High-Density-Lipoprotein-Induced Cholesterol Efflux from Arterial Smooth Muscle Cell Derived Foam Cells: Functional Relationship of the Cholesteryl Ester Cycle and Eicosanoid Biosynthesis†

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ABSTRACT: Eicosanoids have been implicated in the regulation of arterial smooth muscle cell (SMC) cholesteryl ester (CE) metabolism. These eicosanoids, which include prostacyclin (PGI_2), stimulate CE hydrolytic activities. High-density lipoproteins (HDL), which promote cholesterol efflux, also stimulate PGI_2 production, suggesting that HDL-induced cholesterol efflux is modulated by eicosanoid biosynthesis. To ascertain the role of endogenously synthesized eicosanoids produced by arterial smooth muscle cells in the regulation of CE metabolism, we examined the effects of cyclooxygenase inhibition on CE hydrolytic enzyme activities, cholesterol efflux, and cholesterol content in normal SMC and SMC-derived foam cells following exposure to HDL and another cholesterol acceptor protein, serum albumin. Alterations of these activities were correlated with cholesterol efflux in response to HDL or bovine serum albumin (BSA) in the presence or absence of aspirin. HDL stimulated PGI_2 synthesis and CE hydrolases in a dose-dependent manner. Eicosanoid dependency was established by demonstrating that HDL-induced acid cholesteryl ester hydrolase (ACEH) activity was blocked by aspirin. CE enrichment essentially abrogated HDL-induced PGI_2 production in cells which also exhibited decreased lysosomal and cytoplasmic CE hydrolase activities. In CE-enriched cells whose cytoplasmic CE pool was metabolically labeled with [^{3}H]oleate or cLDL containing [^{3}H]cholesteryl linoleate, aspirin did not alter HDL- or BSA-induced net CE hydrolysis or efflux, respectively. Finally, aspirin treatment did not alter the mass of either free or esterified cholesterol content of untreated or CE-enriched SMC following exposure to acceptor proteins. These data demonstrated that CE enrichment significantly reduced HDL-induced activation of CE hydrolytic activity via inhibition of endogenous PGI_2 production. These novel findings support our hypothesis that CE hydrolysis is under eicosanoid metabolic control. We suggest that CE enrichment ablates eicosanoid-dependent control of CE hydrolysis. However, net cholesterol efflux from intracellular pools appeared to be independent of eicosanoid biosynthesis. Therefore, a dissociation exists between eicosanoid-dependent CE hydrolysis and net cholesterol efflux induced by plasma cholesterol acceptors.

The mechanisms by which cellular cholesterol content is regulated have been the subject of intense research. Cellular cholesterol content is stringently maintained through (1) uptake by the low-density lipoprotein (LDL)[1] receptor (Goldstein & Brown, 1977; Steinberg, 1987; Chen et al., 1988), (2) cholesterol synthesis via HMG CoA-reductase (Brown et al., 1981), and (3) efflux, which is mediated by plasma and interstitial acceptors including albumin (Fielding & Moser, 1981; Chau & Geyer, 1978; Bartholow & Geyer, 1982) and high-density lipoprotein (HDL) (Rothblat & Phillips, 1982; Oram, 1983; Stein et al., 1980; Innerarity et al., 1982).

Recent evidence suggests that eicosanoids also influence cellular cholesterol metabolism. First, exogenous prostacyclin (PGI_2) activates CE hydrolytic activities and reduces cellular cholesterol content in intact smooth muscle cells (Hajjar, 1985; Hajjar et al., 1982), thereby rendering the liberated free cholesterol available for removal by interstitial or plasma cholesterol acceptors. Second, HDL stimulates vascular eicosanoid release by providing arachidonate from HDL CE, and by stimulating cellular phospholipases (Van Sickle et al., 1986a,b; Pomerantz et al., 1984, 1985; Fleisher et al., 1982). Third, Iloprost (a stable PGI_2 analogue) and PGE_2 reduce LDL receptor activity (Krone et al., 1988) and HMG CoA-reductase activity in monocytes (Krone et al., 1985, 1988). Fourth, CE-enriched arterial smooth muscle cells (SMC) are deficient in eicosanoid synthetic capacity due primarily to a defect in arachidonate release from membrane phospholipids (Pomerantz & Hajjar, 1989).

In this paper, we have examined novel aspects of cholesterol metabolism in arterial SMC: (1) the influence of lipid enrichment on the cellular activities of the enzymes of the CE cycle; (2) the impact of altered CE metabolic activity on net cellular cholesterol efflux and cholesterol content as a function

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[1] Abbreviations: PGI_2, prostacyclin; CE, cholesteryl ester; LDL, low-density lipoprotein(s); cLDL, cationized LDL; HDL, high-density lipoprotein(s); SMC, smooth muscle cell(s); ACEH, acid cholesteryl ester hydrolase; NCEH, neutral cholesteryl ester hydrolase; ACAT, acyl-CoA:cholesterol acyltransferase; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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of endogenous eicosanoid production. In these experiments, we used the physiological plasma cholesterol acceptors HDL and BSA to distinguish between eicosanoid-dependent and eicosanoid-independent regulation of CE hydrolytic activity and net cholesterol flux.

