Effects of oxidized low density lipoproteins on arachidonic acid metabolism in smooth muscle cells

Hanfang Zhang,* W. Bruce Davis,† Xunsheng Chen,* Kenneth H. Jones, Ronald L. Whisler,† and David G. Cornwell[1,*]**

Departments of Physiological Chemistry,* Internal Medicine,† and Anatomy,** The Ohio State University, Columbus, OH 43210

Abstract The role of oxidized plasma lipoproteins in modifying arachidonic acid (AA) metabolism was studied in smooth muscle cells (SMC). Very low density lipoproteins (VLDL), unoxidized low density lipoproteins (LDL_{BHT}) isolated with butylated hydroxytoluene (BHT), and oxidized LDL (LDL_{OXID}) were separated from human serum. Thiobarbituric acid reactant (TBAR) levels were adjusted by saline incubations. Prostanoids in guinea pig SMC cultures were measured either by radioimmunoassay (RIA) or the isolation by high performance liquid chromatography (HPLC) of labeled prostanoids from SMC prelabeled with [^{14}C]AA. Cell morphology and viability were studied by staining with Giemsa, nile red, and propidium iodide. VLDL and LDL_{BHT} had little effect on prostanoid synthesis. Low-TBAR-LDL_{OXID} enhanced total prostanoid levels and diminished the release of labeled prostanoids. Similar effects were found with exogenous free AA (unlabeled). Low-TBAR-LDL_{OXID} did not affect the release of endogenous phospholipid AA as free AA. Synergism ocurred between LDL_{OXID} and exogenous free AA in prostanoid synthesis. Low-TBAR-LDL_{OXID} evidently enhanced prostanoid levels in SMC both by supplying AA and by stimulating cyclooxygenase. High-TBAR-LDL_{OXID} blocked prostanoid synthesis and enhanced cell death but time and pulse-recovery experiments showed that these effects were unrelated. High-TBAR-LDL_{OXID} stimulated prostanoid synthesis when BHT was added to the incubation media. High-TBAR-LDL_{OXID} also caused massive free AA release and the formation of many nonprostanoid derivatives. High-TBAR-LDL_{OXID} evidently diminished overall prostanoid levels in SMC by inhibiting cyclooxygenase and at the same time stimulating AA release and the formation of other AA derivatives. —**Zhang, H., W. B. Davis, X. Chen, K. H. Jones, R. L. Whisler, and D. G. Cornwell.** Effects of oxidized low density lipoproteins on arachidonic acid metabolism in smooth muscle cells. *J. Lipid Res.* 1990. **31:** 551–565.

Supplementary key words PGI_2 • PGE_2 • fatty acid release • thiobarbituric acid reactants • cytotoxicity • cyclooxygenase • radioimmunoassay • high performance liquid chromatography

Polyunsaturated fatty acids are well known to be involved in the control of cell proliferation (reviewed in 1 and 2). Cells exposed to free arachidonic acid (AA) or agents that promote the release of AA from endogenous phospholipid synthesize prostanoids and lipid peroxides. Low concentrations of prostanoids stimulate cell proliferation whereas lipid peroxides and high concentrations of prostanoids inhibit cell proliferation. The effects of fatty acids on the aorta smooth muscle cell (SMC) have been studied extensively. Numerous studies have shown that positive and negative signals from prostanoids and lipid peroxides influence the growth of SMC (1–15). Prostanoid and lipid peroxide signals from low density lipoprotein (LDL) metabolism are thought to be important determinants of the pathogenesis of atherosclerosis because of their effects on SMC proliferation (16).

The role of LDL in regulating SMC proliferation is controversial. Some studies show that LDL, particularly oxidized LDL (LDL_{OXID}), are cytotoxic (17–21) and prior work from our laboratory shows that LDL_{OXID} inhibit growth of SMC (22). Other investigators have shown that LDL are mitogenic (23–26). Cytotoxicity and mitogenesis are two paradoxical effects that suggest that LDL, like free AA, may act on cells through positive and negative signals provided by prostanoids and lipid peroxides.

The degree of lipid peroxidation controls many of the effects of LDL on SMC. LDL_{OXID} with a low lipid peroxide content inhibited mitogenesis without being cytotoxic (22). LDL_{OXID} with a high lipid peroxide content were cytotoxic. Mitogenesis was restored and cytotoxicity

Abbreviations: AA, arachidonic acid; A23187, calcium ionophore; BHT, butylated hydroxytoluene; EDTA, ethylenediamine tetraacetic acid; FBS, fetal bovine serum; HPLC, high performance liquid chromatography; IM, indomethacin; LPS, lipopolysaccharide; LDL, low density lipoproteins LDL_{BHT}, unoxidized LDL; LDL_{OXID}, oxidized LDL; MDA, malondialdehyde; NL, neutral lipids; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipids; PS, phosphatidylserine; RIA, radioimmunoassay; SMC, smooth muscle cells; TBAR, thiobarbituric acid reactants; TLC, thin-layer chromatography; VLDL, very low density lipoproteins.

[1]To whom correspondence should be addressed.

was prevented by the addition of the antioxidant butylated hydroxytoluene (BHT) to cell cultures. LDL_{OXID} affected AA metabolism in cell cultures but these studies were difficult to interpret because of the formation of lipid oxidation products with cross-reactivity in the radioimmunoassay (RIA) of PGE_2 (22). We have developed a method for the controlled oxidation of LDL, identified some of the lipid oxidation products, and described the characteristics of cross-reacting materials (27). The present investigation examines the effects of unoxidized LDL (LDL_{BHT}) and LDL_{OXID} containing different amounts of lipid peroxides on prostanoid synthesis in SMC cultures.

MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources: [1-^{14}C]AA (54.4 mCi/mmol) and [U-^{14}C]AA (1.0 Ci/mmol) (New England Nuclear, Boston, MA); propidium iodide (Sigma Chemical Co., St. Louis, MO); nile red (Eastman Kodak, Rochester, NY); *Escherichia coli* WEO.27:B8 lipopolysaccharide (LPS) (Difco Laboratories, Detroit, MI); fetal bovine serum (FBS) (Sterile Systems, Logan, UT); other reagents as described elsewhere (27).

Tissue culture

Primary cultures of SMC were established from the dissected medial layer of guinea pig aorta from prepubertal males (28). The cells in these cultures were identified as SMC by their reactivity to antibodies against human umbilical artery F-actin which have been shown to react specifically with muscle actin isoforms (2, 29). The medium for growing cells to confluency (growth medium) and the medium for lipid peroxidation studies with confluent cultures (experimental medium) have been described (4, 6, 12–14). Cells were used at passage level 4. For morphologic studies, cells were fixed in 3.7% phosphate-buffered formalin and stained with filtered Giemsa. For viability studies, unfixed cells were stained with propidium iodide (22, 30). Cultures were then examined with epifluorescence illumination. Nonviable cells fluoresced red because propidium iodide, which was excluded from live cells, entered dead cells and intercalated with nucleic acid forming a red fluorescent complex. The fluorescent lipid probe nile red was used to show lipid accumulation in yellow-gold fluorescent structures when SMC were incubated with LDL_{OXID} (31).

Labeling of cell lipids

SMC were seeded at 1.3×10^4 cells/cm^2 in Corning 35-mm plates. After 3 or 4 days, SMC conditioned medium was collected from some of the plates. An ethanol solution of [^{14}C]AA was evaporated to dryness with N_2. The residue was redissolved in SMC conditioned media and 200 μl was added to a Corning plate. Concentrations were adjusted to 0.6 μM [U-^{14}C]AA or 8 μM [1-^{14}C]AA, based on the specific activity of the labeled AA, and the cultures were incubated for 16 h. Total cellular uptake was estimated by subtracting the label remaining in the medium at the end of the incubation period. Lipids from labeled cells were extracted with chloroform and methanol using a published procedure (7).

Lipids classes were separated by thin-layer chromatography (TLC) as previously described (7, 27, 32). Labeled lipids extracted from cells were detected by a radioscan and radioactive peaks coincided with bands visualized with UV light after spraying with 6-*p*-toluidino-2-naphthalenesulfonic acid. Bands were scraped and counted. The distribution of labeled AA in the different lipid fractions was calculated as percent of total cpm recovered from the TLC plate.

Labeled metabolite release

Confluent SMC prelabeled with [^{14}C]AA were re-fed with fresh experimental media containing different agents. The cells were incubated at 37°C for 24 h. At the end of the incubation period, media was withdrawn and the plate was rinsed once with an equal amount of physiologic saline. The media and saline wash were centrifuged at 2,000 rpm for 3–5 min and transferred to separate tubes. Aliquots of media and saline wash were counted. The total radioactivity in media and wash was divided by the total cellular uptake and expressed as percent of AA label released from the cells.

The [^{14}C]AA metabolites in the culture media were extracted by the addition of ethanol, acidified with formic acid, and extracted with ethyl acetate as previously described (12). Recoveries of radioactive 6-keto-$PGF_{1\alpha}$, PGE_2, and free AA were consistently greater than 90% when these compounds were added to culture media. High performance liquid chromatography (HPLC) was used to separate labeled AA metabolites (12, 27). This procedure used an ultrasphere-ODS reversed phase column and eluting solvents consisting of various mixtures of acetonitrile–isopropanol–aqueous phosphoric acid.

Prostanoids

PGE_2 and 6-keto-$PGF_{1\alpha}$ from cells labeled with [^{14}C]AA were identified by HPLC, and 6-keto-$PGF_{1\alpha}$ from unlabeled cells was then estimated by a standard RIA procedure (3, 9–15, 27). PGE_2 was measured by RIA in a few studies with VLDL and LDL_{BHT} but PGE_2 was not measured by RIA in LDL_{OXID} preparations since material cross-reacting with PGE_2 antibodies was formed during oxidation (22, 27). Antibodies were kindly supplied by Dr. Lawrence Levine (Brandeis University,

Waltham, MA). The cross-reactivity of the 6-keto-$PGF_{1\alpha}$ antibody was: PGE_2, 0.15%; PGD_2, 0.02%; $PGF_{2\alpha}$, 0.10%; AA, 0.005%. The cross-reactivity of the PGE_2 antibody was: 6-keto-$PGF_{1\alpha}$, 4%; PGF_1, 0.76%; PGF_2, 0.31%; PGD_2, 0.051%; AA, 0.00045%. Data for immunoreactive materials are expressed as nmoles/culture.

