pharmac. 76, 3-31.
2. Needleman, P. and Kaley, G. (1978) N.Engl.J.Med. 298, 1122-1128.
3. Moncada, S. and Vane, J.R. (1979) N.Engl.J.Med. 300, 1142-1147.
4. Sinzinger, H., Feigl, W. and Silberbauer, K. (1979) Lancet ii, 469.
5. Gryglewski, R.J., Dembinska-Kiec, A., Zmuda, A.and Gryglewska, T (1978) Atherosclerosis 31, 385-394.
6. Larrue, J., Leroux, C., Daret, D. and Bricaud, H. (1982) Biochim.Biophys.Acta 710, 257-263.
7. Beitz, J. and Förster, W. (1980) Biochim.Biophys.Acta 620, 352-355.
8. Szczeklik, A. and Gryglewski, R.J. (1980) Artery 7, 488-495.
9. Fleisher, L.N., Tall, A.R., Wite, L.D., Miller, R.W. and Cannon, P.J. (1982) J.Biol.Chem. 257, 6653-6655.
10. Gordon, T., Castelli, W.P., Hjortland, M.C., Kannel, W.B. and Dawber, T.R. (1977) Amer.J.Med. 62, 707-714.
11. Hajjar, D.P. and Weksler, B.B. (1983) J.Lipid.Res. 24, 1176-1185.
12. Schaffner, T., Taylor, K., Bartucci, E.J., Fisher-Dzoga, K., Beeson, J.H., Glagov, S. and Wissler, R.W. (1980) Am.J.Pathol. 100, 57-80.
13. Gerrity, R.G. (1981) Am.J.Pathol. 103, 181-200.

14. Vos, J., Aarnoudse, M.W., Dijk, F. and Lamberts, H.B. (1983) Virchows Arch.B 43, 1-16.
15. Brown, M.S., Goldstein, J.L., Krieger, M., Ho Y.K. and Anderson, R.G.W. (1979) J.Cell Biol. 82, 597-613.
16. Redgrave, T.G., Roberts, D.C.K. and West, C. (1975) Anal.Biochem. 65, 42-49.
17. Basu, S., Goldstein, J.L., Anderson, R.G.W. and Brown, M.S. (1976) Proc.Natl. Acad.Sci.USA 73, 3178-3181.
18. Bergmeijer, H.V. (1970) Methoden der enzymatischen Analyse, 2nd Ed., Band I, Verlag Chemie, Weinheim, pp. 533-538.
19. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J.Biol.Chem. 193, 265-275.
20. Bligh, E.G. and Dyer, W.J. (1959) Can.J.Biochem.Physiol. 37, 911-917.
21. Armitage, P. (1971) Statistical methods in medical research. Blackwell Scientific Publications. Oxford. pp. 189-207.
22. Schrör, K., Darius, H., Matzky, R. and Ohlendorf, R. (1981) Naunyn-Schmiedeberg's Arch.Pharmacol. 316, 252-255.
23. Sing, S.P., Shamgar, F.R. and Day, A.J. (1982) Atherosclerosis 42, 109-119.
24. Sing, S.P., Shamgar, F.R. and Day, A.J. (1982) Exp.Molec.Pathol. 37, 74-84.
25. Pawlowski, N.A., Kaplan, G., Hamill, A.Z., Cohn, Z.A. and Scott, W.A. (1983) J. Exp.Med. 158, 393-412.
26. Bailey, D.M. and Casey, F.B. (1982) Ann.Reports Med.Chem. 17, 203-217.