Experimental Procedures

Materials. Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin, and fungizone (amphotericin B) were purchased from Gibco (Grand Island, NY). Fetal bovine serum was purchased from Hyclone (Logan, UT). *N,N*-Dimethyl-1,3-propanediamine was from Kodak Chemicals (Rochester, NY); 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride was from Aldrich Chemicals (Milwaukee, WI). [^{14}C]Oleoyl-coenzyme A (49–53 mCi/mmol), [^{3}H]oleic acid (9.0 Ci/mmol), [^{14}C]cholesterol, [^{3}H]cholesteryl linoleate (97.1 Ci/mmol), and cholesteryl [1-^{14}C]oleate (57.0 mCi/mmol) were purchased from New England Nuclear (Boston, MA). Albumin (fraction V), aspirin, digitonin, and taurocholic acid were obtained from Sigma Chemicals (St. Louis, MO). Neutral lipids were obtained from Nu-Chek Prep (Elysian, MN). Phospholipids were supplied by Avanti (Birmingham, AL). All organic solvents (HPLC grade) and other biochemicals (reagent grade) were obtained from Fisher Scientific (Springfield, NJ).

Preparation of Plasma Lipoproteins and cLDL. Single-unit platelet-poor plasma (PPP) was purchased from the New York Blood Center (New York, NY). Two pooled units of PPP for each lipoprotein preparation were used to isolate LDL (1.019–1.063 g/mL) and HDL (1.063–1.21 g/mL) by preparative ultracentrifugation using NaBr to adjust the density (Havel et al., 1955). The $d > 1.21$ g/mL fraction and the native and derivatized (see below) lipoproteins were dialyzed against 0.9% NaCl, 0.1 mM EDTA, pH 7.4, and 25 mM HEPES and stored under N_2 at 4 °C. Cationized LDL (cLDL) was prepared from LDL by covalent attachment of *N,N*-dimethyl-1,3-propanediamine to aspartate and glutamate residues of native LDL using 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride as catalyst at pH 6.5 (Basu et al., 1976, 1977; Goldstein et al., 1977; Pomerantz & Hajjar, 1989). Demonstration of successful derivatization of LDL to cLDL was accomplished by differential migration of native and cLDL at pH 8.6 by agarose gel electrophoresis. Lipoproteins were dialyzed against 12 L of 0.154 M NaCl, 0.05 M EDTA, and 10 mM HEPES (pH 7.4) for 48 h prior to concentration by ultrafiltration. Lipoproteins were added to tissue culture media just prior to initiation of experiments; lipoproteins were used within 1 month of preparation.

Isolation and Culture of Aortic SMC. SMC were isolated by collagenase/elastase digestion of rabbit thoracic aorta and cultured as previously described (Pomerantz et al., 1984). Cells were determined to be smooth muscle by their characteristic hill-and-valley morphology. All experiments were performed on cells between passages 3 and 7. Cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/fungizone (v/v) at 37 °C in 5% CO_2 in air.

Preparation of CE-Enriched SMC. To induce CE accumulation in SMC, confluent cultures in 25 cm^2 flasks or 35-mm plates were incubated with cLDL (100 μg/mL) in 2 mL of DMEM containing 10% fetal bovine serum for 7 days. Fresh media containing cLDL were added on day 4 (Pomerantz & Hajjar, 1989).

Assay of Prostacyclin. The mass of 6-keto-PGF$_{1\alpha}$, the stable hydrolysis product of PGI_2, was evaluated by standard RIA procedures, as previously described (Pomerantz et al., 1984). Data are expressed as nanograms per milligram of cell protein.

Cholesteryl Ester Hydrolytic Enzyme Assays. (A) Acid Cholesteryl Ester Hydrolase (ACEH) Activity. ACEH activity was assayed as previously described (Hajjar et al., 1982). Cells grown in 35-mm plates (9.61 cm^2) were washed 3 times in PBS and scraped into sucrose buffer (250 mM sucrose, 0.10 mM EDTA, and 10 mM Tris-HCl, pH 7.5). Homogenates were prepared by sonication using a Branson 350 cell disruptor; 13 μM cholesteryl [1-^{14}C]oleate served as the substrate suspended in a lecithin/digitonide dispersion. Substrate blanks were run under identical conditions with sucrose buffer used in place of the enzyme. The final reaction conditions consisted of enzyme (approximately 100 μg of cellular protein), 1.27 mM lecithin, 12.7 μM cholesteryl oleate, 2.0 mM sodium taurocholate, 0.005% digitonin, and 50 mM sodium acetate buffer, pH 3.9. The reaction was terminated after 60 min at 37 °C, and the unhydrolyzed substrate was extracted by addition of methanol/chloroform/heptane (1.4:1.3:1.0 v/v), followed by the addition of 50 mM borate buffer (pH 10.0). The mixture was then agitated and centrifuged to separate the phases. The amount of [^{14}C]oleate in the aqueous phase was determined by scintillation counting of 1.0 mL of supernatant in 10 mL of Hydroflour. Aliquots of the cell homogenate were assayed for protein; ACEH activity is expressed as picomoles of cholesteryl [1-^{14}C]oleate hydrolyzed per milligram of cell protein.