Lipoprotein preparation

Individual units of freshly drawn human plasma were converted to serum and LDL (1.019 to 1.063 g/ml) were isolated by ultracentrifugal flotation (33). LDL_{OXID} and unoxidized LDL (LDL_{BHT}) were prepared as previously described (27) and further characterized by electrophoresis in agarose gel (34). Relative electrophoretic mobility was unchanged for low-TBAR-LDL_{OXID} and was 1.1 for high-TBAR-LDL_{OXID} showing that mild oxidation which generated both low- and high-TBAR-LDL_{OXID} did not have a great effect on the protein moieties of the lipoproteins. Much higher relative electrophoretic mobilities have been reported for highly oxidized LDL preparations (19, 22, 35). Total cholesterol was measured by an established procedure (36) and LDL concentrations were reported as μg cholesterol throughout this study. In some experiments, very low density lipoproteins (VLDL, density $<$ 1.019 g/ml) and LDL were both isolated from the same serum by the two-step ultracentrifugal flotation procedure (33). The triglyceride content of the VLDL fraction was measured by an established procedure (37) and the concentration of VLDL was reported as μg triglyceride. Lipid peroxides were estimated as TBAR generated in the lipoprotein preparations (6, 9, 12–15, 27). Absorbance was measured at 532 nm and converted to nmoles malondialdehyde (MDA) from a standard curve generated with 1,1,3,3-tetramethoxypropane.

Statistics

Data are reported as mean ± SEM. Main and interaction effects are investigated by one-, two-, and three-way analyses of variance.

RESULTS

Prostanoid synthesis in cultured SMC

SMC cultures synthesize both PGE_2 and PGI_2 (6-keto-$PGF_{1\alpha}$) and prostanoid synthesis in these cultures is stimulated by the addition of precursor fatty acids (38) and a number of agents that differ in structure, function, and the duration of their stimulatory effects (9–13, 15, 39). In typical experiments with SMC, we found the following relative increments in 6-keto-$PGF_{1\alpha}$ measured as (treatment)/(media alone) in %: 100 μM dipyridamole, 207%; 100 μM hydralazine, 250%; 10 μM cyclosporine A, 161%; 1 μg/ml LPS, 358%. In present investigation, 1 μg/ml A23187 and 10 μg/ml LPS produced relative increments of 179% and 1260%. Relative increments for PGE_2 and 6-keto-$PGF_{1\alpha}$ with stimulatory agents such as LPS, cyclosporine A, and A23187 were similar when the relative increments were calculated from either RIA data or HPLC data with prelabeled cells (39). These results showed that the SMC cultures were capable of responding to a variety of stimulatory agents for prostanoid synthesis.

Preliminary studies with VLDL and LDL

VLDL and LDL were obtained from the same serum sample by a two-step ultracentrifugal fractionation procedure (33) and dialyzed against 0.15 M NaCl that did not contain either EDTA or BHT. LDL prepared in this way contained 0.7 nmol MDA/200 μg cholesterol (22, 27) and was designated low-TBAR-LDL_{OXID}. Different concentrations of the two lipoprotein fractions were incubated with SMC for 24 h. RIA data showed that VLDL had a small stimulatory effect on 6-keto-$PGF_{1\alpha}$ but not PGE_2 in the absence of LPS, and VLDL had no effect on either prostanoid in the presence of LPS (**Table 1**). Freshly isolated LDL_{OXID} stimulated 6-keto-$PGF_{1\alpha}$ synthesis to a much greater extent than freshly isolated VLDL (Table 1). Additional experiments showed both that LDL were highly susceptible to lipid peroxidation and that oxidation could be controlled when LDL were incubated at 37°C in 0.15 M NaCl (27). VLDL contained less TBAR and VLDL were much less susceptible to lipid peroxidation in 0.15 M NaCl although extensive oxidation of this lipoprotein fraction was catalyzed by Cu^{2+} or Fe^{3+} (data not shown). Since LDL_{OXID} had a significant stimulatory effect on prostanoid synthesis in SMC, and lipid peroxidation in this lipoprotein fraction was readily controlled without the addition of metal ions, LDL was selected for further studies on AA metabolism in SMC.

Unoxidized lipoproteins (LDL_{BHT}) and prostanoid synthesis in SMC

Our previous studies (22, 27, 33) showed that lipid peroxidation in lipoproteins was blocked both by ethylenediamine tetraacetic acid (EDTA) and antioxidants such as BHT. In the present study, we found that EDTA even in low concentration diminished prostanoid synthesis in SMC. For example, the 6-keto-$PGF_{1\alpha}$ level in cultures treated with 54 μM EDTA was only 63% of the 6-keto-$PGF_{1\alpha}$ level in cultures treated with media alone. Consequently, EDTA was not used in studies on AA metabolism. BHT in the 40 to 100 μM range had a small inhibitory effect on prostanoid synthesis (9, 12 and **Table 2**). However, BHT in the 10 to 20 μM range had no effect

TABLE 1. Small effect of VLDL and large effect of LDL_{OXID} on prostanoid synthesis in smooth muscle cells

Treatment	6-keto-$PGF_{1\alpha}$	PGE_2
	nmol/plate	
VLDL (μg) + media		
0	0.136 ± 0.002 (3)[a,c]	0.036 ± 0.005 (3)[a]
200	0.189, 0.175	0.041, 0.052
400	0.210, 0.219	0.043, 0.043
800	0.171, 0.184	0.048, 0.051
VLDL (μg) + 10 μg/ml LPS		
0	1.85 ± 0.12 (3)[b]	1.45 ± 0.11 (3)[b]
200	1.86, 2.06	1.29, 1.66
400	1.64, 1.59	1.21, 1.57
800	1.75, 1.56	1.31, 1.74
LDL_{OXID} (μg) + media		
0	0.136 ± 0.002 (3)[c]	
200	0.285, 0.351	
400	0.386, 0.403	
800	0.447, 0.368	

SMC were incubated for 24 h with different concentrations of VLDL (μg triglyceride/plate) or LDL_{OXID} (μg cholesterol/plate) that were isolated at the same time from the same serum.

[a]A two-way analysis of variance showed that 6-keto-$PGF_{1\alpha}$ was greater than PGE_2 (F 1787, $P < 0.0001$) and that VLDL enhanced the total prostanoid content (F 37.61, $P < 0.0001$). However, an analysis of the interaction (F 23.51, $P < 0.0001$) showed that VLDL affected 6-keto-$PGF_{1\alpha}$ but not PGE_2.

[b]A two-way analysis of variance showed that 6-keto-$PGF_{1\alpha}$ was greater than PGE_2 (F 10.21, $P < 0.0096$) and that VLDL had no effect on the level of either prostanoid (F 0.82, $P < 0.5117$).

[c]A two-way analysis of variance showed that 6-keto-$PGF_{1\alpha}$ was enhanced more by LDL_{OXID} than VLDL (F 107.3, $P < 0.0001$).

TABLE 2. Low concentrations of BHT and LDL_{BHT} have little effect on the 6-keto-$PGF_{1\alpha}$ level in SMC

Treatment	No AA	120 μM AA
	6-keto-$PGF_{1\alpha}$	
	nmol/plate	
BHT (μM)		
0	0.26 ± 0.01 (12)[a,c]	1.20 ± 0.08 (3)[b]
10	0.26 ± 0.02 (9)	1.23 ± 0.04 (3)
20	0.25 ± 0.03 (5)	1.12 ± 0 (3)[c]
30	0.23 ± 0.04 (5)	1.14 ± 0.05 (3)
40	0.22 ± 0 (3)	1.04 ± 0.08 (3)
100	0.16 ± 0.03 (5)	0.98 ± 0.04 (3)
LDL_{BHT} (μg) in low BHT (10 and 20 μM)		
0	0.287 ± 0.014 (6)[c]	1.43 ± 0.025 (4)[c]
200	0.257 ± 0.012 (4)	1.51, 1.41
400	0.247 ± 0.013 (4)	1.49, 1.49
800	0.254 ± 0.015 (4)	1.49, 1.60
	PGE_2	
LDL_{BHT} (μg) in high BHT (100 μM)		
0	0.043 ± 0.04 (6)[d]	
200	0.032 ± 0.03 (6)	
400	0.032 ± 0.03 (6)	
800	0.033 ± 0.03 (5)	

SMC in media alone or 120 μM AA were incubated with different concentrations of BHT or LDL_{BHT} (μg cholesterol/ml).

[a]A one-way analysis of variance showed that BHT affected the 6-keto-$PGF_{1\alpha}$ level (F 2.556, $P < 0.0462$) but no two BHT concentrations differed significantly from each other (Scheffe test).

[b]A two-way analysis of variance showed that LDL_{BHT} did not affect the 6-keto-$PGF_{1\alpha}$ level (F 2.928, P 0.0591).

[c]A two-way analysis of variance showed that AA enhanced (F 6330, P 0.0001) and that LDL_{BHT} had no effect (F 1.29, P 0.3041) on the 6-keto-$PGF_{1\alpha}$ level.

[d]A one-way analysis of variance showed that LDL_{BHT} had no effect on the PGE_2 level (F 2.726, P 0.0728).

on prostanoid synthesis (Table 2) and 10 μM BHT was sufficient to prevent the controlled oxidation of LDL. RIA data showed that LDL_{BHT} prepared in 10 or 20 μM BHT had no effect on 6-keto-$PGF_{1\alpha}$ levels in SMC incubated with media alone or 120 μM AA (Table 2). Similar results were obtained with LDL_{BHT} for PGE_2 levels and since LDL_{BHT} does not cross-react with antibodies to PGE_2 (22, 27), the RIA data obtained with LDL_{BHT} represented true PGE_2 levels (Table 2). The 6-keto-$PGF_{1\alpha}$ and PGE_2 data showed that LDL_{BHT}, an unoxidized lipoprotein preparation, did not affect prostanoid metabolism in SMC.

LDL_{OXID} and RIA of PGI_2

Preliminary experiments were undertaken with LDL_{OXID} and authentic 6-keto-$PGF_{1\alpha}$ in order to validate the RIA assay for this prostanoid. LDL_{OXID} was prepared with 1.8 nmol MDA/200 μg cholesterol and added to media at a concentration of 800 μg/ml or 9.2 nmol MDA/1.3 ml plate. After the plates were incubated at 37°C for 24 h, the 6-keto-$PGF_{1\alpha}$ content (RIA) was 0.0077 ± 0.0002 nmol/plate. After authentic 6-keto-$PGF_{1\alpha}$ was added to media only in other plates and incubated for 24 h, the 6-keto-$PGF_{1\alpha}$ content (RIA) was 0.076 ± 0.0056 nmol/plate. Authentic 6-keto-$PGF_{1\alpha}$ (0.076 nmol/plate) and LDL_{OXID} (0.0077 nmol/plate) were combined and incubated. The 6-keto-$PGF_{1\alpha}$ levels (RIA) in the mixtures were 0.091 ± 0.0054 and 0.078 ± 0.0071 nmol/plate in two separate experiments. Thus 6-keto-$PGF_{1\alpha}$ levels assayed in the mixtures were 108% and 93% of the sum (0.084 nmol/plate) of the authentic prostanoid and LDL_{OXID} alone. These data confirmed our earlier observation the LDL_{OXID} did not cross-react significantly with antibodies to 6-keto-$PGF_{1\alpha}$ (22, 27). Furthermore, LDL_{OXID} did not destroy or interact with authentic 6-keto-$PGF_{1\alpha}$.

LDL_{OXID} and PGI_2 synthesis in SMC

LDL_{OXID} preparations containing different amounts of lipid peroxides measured as TBAR were incubated with confluent SMC. Refractile lipid droplets were seen with phase contrast microscopy and nile red staining showed that these droplets had the yellow-gold fluorescence characteristic of droplets in cells treated with LDL (31). 6-Keto-$PGF_{1\alpha}$ in culture media was measured by RIA. All prostanoid synthesis in these cultures was blocked by 10 μM indomethacin (IM) added to media alone or media containing different concentrations of lipid peroxides (data not shown). Relative 6-keto-$PGF_{1\alpha}$ content, (treatment)/(media alone) in % was reported as a function of the lipid peroxide content (nmol MDA/plate) since preliminary experiments showed that PGI_2 varied with total lipid peroxide rather than total LDL. The relative RIA data (**Fig. 1**) showed that PGI_2 generated from SMC in tissue culture was enhanced by low concentrations of lipid peroxides (low-TBAR-LDL_{OXID}) and diminished by high concentrations of lipid peroxides (high-TBAR-LDL_{OXID}). (Fig. 1 also contains HPLC data which will be discussed in a later section).

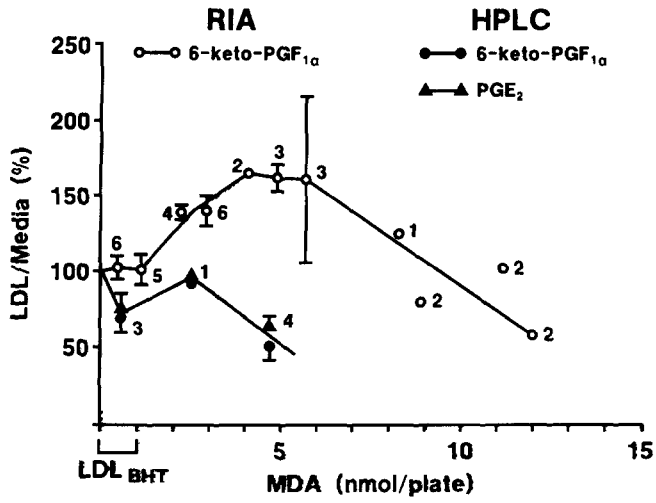

Fig. 1. The relative prostanoid content of SMC cultures is a function of the amount of lipid peroxide supplied by LDL_{OXID}. In RIA experiments, confluent SMC cultures were incubated for 24 h at 37°C with media alone and with LDL_{OXID} preparations which supplied different amounts of lipid peroxides. The 6-keto-$PGF_{1\alpha}$ level in media alone was 0.1.05 ± 0.0056 nmol/plate (mean ± SEM for 26 different incubation studies). Data are reported as the relative prostanoid level (treatment)/(media alone) in %. The number of SMC culture-LDL_{OXID} preparations for each data point is listed in the figure. Each preparation was generally incubated with two or three culture plates. In HPLC experiments, labeled cells were incubated for 24 h at 37°C with media alone or media containing LDL_{OXID}. Metabolites were extracted, separated, and counted. Data are reported as relative PGE_2 and 6-keto-$PGF_{1\alpha}$ levels calculated in %. The number of preparations is listed in the figure. Each preparation was incubated with one culture plate.

SMC viability and PGI_2 synthesis

Previous studies from our laboratory and elsewhere found that high-TBAR-LDL_{OXID} damaged cells increasingly over a 24-h period and ultimately caused cell death (17, 18, 20–22, 40). This observation suggested that diminished prostanoid synthesis with high-TBAR-LDL_{OXID} could be related to a general effect on viability. The observation could also be explained by a specific effect on prostanoid synthesis since the enzyme activity of the cyclooxygenase complex is susceptible to inhibition by oxidants (41–46).

Since BHT protects SMC viability (22, 40) PGI_2 synthesis was measured in cultures treated with LDL_{OXID} and several concentrations of the antioxidant combined in two groups, low-BHT and high-BHT, which had little effect on absolute PGI_2 synthesis (Table 2). Morphologic studies showed that extensive cell death occurred at 24 h with high-TBAR-LDL_{OXID} in media alone. Viability and morphology improved with low-BHT and, as previously observed (22, 40), all cells remained viable in high-BHT.

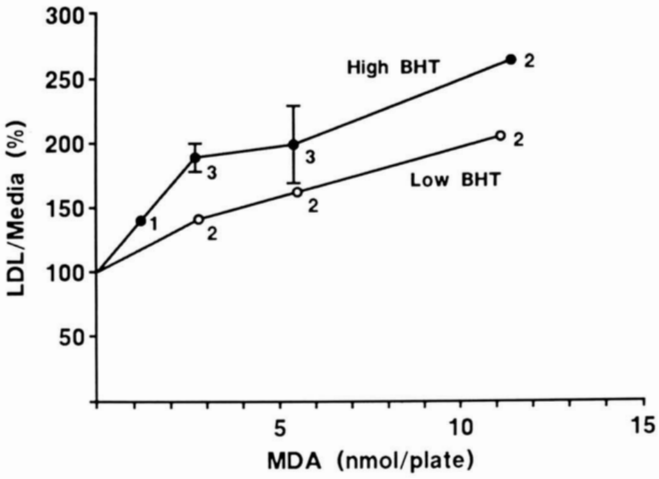

Fig. 2. High-TBAR-LDL_{OXID} levels do not inhibit PGI_2 synthesis when SMC incubated with either 10 or 20 μM BHT (Low BHT) and either 40 or 100 μM BHT (High BHT). LDL_{OXID} was added to confluent cultures and incubated for 24 h at 37°C with media containing different concentrations of BHT. 6-Keto-$PGF_{1\alpha}$ was assayed by RIA and reported as the relative prostanoid level (treatment)/(media containing only BHT) in %. The number of measurements is explained in Fig. 1.

In these studies, high-BHT reduced LDL_{OXID} TBAR in the cultures to 57.8 ± 5.6% of the lipid peroxide levels in the absence of BHT. A similar reduction, but not the elimination of TBAR, was found in other studies with LDL_{OXID} and BHT (27).

The relative PGI_2 content after a 24-h incubation period increased with increasing lipid peroxide levels in both low-BHT and high-BHT groups (**Fig. 2**). These data showed that diminished PGI_2 synthesis in cultures containing high levels of lipid peroxides (Fig. 1) was related to lipid peroxide levels. BHT lowered but did not eliminate lipid peroxides which evidently remained at levels sufficient to stimulate prostanoid synthesis. The 24-h BHT data did not distinguish between a general oxidant effect on viability and a specific oxidant effect on PGI_2 synthesis, but most PGI_2 synthesis in SMC occurred during the early phase of the incubation period (9) and preliminary experiments suggested that cells were viable during that time.

Diminished prostanoid synthesis with high-TBAR-LDL_{OXID} was separated from a general effect on viability

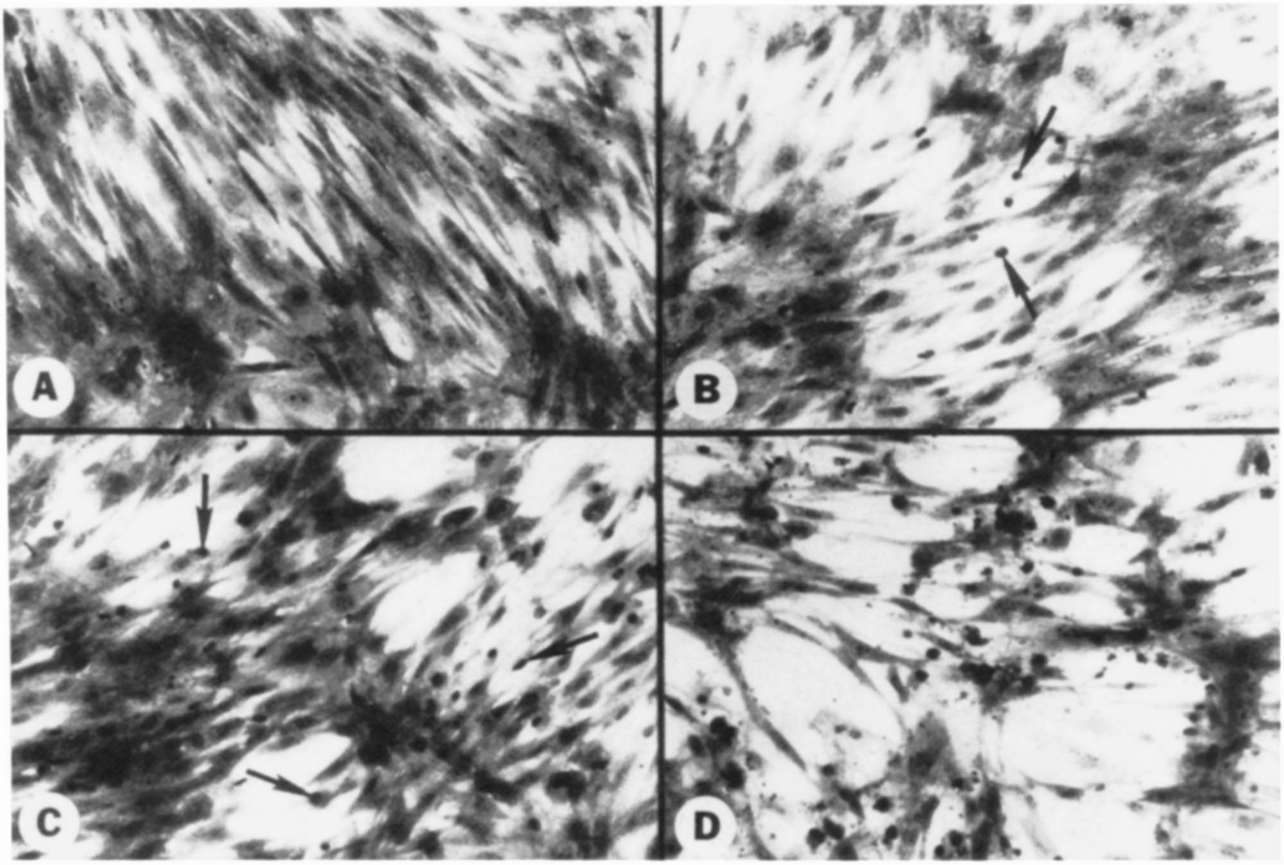

Fig. 3. High-TBAR-LDL_{OXID} (12.3 nmol MDA/plate) damages SMC and diminishes viability during a 24-h incubation period. Confluent SMC cultures were incubated with LDL_{OXID}, fixed, stained with filtered Giemsa at 2, 4, 8, and 24 h. A(2 h): no differences were seen compared to media alone (see Fig. 4-A). B(4 h): most cells appeared viable and healthy but there was a slight increase in the number of pyknotic nuclei (arrows); propidium iodide staining increased from less than 5% in media to 10% in LDL_{OXID}. C(8 h): increased cell damage was evidenced by frequent pyknotic nuclei (arrows) and areas where dead or damaged cells had detached from the plate; propidium iodide staining increased to 30–35%. D(24 h): extensive cell damage and death were evidenced by pyknotic nuclei and large areas where cells had detached; most attached cells stained with propidium iodide. × 160 original magnification.