(B) Effect of Native LDL on Apparent ACEH Activity. To ascertain the possible influence of endogenous lipid on the apparent activity of ACEH, smooth muscle cell homogenates were assayed for ACEH activity in the presence of native LDL (0–30 μg of LDL CE); native LDL was used as the source of CE since SMC exposed to cLDL accumulate CE principally in the lysosomal compartment in the form of unhydrolyzed LDL-derived CE (Stein et al., 1979; Pomerantz & Hajjar, 1989). Estimation of the apparent ACEH activity of CE-enriched cells was then corrected on the basis of the amount of CE that was added to the assay system after exposure of cells to cLDL.

(C) Neutral Cholesteryl Ester Hydrolase (NCEH) Activity. NCEH activity was assayed as previously described (Hajjar et al., 1983; Fabricant et al., 1981) and is essentially the same as described for the assay for ACEH, with the following modifications: Cholesteryl [1-^{14}C]oleate was prepared as a mixed micelle consisting of cholesteryl oleate, lecithin, and sodium taurocholate at a final 1:4:2 molar ratio in the reaction mixture. An incubation mixture was prepared by the addition of 50 μL of micellar cholesteryl [^{14}C]oleate to 800 μL of 100 mM potassium phosphate buffer (pH 7.0) containing 0.05% bovine serum albumin. The reaction was initiated by the addition of 250 μL of cell homogenate in sucrose buffer (150 μg of protein) prepared as described above. The reaction conditions, as well as extraction and quantitation, were otherwise similar to the ACEH assay. All reaction conditions to assay NCEH activities have been optimized in our cell system in preliminary experiments and clearly differentiate NCEH from ACEH activities (Hajjar et al., 1983; Hajjar, 1986).

Studies on Cellular Cholesterol Efflux. (A) Flux of [^{3}H]Oleic Acid through Cytoplasmic CE. To confluent SMC cultures in 35-mm wells was added a [^{3}H]oleic acid/albumin mixture (specific activity 40 000 dpm/nmol) to give a final concentration of 100 μM oleate/20 μM albumin in the absence of serum for 24 h at 37 °C (Brown et al., 1980). Cells were then washed 3 times in PBS containing 0.1% BSA, followed by three washes in PBS. Cells were then extracted twice into

2.0 mL each of hexane/2-propanol (3:2 v/v) (Hara & Randin, 1978). To the first cell lipid extracts was added 30 μg each of unlabeled cholesteryl oleate, cholesterol, and oleic acid and 40 000 dpm of [^{14}C]cholesterol to monitor recovery. The distribution of radioactivity in SMC lipids was determined by TLC on silica gel 60 (E. Merck, Co.), by sequential development in diethyl ether/acetic acid/petroleum ether (100:3:97 v/v) and diethyl ether/petroleum ether (3:97 v/v) (Hajjar et al., 1980). Extracted cells remaining in dishes were solubilized in 0.2 N NaOH and assayed for protein.

To estimate subsequent hydrolysis of cellular cholesteryl [^{3}H]oleate, untreated or CE-enriched cells exposed to [^{3}H]oleic acid/albumin (final concentration of 100 μM oleate/20 μM albumin) were then washed and exposed to serum-free DME alone or to serum-free DME containing either HDL (400 μg of protein/mL) or BSA (1.0 mg/mL) for 24 h. At selected time intervals, media were removed, and the radioactivity of cellular CE was then quantitated as described above (Brown et al., 1980).

(B) Measurement of Cellular Total Cholesterol Efflux from CE-Enriched Cells following Exposure to Reconstituted cLDL Containing [^{3}H]Cholesteryl Linoleate. Reconstituted cLDL containing [^{3}H]cholesteryl linoleate (henceforth called [^{3}H]cLDL, specific activity = 9635 dpm/μg of protein, 100 μg of protein/mL) was prepared as described by Brown et al. (1975). Cells were exposed to [^{3}H]cLDL for 7 days with one media change at 4 days. Cells were then washed and then exposed to serum-free DME, or DME containing HDL (400 μg/mL) or BSA (1.0 mg/mL) in the presence and absence of aspirin. Efflux of total cholesterol was then monitored by the reduction in cellular radioactivity after 24 h; media were removed, and the cells were washed as described above. Following digestion in 0.2 N NaOH, aliquots were taken for cellular radioactivity and protein. Data are expressed as dpm per milligram of protein (Brown et al., 1980).