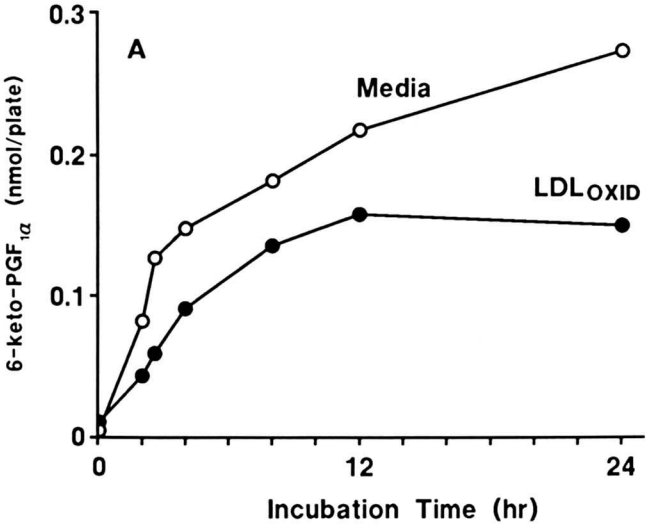

Fig. 4. High-TBAR-LDL_{OXID} (12.3 nmol MDA/plate) diminishes PGI_2 synthesis during the early phase of the incubation period when SMC remain viable. SMC were incubated with media alone and media containing LDL_{OXID}. 6-Keto-$PGF_{1\alpha}$ was assayed by RIA at different times during the incubation period and reported as the actual prostanoid level. The same cultures were used for the prostanoid level and morphologic examination (Fig. 3). Some of these data were used in Table 3.

in two other studies. In the first study, SMC were treated with high-TBAR-LDL_{OXID} and both morphology and PGI_2 synthesis were examined over a 24-h incubation period. In the second pulse-recovery experiment, SMC were treated with high-TBAR-LDL_{OXID} for 2.5 h and then incubated with fresh media alone for a 24-h period. Morphology and PGI_2 synthesis were also examined in the pulse-recovery study.

SMC treated with high-TBAR-LDL_{OXID} for 2 h were viable and had the same morphology as untreated cells (**Fig. 3**) and yet PGI_2 synthesis was diminished in these cells (**Fig. 4**). Overall culture viability was not greatly changed at 4 h, although occasional cell damage was seen (Fig. 3), and yet PGI_2 synthesis remained depressed (Fig. 4). Increased cell damage and markedly decreased viability were observed at 8 h and 24 h (Fig. 3). PGI_2 levels remained depressed but it was clear that PGI_2 levels were decreased most significantly during the early phase of the time study when cells remained viable (Fig. 4). Thus PGI_2 levels were affected by LDL_{OXID} before cell death.

In the pulse-recovery experiment, SMC incubated for 2.5 h with LDL_{OXID} contained less PGI_2 than SMC in-

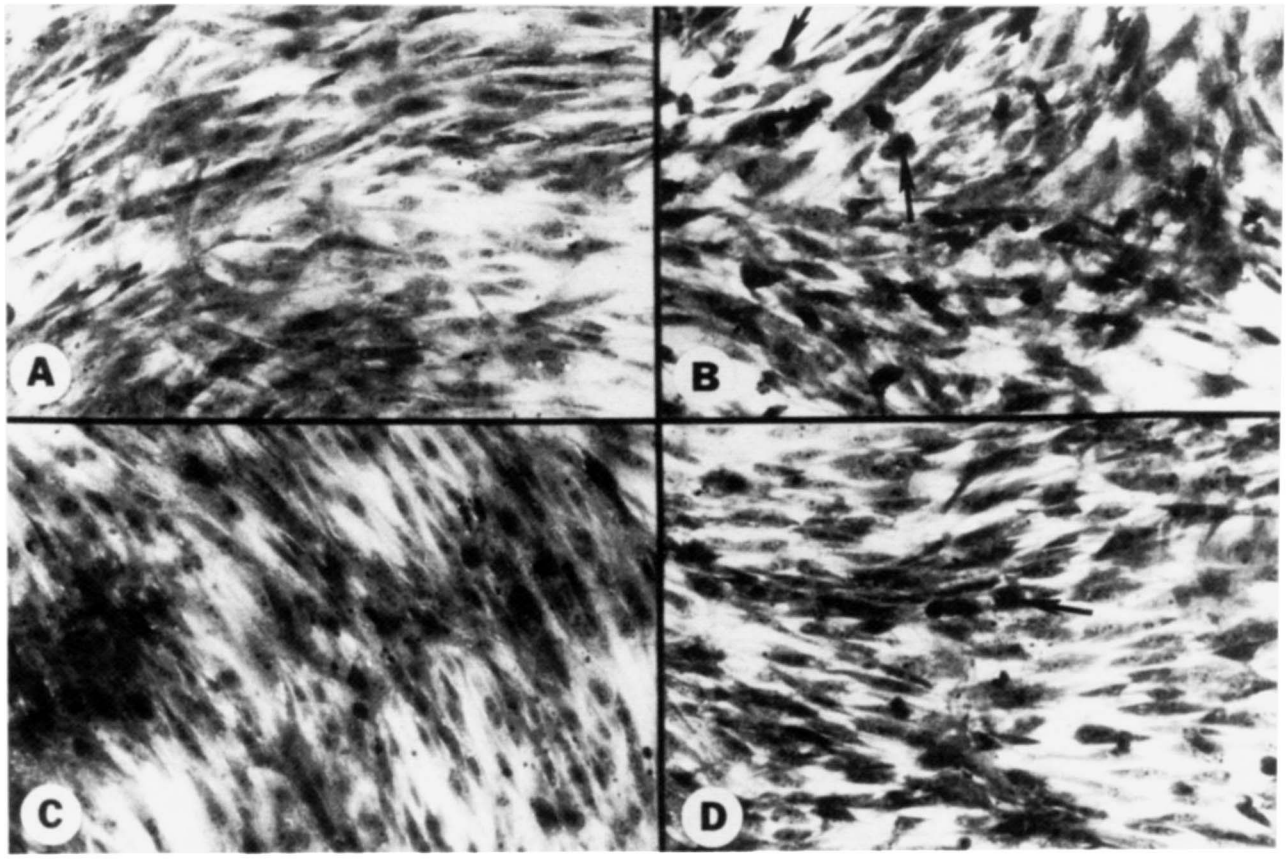

Fig. 5. SMC are rescued from high-TBAR-LDL_{OXID} (12.3 nmol MDA/plate) when cells are incubated for 2.5 h with LDL_{OXID} and then incubated with fresh media in a pulse-recovery experiment. A(2.5 h media alone): cells were undamaged and viable. B(2.5 h media–24 h fresh media): cells were undamaged and had mitotic figures (arrows) that averaged 8.9 ± 1.19/250 × field. C(2.5 h LDL_{OXID}): no differences were seen compared to cultures treated with media alone (A). D(2.5 h LDL_{OXID}–24 h fresh media): viability and density were similar to cultures treated with media alone (B) but mitotic figures (arrow) in treated cells were only 0.6 ± 0.7/250 × field; treated cells did not stain with propidium iodide. × 160 original magnification.

cubated with media alone (Fig. 4). These SMC were rescued when the media containing LDL_{OXID} was replaced by fresh media alone and viability and morphology were maintained when cells were incubated for an additional 24 h (**Fig. 5**). Viable cells rescued in the pulse-recovery experiment had fewer mitotic figures, reflecting the inhibitory effect of lipid peroxides on SMC proliferation that we had previously noted (1, 2, 4, 8, 9, 13, 14). However, PGI_2 levels in high-TBAR-LDL_{OXID}-treated cells that were rescued with fresh media actually exceeded PGI_2 levels in untreated cells at all subsequent incubation times (**Fig. 6**). These data confirmed our conclusion that the inhibition of PGI_2 synthesis with high-TBAR-LDL_{OXID} was not related to cell death.

Synergism between LDL_{OXID} and free AA in PGI_2 synthesis

Prostanoid synthesis in SMC is enhanced by the addition of free AA to the cultures (9, 11, 13, 38, 39). Agents that promote endogenous AA release from SMC have little effect on prostanoid synthesis in the presence of large amounts of exogenous free AA, whereas agents that act on cyclooxygenase have a synergistic effect on prostanoid synthesis in the presence of exogenous AA (39). Thus studies with exogenous free AA provide evidence for the site of action for a stimulatory agent on prostanoid synthesis.

Low-TBAR-LDL_{OXID} stimulated prostanoid synthesis in the absence of exogenous free AA (**Table 3-A**). Free AA greatly enhanced PGI_2 synthesis, and the stimulatory effect of LDL_{OXID} in the presence of free AA was much greater than the stimulatory effect of LDL_{OXID} in media alone. Similar results were obtained with high-TBAR-LDL_{OXID} and free AA. High-TBAR-LDL_{OXID} inhibited prostanoid synthesis in media alone but synergism between free AA and high-TBAR-LDL_{OXID} greatly enhanced PGI_2 synthesis overcoming the inhibitory effect (Table 3-B). These results were further confirmed in a pulse-recovery experiment where synergism between high-TBAR-LDL_{OXID} supplied in the pulse phase and free AA supplied in the recovery phase again enhanced PGI_2 synthesis (Table 3-C). These data showed that synergism occurred between free AA and LDL_{OXID}.

A recent study from our laboratory showed that prostanoid synthesis in SMC cultures was stimulated with low concentrations of exogenous hydroperoxy fatty acids and inhibited with high concentrations of these compounds (47). The stimulatory effect obtained with hydroperoxy fatty acids was greatly enhanced by free AA and a recalculation of the data from this study showed that synergism occurred between free AA and the hydroperoxy fatty acids (**Table 4**). These data indicated that the stimulation of prostanoid synthesis and a synergistic effect associated with this stimulation were general properties of exogenous lipid peroxides and not unique properties of LDL_{OXID}. These data may help to explain synergism in the pulse-recovery experiment (Table 3-C) since high-TBAR-LDL_{OXID} probably supplied lipid peroxides to SMC during incubation in the pulse phase.

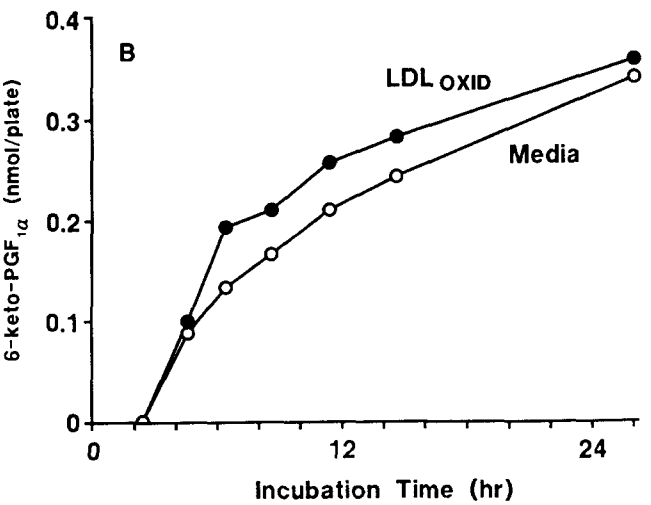

Fig. 6. High-TBAR-LDL_{OXID} (12.3 nmol MDA/plate) does not inhibit PGI_2 synthesis when SMC are rescued by a pulse-recovery experiment. SMC were incubated with media alone and media containing LDL_{OXID} for 2.5 h. 6-Keto-$PGF_{1\alpha}$ was assayed by RIA (see Fig. 4) and the medium in each culture was replaced with fresh medium which was assayed immediately and at different times during the subsequent incubation period. Actual prostanoid levels are reported. The same cultures were used for the prostanoid level and morphologic examination (Fig. 5). Some of these data were used in Table 3.

Labled AA uptake in SMC

SMC prelabeled with [^{14}C]AA have been used in several studies from our laboratory on AA metabolism (7, 39, 48). In these studies, AA was added to cultures by replacing the media with fresh media containing the labeled AA. Fresh media used in this protocol stimulated prostanoid synthesis during the labeling process. In the present study, labeled AA was dissolved in 200 μl of media that had been preconditioned by a 3- to 4-day incubation with SMC and this mixture was added to cultures without a media change. The new protocol with conditioned media did not stimulate prostanoid synthesis during the labeling process, resulted in a greater incorporation into phospholipids (PL), and altered the relative distribution of labeled AA into phosphatidylcholine (PC) and phosphatidylethanolamine (PE).

AA uptake from preconditioned media was rapid for the first 6 h and reached a plateau at 16 h. Over 90% of the label had disappeared from the media at the end of the 16-h incubation period and over 90% of the radioactivity taken up by the cells was recovered in the lipid extract. TLC showed that only 7.1 ± 0.4% of the radioactivity was recovered in the neutral lipid (NL) fraction. Previous labeling experiments with fresh media resulted in 41.6%

TABLE 3. LDL_{OXID} and free AA have a synergistic effect on PGI_2 synthesis in SMC

Treatment	Incubation Time	No AA	AA
	h	*nmol 6-keto-PGF$_{1\alpha}$*/plate	
A. LDL_{OXID}	24	0.142 ± 0.005 (3)	0.583 ± 0.019 (3)
Media		0.112 ± 0.008 (3)	0.408 ± 0.009 (3)
B. LDL_{OXID}	2	0.045, 0.042	0.164, 0.189
Media		0.079, 0.085	0.195, 0.189
LDL_{OXID}	4	0.086, 0.095	0.368, 0.347
Media		0.154, 0.142	0.459, 0.389
LDL_{OXID}	8	0.143, 0.130	0.614, 0.716
Media		0.179, 0.184	0.632, 0.596
LDL_{OXID}	12	0.153, 0.164	0.768, 0.789
Media		0.213, 0.224	0.768, 0.684
LDL_{OXID}	24	0.151	0.713
Media		0.274	0.746
C. LDL_{OXID}	2	0.105, 0.096	0.330, 0.474
Media		0.088, 0.088	0.395, 0.342
LDL_{OXID}	4	0.197, 0.190	0.658, 0.621
Media		0.145, 0.123	0.658, 0.571
LDL_{OXID}	6	0.219, 0.202	0.765, 0.744
Media		0.180, 0.155	0.660, 0.674
LDL_{OXID}	9	0.253, 0.263	0.987, 0.987
Media		0.216, 0.205	0.722, 0.803
LDL_{OXID}	12	0.283	1.042
Media		0.245	0.893
LDL_{OXID}	24	0.360	1.162
Media		0.342	1.053

A: SMC were incubated with media or 30 μM AA, and low-TBAR-LDL_{OXID} (2.9 nmol MDA/plate) with media or 30 μM AA. A two-way analysis of variance showed that both 30 μM AA (F 1163, $P < 0.0001$) and LDL_{OXID} (F 88.67, $P < 0.0001$) enhanced the 6-keto-$PGF_{1\alpha}$ level and that LDL_{OXID} and AA interacted (F 44.53, P 0.0002) to enhance the 6-keto-$PGF_{1\alpha}$ level.

B: SMC were incubated for different times with media or 60 μM AA, and high-TBAR-LDL_{OXID} (12.3 nmol MDA/plate) with media or 60 μM AA. A three-way analysis of variance showed that both 60 μM AA (F 1584, $P < 0.0001$) and incubation time (F 206.8, $P < 0.0001$) enhanced, and LDL_{OXID} (F 11.86, P 0.0033) lowered the 6-keto-$PGF_{1\alpha}$ level. LDL_{OXID} and AA interacted (F 9.98, P 0.0061) to enhance the 6-keto-$PGF_{1\alpha}$ level.

C: SMC were preincubated with media or high-TBAR-LDL_{OXID} (12.3 nmol MDA/plate) for 2.5 h. In this pulse-recovery experiment, media and LDL_{OXID} were removed and cultures were incubated for different times with fresh media or 60 μM AA. A three-way analysis of variance showed that 60 μM AA (F 2564, $P < 0.0001$), incubation time (F 158.97, $P < 0.0001$) and preincubation with LDL_{OXID} (F 37.72, $P < 0.0001$) all enhanced the 6-keto-$PGF_{1\alpha}$ level. LDL_{OXID} and AA interacted (F 8.27, P 0.0110) to enhance the 6-keto-$PGF_{1\alpha}$ level.

uptake in the NL fraction (7, 48). The distribution of radioactivity from preconditioned media was: phosphatidylinositol (PI), 15.2 ± 1.1%; PC, 23.1 ± 0.7%; and PE, 44.9 ± 1.1%. Differences in the distribution of radioactivity when SMC were incubated with preconditioned media and fresh media are shown in **Fig. 7**. TLC separations used in the earlier study (48) did not separate PI from phosphatidic acid (PA) and phosphatidylserine (PS). However, a subsequent separation on a ammonium nitrate plate showed that only traces of PS and PA were present in this fraction (48). It should also be noted that the distribution of radioactivity is affected by both incubation time and media. For example, the relative uptake in PI was much greater after short 1-min and 30-min incubation periods (48).

LDL_{OXID} and endogenous AA metabolism

Previous HPLC studies from our laboratory showed that labeled 6-keto-$PGF_{1\alpha}$, PGE_2, and free AA were found in the media when prelabeled SMC were incubated with fresh media for 24 h (12, 39). Similar data were obtained in the present study with [1-^{14}C]AA and [U-^{14}C]AA. The radioactivity released in prostanoid and free AA fractions (HPLC) from prelabeled cultures treated with media

TABLE 4. Hydroperoxy fatty acids and free AA have a synergistic effect on PGI_2 synthesis in SMC

Treatment	No AA	120 μM AA
	nmol 6-keto-$PGF_{1\alpha}$/plate	
15-HPEPE		
Media	0.36 ± 0.01 (12)[a]	1.46 ± 0.10 (3)[a]
5 μM	0.45 ± 0.02 (11)	2.47, 2.14
15-HPETE		
Media	0.26 ± 0.03 (8)[b]	0.74 ± 0.04 (4)[b]
5 μM	0.37 ± 0.05 (4)	0.98, 0.86
25 μM	0.43 ± 0.02 (4)	1.16, 1.19
50 μM	0.42 ± 0.03 (4)	1.47, 1.47

SMC were incubated for 24 h in media alone and media containing [5Z, 8Z, 11Z, 13E, 15(s)]-15-hydroperoxyeicosatetraenoic acid (15-HPETE) or [5Z, 8Z, 11Z, 13E, 15(S), 17Z]-15-hydroperoxyeicosapentaenoic acid (15-HPEPE) in the presence and absence of 120 μM AA.

[a]A two-way analysis of variance showed that both 15-HPEPE (F 122.02, $P < 0.0001$) and AA (F 1215.95, $P < 0.0001$) enhanced the 6-keto-$PGF_{1\alpha}$ level. 15-HPEPE and AA interacted (F 77.99, $P < 0.0001$) to enhance the 6-keto-$PGF_{1\alpha}$ level.

[b]A two-way analysis of variance showed that both 15-HPETE (F 43.27, $P < 0.0001$) and AA (F 479.45, $P < 0.0001$) enhanced the 6-keto-$PGF_{1\alpha}$ level. 15-HPETE and AA interacted (F 16.62, $P < 0.0001$) to enhance the 6-keto-$PGF_{1\alpha}$ level.

alone is reported in **Table 5**. The radioactivity released in prostanoid fractions was lowered when prelabeled cultures were incubated with media containing low-TBAR-LDL_{OXID} (Table 5) and, as a consequence, the relative prostanoid content estimated by HPLC was much lower than the relative prostanoid content measured by RIA (Fig. 1). Similarly, the radioactivity released in prostanoid fractions was lowered by unlabeled exogenous AA (Table 5) even though total prostanoid levels were increased significantly when SMC cultures were incubated with exogenous AA (Tables 2, 3, and 4). Thus exogenous AA (unlabeled) and low-TBAR-LDL_{OXID} had the same overall effect on relative prostanoid levels measured by the two procedures. In contrast to exogenous AA (unlabeled) and low-TBAR-LDL_{OXID}, relative prostanoid levels measured by HPLC were increased significantly by the fatty acid releasing agent A23187 (Table 5).