(C) Effect of HDL and BSA on Cellular Cholesterol Content: Effect of Aspirin Treatment. To determine if cholesterol acceptors remove cellular cholesterol by an eicosanoid-dependent mechanism, untreated and aspirin-treated normal and CE-enriched SMC were exposed to HDL or BSA for 24 h. SMC were extracted into hexane/2-propanol as described above. Free content and total cholesterol content of normal and CE-enriched SMC were determined by gas–liquid chromatography (3% OV-17 on 100–120-mesh WHP, Supelco) with β-sitosterol (30 μg) as an internal standard (Ishikawa et al., 1975; Pomerantz & Hajjar, 1989).

(D) Protein Assay. Cellular and lipoprotein protein content was measured by the method of Lowry et al. (1951).

(E) Statistical Analysis. All data are expressed as mean ± SEM and analyzed by either the Student's *t* test or analysis of variance, followed by the Newman–Keuls test for statistical significance.

Results

Effect of CE Enrichment on HDL-Induced PGI_2 Synthesis. To determine if CE enrichment altered the ability of HDL to stimulate PGI_2 synthesis, normal and CE-enriched SMC were exposed to increasing concentrations of HDL (0.1–1.0 mg of protein/mL) for 24 h. The calcium ionophore A-23187 and arachidonic acid (AA) were used as comparative agonists (37 °C, for 30 min). First, the 24-h basal production of 6-keto-$PGF_{1\alpha}$ (the stable hydrolytic product of PGI_2) was significantly decreased (70%, $p < 0.01$) in CE-enriched cells versus untreated cells. Second, HDL stimulated 6-keto-$PGF_{1\alpha}$ production in a dose-dependent manner in untreated SMC, resulting in a greater than 2-fold ($p < 0.05$) increase over basal

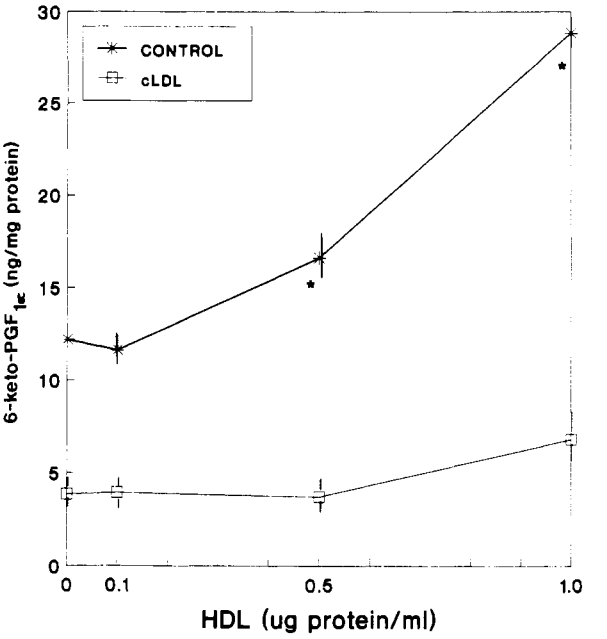

FIGURE 1: Effect of lipid enrichment on HDL-induced PGI_2 production: SMC were grown to confluent density in 24-well cluster plates. Cells were then exposed to DMEM/10% FBS (controls) or to DMEM containing cLDL (100 μg/mL) for 1 week with one media change. Cells were then exposed to serum-free DMEM containing increasing concentrations of HDL (0–1.0 mg/mL) for 24 h at 37 °C. Parallel cultures of untreated and lipid-enriched SMC were exposed to A-23187 (5.0 μM) or AA (10 μM) for 30 min at 37 °C (see Results). PGI_2 was measured as its stable hydrolysis product, 6-keto-$PGF_{1\alpha}$, in the supernatants by radioimmunoassay. The cell monolayer was digested in 0.2 N NaOH, and aliquots were taken for protein determination. Each treatment was performed in triplicate; each supernatant was assayed in duplicate for three separate experiments. Data are expressed as mean ± SEM; asterisks, $p < 0.05$.

levels, but CE enrichment significantly reduced 6-keto-$PGF_{1\alpha}$ production by 79.6% relative to untreated cells exposed to 1.0 mg of protein/mL of HDL ($p < 0.05$, Figure 1). Third, CE-enriched SMC synthesized significantly less 6-keto-$PGF_{1\alpha}$ following exposure to A-23187 (26.25 ± 0.92 ng of 6-keto-$PGF_{1\alpha}$/mg of protein by untreated cells vs 8.03 ± 0.82 ng of 6-keto-$PGF_{1\alpha}$/mg of protein by CE-enriched cells, $p < 0.05$) and AA (24.06 ± 1.06 ng of 6-keto-$PGF_{1\alpha}$/mg of protein by untreated cells vs 10.14 ± 0.18 ng of 6-keto-$PGF_{1\alpha}$/mg of protein by CE-enriched cells, $p < 0.05$).