Low-TBAR-LDL_{OXID} had no effect on radioactivity released in the free AA fraction (Table 5). Thus free AA radioactivity was similar in cultures incubated with media alone and cultures incubated with low-TBAR-LDL_{OXID}. Furthermore, radioactivity in the free AA fraction was only increased by 241 cpm (average of two experiments) when prostanoid synthesis in low-TBAR-LDL_{OXID} cultures was totally blocked by IM. The fatty acid-releasing agent A23187 caused a large increase in the radioactivity of free AA (Table 5). Furthermore, radioactivity was, as expected (39), increased by 6,710 cpm (average of two experiments) when prostanoid synthesis in A23187 cultures was totally blocked by IM and free AA could not be metabolized by the cyclooxygenase pathway. These data showed that low-TBAR-LDL_{OXID} had, in contrast to A23187, little effect on AA release from cellular PL.

High-TBAR-LDL_{OXID} and low-TBAR-LDL_{OXID} had very different effects on endogenous AA metabolism (**Fig. 8**). As anticipated from RIA data (Fig. 1), very little radioactivity was released in prostanoid fractions when prelabeled cultures were incubated with high-TBAR-LDL_{OXID} (Fig. 8-B). Furthermore, a very large amount of labeled free AA was released and significant amounts of a number of other labeled AA derivatives were formed in the presence of high-TBAR-LDL_{OXID}. IM did not block the formation of labeled AA derivatives when SMC were incubated with LDL_{OXID} (data not shown). These data showed that high TBAR-LDL_{OXID} (Fig. 8-B), unlike low-TBAR-LDL_{OXID} (Fig. 8-A), had profound effects on both endogenous fatty acid release and the formation of many labeled AA derivatives.

DISCUSSION

Previous investigations of the effects of LDL on the biosynthesis of prostanoids have yielded differing results. Some studies showed that LDL stimulated while other studies showed that LDL inhibited prostanoid synthesis (49–53). Our results showed that the degree of lipid peroxidation is an important determinant of the effects of LDL on prostanoid synthesis. Unoxidized LDL_{BHT} had no effect on prostanoid synthesis in cell cultures (Table 2 and Fig. 1) whereas low-TBAR-LDL_{OXID} stimulated and high-TBAR-LDL_{OXID} inhibited prostanoid synthesis (Fig. 1). The data indicated that the effects of low-TBAR-LDL_{OXID} and high-TBAR-LDL_{OXID} were explained by the scheme outline in **Fig. 9**. It must be emphasized that the high-TBAR-LDL_{OXID} in this scheme did not have greatly increased electrophoretic mobilities and generally

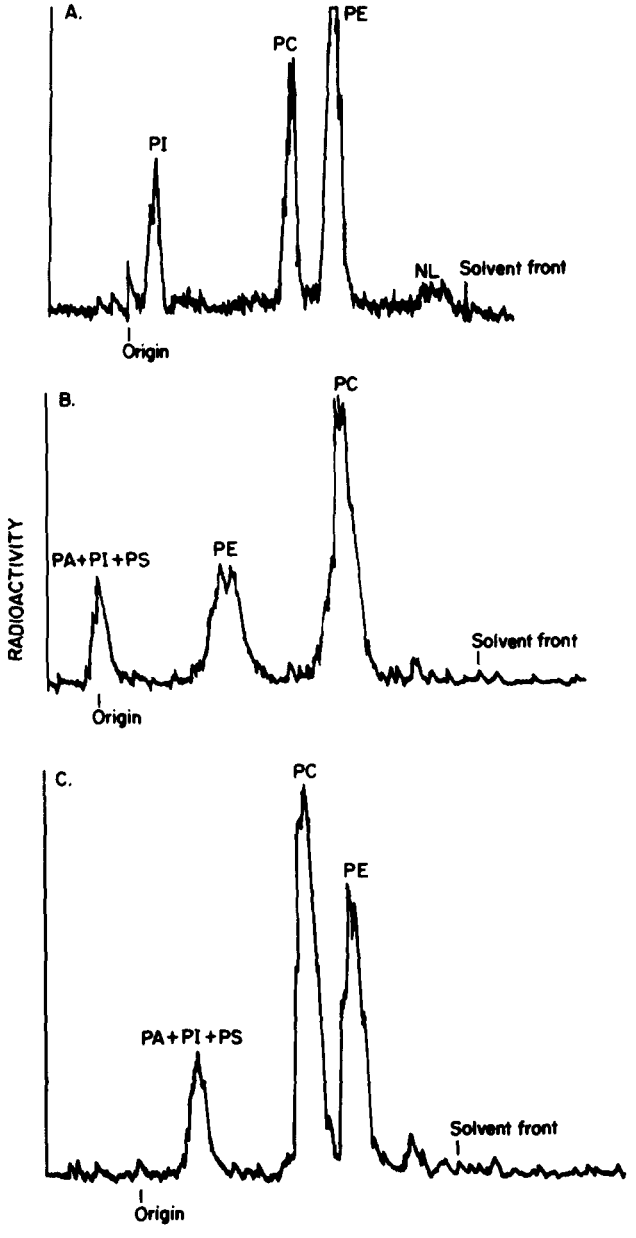

Fig. 7. The relative distribution of radioactivity in PE is much greater after incubation with preconditioned media (A) than fresh media (B, C). Lipid extracts from labeled cells were separated by the following TLC systems: A, Whatman LK5D plate with chloroform–methanol–40% methylamine 60:20:5 (v/v) (32); B, alumina plate with chloroform–methanol–water–pyridine–ammonia 65:27.5:4:2:2 (v/v) (48); C; Whatman LK5D plate with chloroform–methanol–ammonia–water 70:30:4:1 (48).

had a lower MDA content than LDL incubated with metal ions and other cells (19, 22, 27, 35).

The difference between relative prostanoid levels found by RIA and HPLC for both low-TBAR-LDL$_{OXID}$ and 30 μM exogenous AA deserves comment. Other agents such as A23187 do not shown this difference. Lipoproteins contain esterified AA, and LDL$_{OXID}$ at concentrations used in the present study, 400 to 1,600 μM cholesterol, supplied cultures with from ca. 25 to more than 100 μM esterified AA (33, 54). We propose that unlabeled AA esters in low-TBAR-LDL$_{OXID}$ diluted the specific activity of the total AA ester pool (Fig. 9) so that less labeled prostanoid was recovered by HPLC even though more total prostanoid was formed as the result of enhanced cyclooxygenase activity (see below).

Enhanced LDL phospholipase activity is characteristic of LDL$_{OXID}$ (27, 55, 56). However, low-TBAR-LDL$_{OXID}$, unlike the releasing agent A23187, did not increase the release of radioactivity in the free AA fraction (Table 5) and, more importantly, did not result in the accumulation of radioactivity when prostanoid synthesis was blocked by IM. These data showed that low-TBAR-LDL$_{OXID}$ did not have a measurable effect on endogenous AA release (Fig. 9).

Studies with LDL$_{OXID}$ and exogenous free AA suggested that LDL$_{OXID}$ interacted with cyclooxygenase (Table 3-A). Previous studies from our laboratory found that synergism existed between agents that acted primarily through the cyclooxygenase step in prostanoid synthesis and exogenous free AA, but synergism did not exist between fatty acid releasing agents and exogenous AA (39). Other studies have shown that low concentrations of exogenous lipid peroxides promoted prostanoid synthesis probably through an effect on cyclooxygenase (1, 2, 41–43), and a recalculation of data from a recent study from our laboratory showed a synergistic effect between hydroperoxy fatty acids and free AA (Table 4). Since LDL$_{OXID}$ contained peroxidized cholesteryl esters and triglycerides and very small amounts of free fatty acid oxidation products (27), the synergism between LDL$_{OXID}$ and exogenous free AA (Table 3) is explained by a stimulatory effect, possibly a priming reaction, of exogenous lipid peroxides in LDL$_{OXID}$ on cyclooxygenase (Fig. 9).

High-TBAR-LDL$_{OXID}$ have very different effects on AA metabolism in SMC (Fig. 9). Unlike low-TBAR-LDL$_{OXID}$ these lipoproteins promoted the release of very large amounts of endogenous free AA (Fig. 8) and these lipoproteins also stimulated the formation of several AA derivatives that have not been characterized beyond their appearance as distinct peaks on HPLC (Fig. 8). The releasing agent A23187 also promoted the formation of a number of AA derivatives (39), but compounds eluting just before and just after free AA appeared to be unique products of high-TBAR-LDL$_{OXID}$ interactions with SMC. The identification and biological properties of these compounds deserve further study since they may possibly include chemotactic and cytotoxic agents that are formed through the action of LDL$_{OXID}$ on cells rather than the oxidation of LDL itself.

TABLE 5. Low-TBAR-LDL$_{OXID}$ and unlabeled exogenous AA diminish the release of labeled prostanoids but not free AA from SMC prelabeled with [^{14}C]AA while the releasing agent A23187 enhances both labeled prostanoids and free AA

Treatment	6-keto-PGF$_{1\alpha}$	PGE$_2$	Free AA
		cpm	
Media alone	13,500 ± 2,150 (4)[a]	5,520 ± 1,110 (4)[a]	1,200 ± 125 (4)[b]
LDL$_{OXID}$ (4.7 nmol MDA/plate)	6,170 ± 1,260 (4)	3,100 ± 393 (4)	1,040 ± 133 (4)
Exogenous AA (30 μM)	8,220 ± 90 (2)	3,430 ± 304 (2)	1,240 ± 446 (2)
A23187 (1 μg/ml)	24,200 ± 4,600 (2)	9,480 ± 1,270 (2)	10,800 ± 2,930 (2)

SMC cultures were prelabeled by incubation with [^{14}C]AA for 16 h. After a complete change of fresh media alone or fresh media containing low-TBAR-LDL$_{OXID}$, unlabeled exogenous AA or A23187, prelabeled SMC were incubated for an additional 24 h. Media were extracted, equal aliquots were separated by HPLC and counted.

[a]A two-way analysis of variance showed that differences in total prostanoid levels were significant (F 15.03, P 0.00001) and the Scheffe test showed that LDL$_{OXID}$ and media differed significantly in total postanoid levels.

[b]A one-way analysis of variance showed that differences in free AA levels were significant (F 23.1636, P 0.0003) and the Scheffe test showed that A23187 differed significantly from all the other groups.

Data reported in the present study showed that high-TBAR-LDL$_{OXID}$ inhibited prostanoid synthesis (Figs. 1 and 8) through a specific effect rather than a generalized effect associated with cell death. As previously reported (22, 40) high-TBAR-LDL$_{OXID}$ had pronounced cytotoxicity on SMC in culture (Fig. 3). BHT overcame cytotoxicity and prostanoid synthesis was very much enhanced by high-TBAR-LDL$_{OXID}$ in the presence of BHT (Fig. 2). The BHT effect could be explained by protection against cytotoxicity, but this effect was also consistent with the protection afforded to cyclooxygenase by antioxidants in the presence of high concentrations of exogenous lipid peroxides (1, 2, 41–43). High-TBAR-LDL$_{OXID}$ actually contained lipid peroxides that inhibited prostanoid synthesis (Fig. 1 and Table 3) and lipid peroxides that stimulated prostanoid synthesis through a synergistic effect with free AA (Table 3-B and 3-C). The dominant inhibitory effect was evidently blocked by BHT leading to greatly enhanced prostanoid synthesis with high-TBAR-LDL$_{OXID}$ in the presence of BHT (Fig. 2).

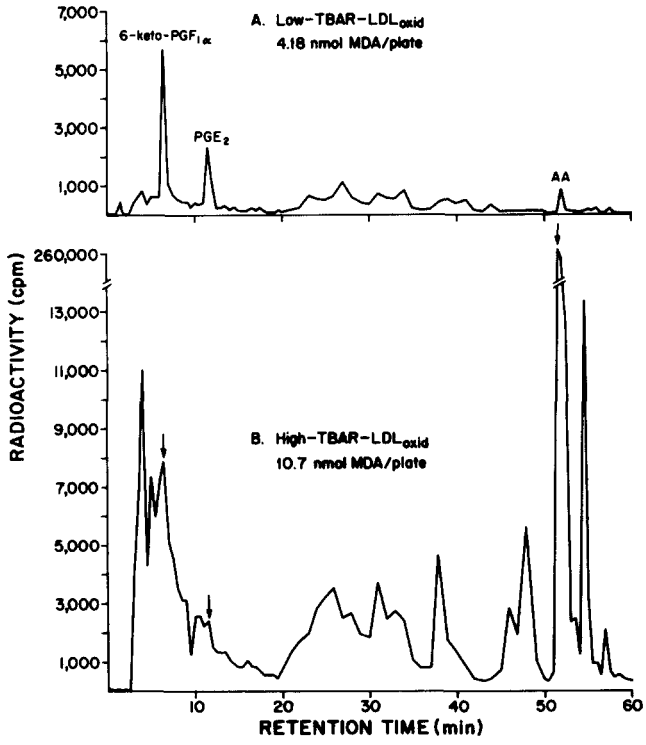

Fig. 8. High-TBAR-LDL$_{OXID}$ in contrast to low-TBAR-LDL$_{OXID}$ diminishes radioactivity released in prostanoid fractions and greatly enhances radioactivity released as free AA and other labeled AA derivatives. SMC cultures were prelabeled by incubation with [U-^{14}C]AA for 16 h. After a complete change of fresh media containing low-TBAR-LDL$_{OXID}$ (A) or high-TBAR-LDL$_{OXID}$ (B), prelabeled SMC were incubated for an additional 24 h. Metabolites were separated by HPLC using mixtures of acetonitrile–aqueous phosphoric acid (pH 2). Known peaks are labeled or marked by arrows.

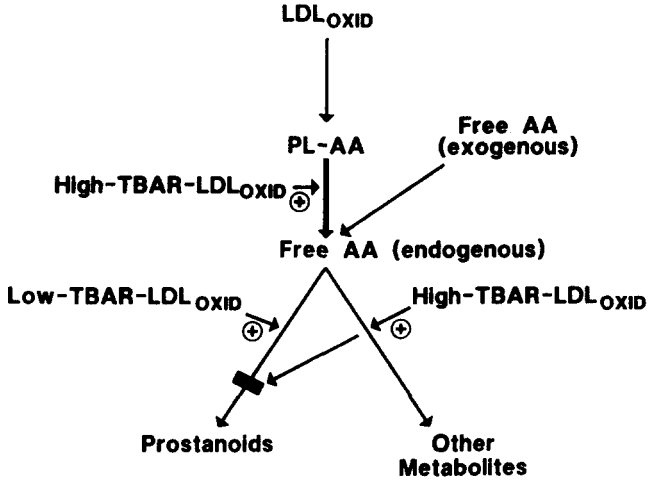

Fig. 9. Schematic diagram of the effects of low-TBAR-LDL$_{OXID}$ and high-TBAR-LDL$_{OXID}$ on AA metabolism in SMC.

Several experiments were able to distinguish between a specific high-TBAR-LDL$_{OXID}$ effect and generalized cytotoxicity. Thus short time incubations showed that prostanoid synthesis was diminished before cultures evidence morphologic changes associated with cell damage (Figs. 3 and 4). Furthermore, pulse-recovery experiments with high-TBAR-LDL$_{OXID}$ and fresh media rescued inhibited cells which then synthesized more prostanoid than cells incubated in media alone (Figs. 5 and 6). These data for high-TBAR-LDL$_{OXID}$ are consistent with a reversible metabolic effect that precedes cell death rather than a generalized inhibitory effect that results from cytotoxicity and cell death.

A number of earlier reports have shown that LDL are either cytotoxic (17–22) or mitogenic (23–26). Our experiments may help to explain these data. High-TBAR-LDL$_{OXID}$, like lipid peroxides in high concentrations, functions as both a cytotoxin and an inhibitor of cell proliferation, whereas low-TBAR-LDL$_{OXID}$ could function as a proliferative factor when the ratio of antioxidant to lipid peroxides prevented cytotoxicity and still stimulated the formation of mitogenic prostanoids (1–15).

We have recently proposed as a hypothesis that the different effects of low-TBAR-LDL$_{OXID}$ and high-TBAR-LDL$_{OXID}$ on prostanoid synthesis explain the vitamin E or antioxidant paradox (22, 40). Studies from our laboratory and elsewhere show that neither the omission of vitamin E nor the addition of vitamin E in a high concentration has any significant effect on prostanoid synthesis by isolated microsomes (44–46) or cells in cultures (4, 9–15). Paradoxically, prostanoids such as PGE_2, $PGF_{2\alpha}$, and TXA_2 are elevated in the tissues and serum of animals and humans with vitamin E deficiencies, and prostanoid levels return to normal when vitamin E is restored to the diet (45, 46, 57, 58). Our data support the hypothesis that dietary vitamin E may act indirectly on prostanoid synthesis, in vivo, by decreasing the formation of low-TBAR-LDL$_{OXID}$ and tissue-specific lipid peroxides that have a stimulatory effect on prostanoid synthesis.

We are grateful to Dr. R. V. Panganamala for suggestions, Dr. Arthur Strauch for the gift of antibodies to human umbilical artery F-actin, and Dr. Larry Sachs for statistical analyses. This study was supported in part by a Research Challenge Grant from The Ohio State University.

Manuscript received 12 October 1987, in revised form 25 July 1988, and in re-revised form 10 November 1989.

REFERENCES

1. Cornwell, D. G., and N. Morisaki. 1984. Fatty acid paradoxes in the control of cell proliferation: prostaglandins, lipid peroxides and co-oxidation reactions. *In* Free Radicals in Biology. Vol. VI. W. A. Pryor, editor. Academic Press, New York. 95–147.
2. Cornwell, D. G., and H. Zhang. 1988. Fatty acid metabolism and cell proliferation. *In* Lipid Peroxidation in Biological Systems. A. Sevanian, editor. Am. Oil Chemists' Soc., Champaign, IL. 163–195.
3. Huttner, J. J., E. T. Gwebu, R. V. Panganamala, G. E. Milo, D. G. Cornwell, H.M. Sharma, and J. C. Geer. 1977. Fatty acids and their prostaglandin derivatives: inhibitors of proliferation in aortic smooth muscle cells. *Science.* **197:** 289–291.
4. Cornwell, D. G., J. J. Huttner, G. E. Milo, R. V. Panganamala, H. M. Sharma, and J. C. Geer. 1979. Polyunsaturated fatty acids, vitamin E and the proliferation of aortic smooth muscle cells. *Lipids.* **14:** 194–207.
5. Miller, J. S., V. C. Gavino, G. A. Ackerman, H. M. Sharma, G. E. Milo, J. C. Geer, and D. G. Cornwell. 1980. Triglycerides, lipid droplets, and lysosomes in aorta smooth muscle cells during the control of cell proliferation with polyunsaturated fatty acids and vitamin E. *Lab. Invest.* **42:** 495–506.
6. Gavino, V. C., J. S. Miller, S. P. Ikharebha, G. E. Milo, and D. G. Cornwell. 1981. Effect of polyunsaturated fatty acids and antioxidants on lipid peroxidation in tissue cultures. *J. Lipid Res.* **22:** 763–769.
7. Gavino, V. C., J. S. Miller, J. M. Dillman, G. E. Milo, and D. G. Cornwell. 1981. Polyunsaturated fatty acid accumulation in the lipids of cultured fibroblasts and smooth muscle cells. *J. Lipid Res.* **22:** 57–62.
8. Gavino, V. C., G. E. Milo, and D. G. Cornwell. 1982. Image analysis for the automated estimation of clonal growth and its application to the growth of smooth muscle cells. *Cell Tissue Kinet.* **15:** 225–231.
9. Morisaki, N., J. M. Stitts, L. Bartels-Tomei, G. E. Milo, R. V. Panganamala, and D. G. Cornwell. 1982. Dipyridamole: an antioxidant that promotes the proliferation of aorta smooth muscle cells. *Artery.* **11:** 88–107.
10. Morisaki, N., J. A. Lindsey, G. E. Milo, and D. G. Cornwell. 1983. Fatty acid metabolism and cell proliferation. III. Effect of prostaglandin biosynthesis either from exogenous fatty acid or endogenous fatty acid release with hydralazine. *Lipids.* **18:** 348–352.
11. Lindsey, J. A., N. Morisaki, J. M. Stitts, R. A. Zager, and D. G. Cornwell. 1983. Fatty acid metabolism and cell proliferation. IV. Effect of prostanoid biosynthesis from endogenous fatty acid release with cyclosporin-A. *Lipids.* **18:** 566–569.
12. Morisaki, N., J. A. Lindsey, J. M. Stitts, H. Zhang, and D. G. Cornwell. 1984. Fatty acid metabolism and cell proliferation. V. Evaluation of pathways for the generation of lipid peroxides. *Lipids.* **19:** 391–394.
13. Cornwell, D. G., J. A. Lindsey, H. Zhang, and N. Morisaki. 1984. Fatty acid metabolism and cell proliferation. VI. Properties and antithrombotic agents that influence metastasis. *In* Icosanoids and Cancer. H. Thaler-Dao, A. C. de Paulet, and R. Paoletti, editors. Raven Press, New York. 205–222.
14. Lindsey, J. A., H. Zhang, H. Kaseki, N. Morisaki, T. Sato, and D. G. Cornwell. 1985. Fatty acid metabolism and cell proliferation. VII. Antioxidant effects of tocopherols and their quinones. *Lipids.* **20:** 151–157.
15. Morisaki, N., L. D. Tomei, G. E. Milo, and D. G. Cornwell. 1985. Role of prostanoids and lipid peroxides as mediators of the 12-O-tetradecanoylphorbol-13-acetate effect on cell growth. *Lipids.* **20:** 602–610.
16. Cornwell, D. G., and R. V. Panganamala. 1982. Atherosclerosis: an intracellular deficiency in essential fatty acids. *Prog. Lipid Res.* **20:** 365–376.