Effect of CE Enrichment on CE Hydrolytic Activities. The effects of CE enrichment on the activities of the lysosomal (acid) and cytoplasmic (neutral) CE hydrolases were examined over a 1-week period (Figure 2). On day 0, confluent SMC possessed approximately 6-fold greater ACEH than NCEH activities [1394 ± 147 and 88 ± 8 pmol of cholesteryl oleate hydrolyzed (mg of protein)$^{-1}$ h^{-1}]. ACEH activity in untreated cells declined by 78% to 298 ± 8 pmol (mg of protein)$^{-1}$ h^{-1} over the 7-day period of incubation. In contrast, NCEH activity in untreated cells remained unchanged throughout the 7-day incubation period. As cells became CE enriched, the apparent activities of the ACEH and NCEH were reduced relative to control SMC, with a significantly more pronounced effect on the apparent ACEH activity than NCEH activity. The reduction of the activities of apparent ACEH and NCEH occurred *before* significant intracellular CE accretion. The inhibitory effect of CE enrichment on apparent ACEH and NCEH activity was sustained over the 7-day time course of study (Figure 2). However, endogenous CE (in the form of native LDL) greater than 1.0 μg inhibited ACEH activity in a dose-dependent manner, when added to arterial smooth

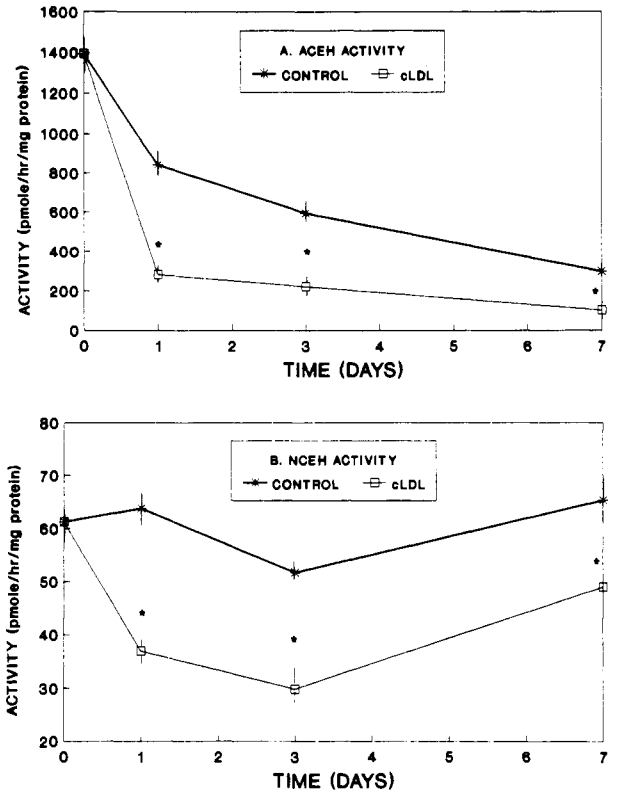

FIGURE 2: Effect of lipid enrichment on ACEH (panel A) and NCEH (panel B) activities. SMC were grown to confluent density in 35-mm dishes. Cells were then exposed to DME containing 10% FBS or to these media containing cLDL (100 μg/mL) for 1 week with one media change. Cells were washed in PBS, scraped into 500 μL of sucrose buffer, and sonicated. Activities of NCEH and ACEH were determined on 250-μL aliquots on days 0, 1, 3, and 7 as described under Experimental Procedures. Duplicate 50-μL aliquots were taken for measurement of protein. Enzyme activities are expressed as picomoles of substrate hydrolyzed per milligram of cell protein per hour. Each sample was measured in triplicate, and is representative of six separate experiments. Data are expressed as mean ± SEM; asterisks, $p < 0.05$.

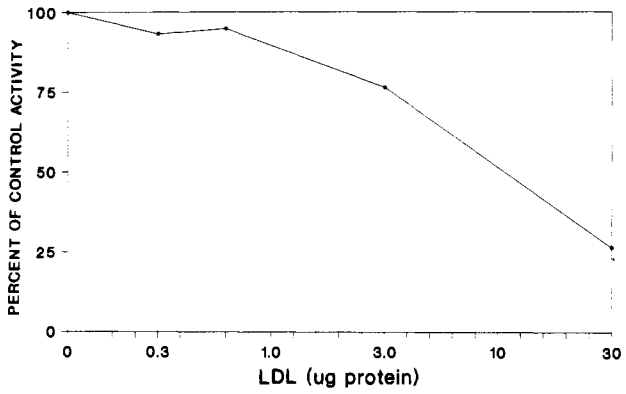

FIGURE 3: Effect of native LDL on apparent ACEH activity. Arterial smooth muscle cell homogenates (85 μg of protein) were incubated in the standard ACEH assay system as described under Experimental Procedures in the presence of native LDL (0–30 μg of CE). Each point is the mean of triplicate samples (SEM < 2.5%).

muscle cell homogenates (Figure 3). Thus, endogenous lipid equivalent in amounts of CE in CE-enriched cells (2–5 μg) reduces the apparent ACEH activity approximately 30%. Data on ACEH activity in CE-enriched cells have been corrected for this artifact.

Effect of Plasma Cholesterol Acceptors on Apparent ACEH Activity and PGI_2 Production: Effect of Aspirin. The effects of HDL (400 μg/mL) and BSA (1.0 mg/mL) on ACEH activity (Figure 4A) and PGI_2 production (Table I) were evaluated in both normal and CE-enriched SMC in the

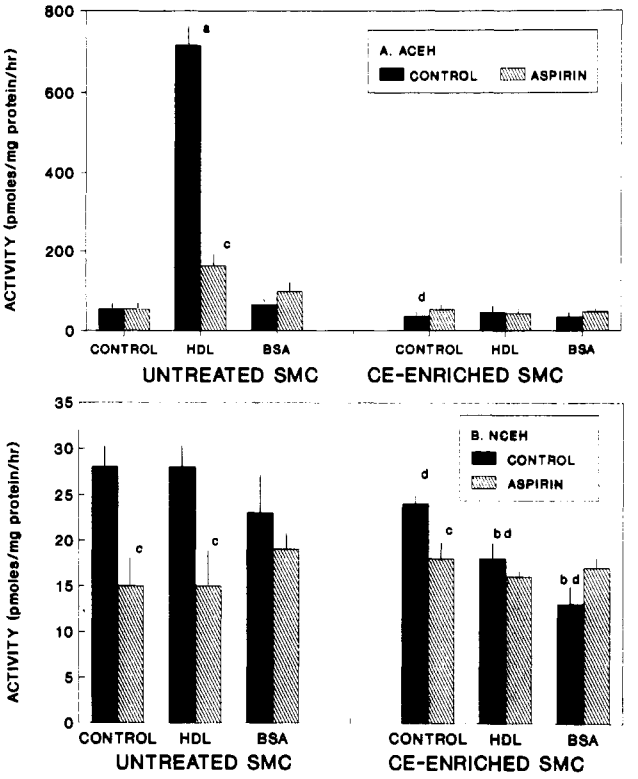

FIGURE 4: Effect of HDL on smooth muscle cell CE hydrolytic activity: effect of CE enrichment and aspirin pretreatment (panel A, ACEH activity; panel B, NCEH activity). Cells grown to confluent density in 35-mm plates were exposed to DME containing 10% FBS or to the same medium containing cLDL (100 μg of protein/mL) for 7 days with one media change on day 4. On day 7, untreated and CE-enriched cells were exposed to serum-free DME or serum-free DME containing HDL (400 μg of protein/mL) or BSA (1.0 mg/mL) in the absence or presence of aspirin (100 μM) for 24 h. Supernatants were then removed and assayed for 6-keto-$PGF_{1\alpha}$ by RIA. After being washed, cells were scraped into sucrose buffer and sonicated. Cell homogenates were then assayed for ACEH (panel A) and NCEH (panel B). Enzyme activity is reported as picomoles of substrate hydrolyzed per milligram of protein per hour. Data are expressed as mean ± SEM of triplicate samples. This figure is representative of two such experiments. Statistical comparisons are made by using the legend of Table I.

presence and absence of aspirin (a specific cyclooxygenase inhibitor). Aspirin did not alter ACEH activity in untreated cells exposed to DMEM alone. However, HDL stimulated ACEH activity (12-fold, $p < 0.05$, Figure 4A) and PGI_2 production (Table I) relative to untreated SMC exposed to DMEM alone. Also, the stimulation of ACEH activity by HDL was attenuated by aspirin. In contrast to untreated cells, CE-enriched cells were incapable of augmenting apparent ACEH activity in the presence of HDL. In addition, both basal and HDL-induced 6-keto-$PGF_{1\alpha}$ production was reduced by CE enrichment. Finally, BSA did not alter the apparent ACEH activity in either untreated or CE-enriched SMC (independent of aspirin treatment) but reduced basal 6-keto-$PGF_{1\alpha}$ production significantly in untreated cells.

Effect of Plasma Cholesterol Acceptors on NCEH Activity: Effect of Aspirin. The effect of HDL (400 μg/mL) and BSA (1.0 mg/mL) on the cytoplasmic CE hydrolase activity of untreated and CE-enriched SMC in the presence and absence of aspirin was also evaluated in identical experiments with those described immediately above (Figure 4B). In both untreated cells and CE-enriched cells, aspirin significantly ($p < 0.05$) reduced cytoplasmic CE hydrolytic activity. However, HDL and ASA did not stimulate NCEH activity in untreated cells, but rather significantly reduced NCEH activity in CE-enriched SMC.