17. Hessler, J. R., D. W. Morel, L. J. Lewis, and G. M. Chisolm. 1983. Lipoprotein oxidation and lipoprotein-induced cytotoxicity. *Arteriosclerosis.* **3:** 215–222.
18. Morel, D. W., J. R. Hessler, and G. M. Chisolm. 1983. Low density lipoprotein cytotoxicity induced by free radical peroxidation in lipids. *J. Lipid Res.* **24:** 1070–1076.
19. Morel, D. W., P. E. DiCorleto, and G. M. Chisolm. 1984. Endothelial and smooth muscle cells alter low density lipoprotein in vitro by free radical oxidation. *Arteriosclerosis.* **4:** 357–364.
20. Henriksen, T., S. A. Evensen, and B. Carlander. 1979. Injury to human endothelial cells in culture induced by low density lipoproteins. *Scand. J. Clin. Lab. Invest.* **39:** 361–368.
21. Hessler, J. R., A. L. Robertson, and G. M. Chisolm. 1979. LDL-induced cytotoxicity and its inhibition by HDL in human vascular smooth muscle and endothelial cells in culture. *Atherosclerosis.* **32:** 213–229.
22. Zhang, H., K. H. Jones, W. B. Davis, R. L. Whisler, R. V. Panganamala, and D. G. Cornwell. 1987. Oxidized low density lipoproteins in smooth muscle cell cultures: differential effects on prostanoid synthesis and viability. *In* Clinical and Nutritional Aspects of Vitamin E. O. Hayaishi and M. Mino, editors. Elsevier Science Publishers B. V., Amsterdam. 89–100.
23. Brown, B. G., R. Mahley, and G. Assman. 1976. Swine aortic smooth muscle in tissue culture (some effects of purified swine lipoprotein on cell growth and morphology). *Circ. Res.* **39:** 415–424.
24. Fischer-Dzoga, K., R. A. Fraser, and R. W. Wissler. 1976. Stimulation of proliferation in stationery primary cultures of monkey and rabbit aortic smooth muscle cells. I. Effects of lipoprotein fractions of hyperlipemic serum and lymph. *Exp. Mol. Pathol.* **24:** 346–359.
25. Layman, D. L., B. L. Jelen, and D. R. Illingworth. 1980. Inability of serum from abetalipoproteinemia subjects to stimulate proliferation of human smooth muscle cells and dermal fibroblasts in vitro. *Proc. Natl. Acad. Sci. USA.* **77:** 1511–1515.
26. Tauber, J. P., J. Cheng, and D. Gospodarowicz. 1980. Effect of high and low density lipoproteins on proliferation of cultured bovine vascular endothelial cells. *J. Clin. Invest.* **66:** 696–708.
27. Zhang, H., W. B. Davis, X. Chen, R. L. Whisler, and D. G. Cornwell. 1989. Studies on oxidized low density lipoproteins. Controlled oxidation and a prostaglandin artifact. *J. Lipid Res.* **30:** 141–148.
28. Huttner, J. J., D. G. Cornwell, and G. E. Milo. 1977. Preparation of primary cultures of smooth muscle cells from prepubertal guinea-pig aorta. *T. C. A. Man.* **3:** 633–639.
29. Lessard, J. L. 1988. Two monoclonal antibodies to actin: one muscle selective and one generally reactive. *Cell Motil. Cytoskeleton.* **10:** 349–362.
30. Jones, K. H., and J. A. Senft. 1985. An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. *J. Histochem. Cytochem.* **33:** 77–79.
31. Greenspan, P., and S. D. Fowler. 1985. Spectrofluorometric studies of the lipid probe, nile red. *J. Lipid Res.* **26:** 781–789.
32. Hadjiagapiou, C., and A. A. Spector. 1987. Docosahexaenoic acid metabolism and effect on prostacyclin production in endothelial cells. *Arch. Biochem. Biophys.* **253:** 1–12.
33. Oncley, J. L., K. W. Walton, and D. G. Cornwell. 1957. A rapid method for the bulk isolation of β-lipoproteins from human plasma. *J. Am. Chem. Soc.* **79:** 4666–4671.
34. Noble, R. P. 1968. Electrophoretic separation of plasma lipoproteins in agarose gel. *J. Lipid. Res.* **9:** 693–700.
35. Steinbrecher, U. P., S. Parthasarathy, D. S. Leake, J. L. Witztum, and D. Steinberg. 1984. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc. Natl. Acad. Sci. USA.* **81:** 3883–3887.
36. Witte, D. L., D. A. Barrett, and D. A. Wycoff. 1974. Evaluation of an enzymatic procedure for determination of serum cholesterol with the Abbott ABA-100. *Clin. Chem.* **20:** 1282–1286.
37. Sampson, E. J., L. M. Demers, and A. F. Krieg. 1975. Faster enzymatic procedure for serum triglycerides. *Clin. Chem.* **21:** 1983–1985.
38. Morisaki, N., H. Sprecher, G. E. Milo, and D. G. Cornwell. 1982. Fatty acid specificity in the inhibition of cell proliferation and its relationship to lipid peroxidation and prostaglandin biosynthesis. *Lipids.* **17:** 893–899.
39. Zhang, H., H. Kaseki, W. B. Davis, R. L. Whisler, and D. G. Cornwell. 1989. Mechanisms for the stimulation of prostanoid synthesis by cyclosporine and bacterial lipopolysaccharide. *Transplantation.* **47:** 864–871.
40. Cornwell, D. G., H. Zhang, W. B. Davis, R. L. Whisler, and R. V. Panganamala. 1990. Paradoxical effects of vitamin E: oxidized lipoproteins, prostanoids and the pathogenesis of atherosclerosis. *In* Action of Free Radicals and Active Forms of Oxygen on Lipoproteins and Membrane Lipids: Cellular Interactions and Atherogenesis. H. Thaler-Dao, A. Crastes de Paulet, L. Douste-Blazy, and R. Paoletti, editors. Plenum Press, New York.
41. Panganamala, R. V., V. C. Gavino, and D. G. Cornwell. 1979. Effect of low and high methional concentrations on prostaglandin biosynthesis in microsomes from bovine and sheep vesicular glands. *Prostaglandins.* **17:** 155–162.
42. Gale, P. H., and R. W. Egan. 1984. Prostaglandin endoperoxide synthase-catalyzed oxidation reactions. *In* Free Radicals in Biology. Vol. VI. W. A. Pryor, editor. Academic Press, New York. 1–38.
43. Lands, W. E. M., R. J. Kulmacz, and P. J. Marshall. 1984. Lipid peroxide actions in the regulation of prostaglandin biosynthesis. *In* Free Radicals in Biology. Vol. VI. W. A. Pryor, editor. Academic Press, New York. 39–61.
44. Panganamala, R. V., J. S. Miller, E. T. Gwebu, H. M. Sharma, and D. G. Cornwell. 1977. Differential inhibitory effects of vitamin E and other antioxidants on prostaglandin synthetase, platelet aggregation and lipoxidase. *Prostaglandins.* **14:** 261–271.
45. Panganamala, R. V., and D. G. Cornwell. 1982. The effect of vitamin E on arachidonic acid metabolism. *Ann. NY Acad. Sci.* **393:** 376–390.
46. Carpenter, M. P. 1982. Antioxidants and prostaglandin synthesis: in vivo and in vitro effects. *Prog. Lipid Res.* **20:** 143–149.
47. Zhang, H., K. H. Jones, W. B. Davis, R. L. Whisler, R. V. Panganamala, and D. G. Cornwell. 1990. Heterogeneity in lipid peroxides: cellular arachidonic acid metabolism and DNA synthesis. *In* The Pharmacological Effects of Lipids. Vol. III. J. J. Kabara, editor. Am. Oil Chemists' Soc., Champaign, IL. 257–267.
48. Gavino, V. C. 1981. Polyunsaturated fatty acids, lipid accumulation, and oxidant stress in cells in culture. Dissertation, The Ohio State University, Columbus, Ohio.
49. Szczeklik, A., and R. J. Gryglewski. 1980. Low density lipoproteins (LDL) are carriers for lipid peroxides and inhibit prostacyclin (PGI_2) biosynthesis in arteries. *Artery.* **7:** 488–495.

50. Pomerantz, K. B., A. R. Tall, S. J. Feinmark, and P. J. Cannon. 1984. Stimulation of vascular smooth muscle cell prostacyclin and prostaglandin E_2 synthesis by plasma high and low density lipoproteins. *Circ. Res.* **54:** 554–565.

51. Giessler, C., J. Beitz, P. Mentz, and W. Förster. 1986. The influence of lipoproteins (LDL and HDL) on PGI_2 formation by isolated aortic preparations of rabbits. *Prostaglandins Leukotrienes Med.* **22:** 221–234.

52. Yokode, M., T. Kita, Y. Kikawa, T. Ogorochi, S. Narumiya, and C. Kawai. 1988. Stimulated arachidonate metabolism during formal cell transformation of mouse peritoneal macrophages with oxidized low density lipoprotein. *J. Clin. Invest.* **81:** 720–729.

53. Triau, J. E., S. N. Meydani, M. Meydani, P. Libby, and E. J. Schaefer. 1986. Oxidized low density lipoproteins (OX-LDL) stimulate prostacyclin (PGI_2) production by adult human vascular endothelial cells. *Fed. Proc.* **45:** 347 (Abstract).

54. Cornwell, D. G. 1967. Lipoproteins. *In* Lipids and Lipidoses. G. Schettler, editor. Springer-Verlag, New York. 168–189.

55. Parthasarathy, S., U. P. Steinbrecher, J. Barnett, J. L. Witztum, and D. Steinberg. 1985. Essential role of phospholipase A_2 activity in endothelial cell-induced modification of low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **82:** 3000–3004.

56. Steinbrecher, U. P., and P. H. Pritchard. 1989. Hydrolysis of phosphatidylcholine during LDL oxidation is mediated by platelet-activating factor acetylhydrolase. *J. Lipid. Res.* **30:** 305–315.

57. Hope, W. C., C. Dalton, L.J. Machlin, R. J. Filipski, and F. M. Vane. 1975. Influence of dietary vitamin E on prostaglandin biosynthesis in rat blood. *Prostaglandins.* **10:** 557–571.

58. Kitabchi, A. E. 1980. Hormonal status in vitamin E deficiency. *In* Vitamin E. L. J. Machlin, editor. Marcel Dekker, New York. 348–371.