Table I: Effect of Cholesterol Acceptors on PGI_2 Production: Effect of CE Enrichment and Inhibition of Cyclooxygenase[a]

	treatment					
	control		HDL		BSA	
cells	−	+	−	+	−	+
untreated SMC	4.2 ± 0.3 (a)	ND (c)	17.3 ± 2.0 (a)	ND (c)	2.0 ± 0.1 (a)	ND (c)
CE-enriched SMC	1.0 ± 0.1 (b, d)	ND (c)	2.5 ± 0.2 (b, d)	ND (c)	ND (b, d)	ND (c)

[a] Cells grown to confluent density in 35-mm plates were exposed to DME containing 10% FBS or the same medium containing cLDL (100 μg of protein/mL) for 7 days with one medium change on day 4. On day 7, untreated and CE-enriched cells were exposed to serum-free DME or serum-free DME containing HDL (400 μg of protein/mL) or BSA (1.0 mg/mL) in the absence or presence of aspirin (100 μM) for 24 h. Supernatants were assayed for 6-keto-$PGF_{1\alpha}$ by RIA. Data are expressed as nanograms of 6-keto-$PGF_{1\alpha}$ per milligram of protein, mean ± SEM of triplicate samples. This is representative of two such experiments. Comparisons are as follows: (a) $p < 0.05$, control vs HDL or BSA, untreated SMC. (b) $p < 0.05$, control vs HDL or BSA, CE-entiched SMC. (c) $p < 0.05$, (−) vs (+) aspirin within any treatment group. (d) $p < 0.05$, untreated SMC vs CE-enriched SMC.

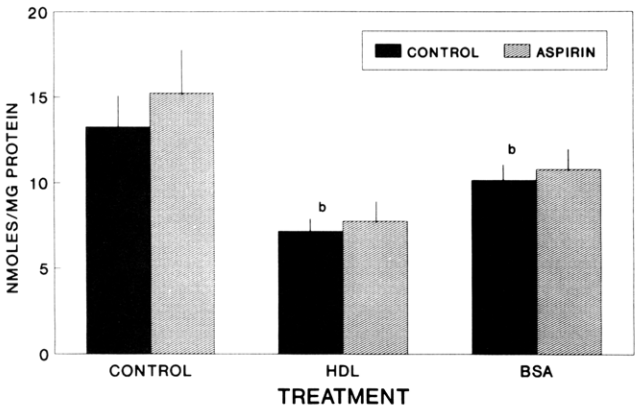

FIGURE 5: Effect of aspirin on HDL-induced CE hydrolysis from arterial SMC. Smooth muscle cells grown to confluent density in 35-mm wells were exposed to cLDL (100 μg of protein/mL) for 7 days with one media change. Cells were then exposed to serum-free DME containing 100 μM oleate/20 μM albumin supplemented with [^{3}H]oleate (specific activity = 40000 dpm/nmol) overnight. The cells were then exposed to serum-free DME or to DME containing HDL (400 μg of protein/mL) or BSA (1.0 mg/mL) in the presence and absence of aspirin (100 μM) for 24 h. Cellular radioactivity in CE was determined after lipid extraction and TLC to separate CE from other cellular lipids. Cell protein was determined on aliquots of 0.2 N NaOH digests. Data are expressed as nanomoles of CE per milligram of cell protein (mean ● SEM; each treatment performed in triplicate, for two different experiments). Statistical comparisons are made by using the legend of Table I.

Effect of Aspirin on the Rate of CE Hydrolysis Induced by Cholesterol Acceptors. To determine if eicosanoids mediate the rate of CE hydrolysis in CE-enriched SMC, the cytoplasmic CE pool was isotopically labeled by exposure of CE-enriched cells to [^{3}H]oleate/albumin prior to exposure to media alone, or media containing HDL or BSA in the presence and absence of aspirin. Hydrolysis of labeled cellular CE was then determined (Figure 5). HDL and BSA promoted CE hydrolysis, with HDL being the more efficacious cholesterol acceptor protein. However, aspirin did not significantly alter CE hydrolysis induced by either HDL or BSA.

Effect of Aspirin on SMC CE Efflux Induced by Cholesterol Acceptors. To determine if eicosanoids mediate cholesterol efflux from CE-enriched SMC, the SMC cholesterol pool was labeled by exposure to cLDL containing [^{3}H]-cholesteryl linoleate prior to exposure to media alone, or media containing HDL or BSA in the presence and absence of aspirin (Figure 6). Prior to efflux, CE-enriched cells contained (61.9 ± 0.24) × 10^3 dpm of total [^{3}H]cholesterol/mg of cell protein. In the absence of a cholesterol acceptor (serum-free DME), no efflux of cholesterol was observed. However, both HDL and BSA induced cholesterol efflux. Aspirin treatment did not alter the rate by which HDL or BSA enhanced cholesterol removal.

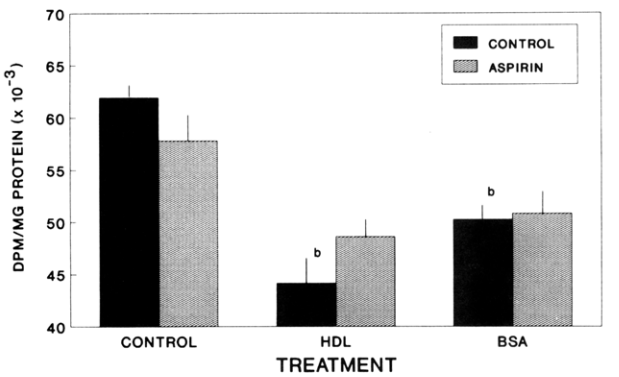

FIGURE 6: Effect of aspirin on HDL-induced cholesterol efflux from arterial SMC. Cells were exposed to cLDL containing [^{3}H]cholesteryl oleate for 7 days. Cells were then exposed to serum-free media or to media containing HDL or BSA for 24 h. Cells were then washed and digested in 0.2 N NaOH. Aliquots were taken for radioactivity and cell protein. Data are expressed as dpm per milligram of cell protein (mean ± SEM, each treatment performed in triplicate). This is representative of three different experiments. Statistical comparisons are made by using the legend of Table I.

Effect of Aspirin on SMC Cholesterol Content following Exposure to Plasma Cholesterol Acceptors. To determine if cholesterol acceptors remove cellular cholesterol by an eicosanoid-dependent mechanism, untreated and aspirin-treated control and CE-enriched cells were exposed to HDL and BSA for 24 h. Both free (Figure 7A) and esterified (Figure 7B) cholesterol were reduced in both normal and CE-enriched SMC. However, aspirin treatment did not alter either HDL- or BSA-induced reductions in cholesterol content.

Discussion

The mediation of arterial cellular CE content by eicosanoids is suggested by previous observations that exogenous PGI_2 and its metabolites mobilize CE from normal arterial smooth muscle cells (Etingin et al., 1986; Hajjar et al., 1982). In addition, HDL, the most efficacious of plasma cholesterol acceptors (Phillips et al., 1980, 1987), also stimulates PGI_2 synthesis (Pomerantz et al., 1984, 1985). Thus, the ability of HDL to promote net cholesterol efflux may be due in part to its ability to synthesize PGI_2. These observations also imply that the ability of HDL to induce cholesterol efflux from cells deficient in eicosanoid synthetic capacity may also be impaired. To test this hypothesis, we characterized the functional aspects of endogenous eicosanoids on CE hydrolysis and CE content of normal and CE-enriched SMC following exposure to HDL and albumin.

First, CE enrichment significantly reduces HDL-induced PGI_2 production (Figure 1). The inability of CE-enriched SMC to synthesize PGI_2 in response to HDL supports previous observations that CE-enriched cells cannot metabolize exogenous or endogenously released AA to PGI_2 (Pomerantz &

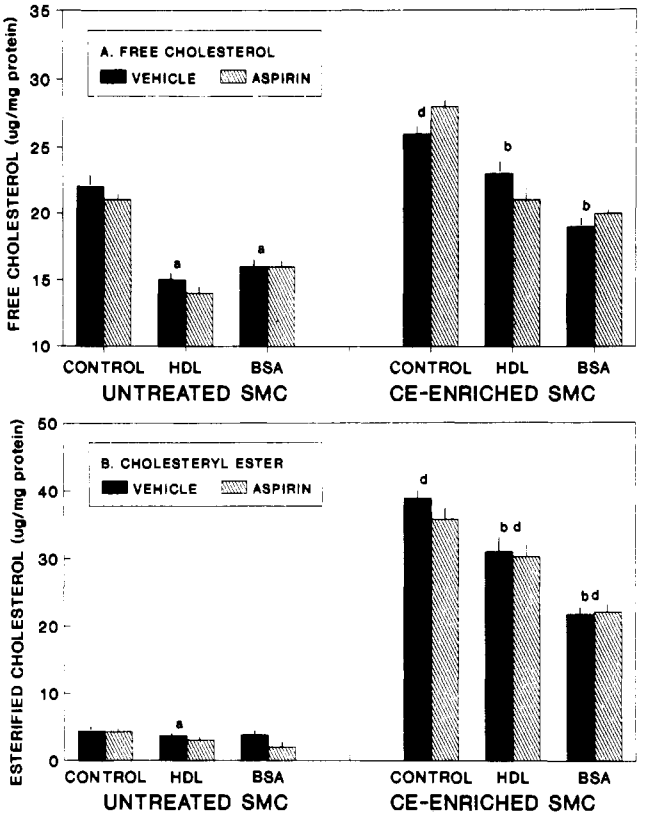

FIGURE 7: Effect of aspirin on the cholesterol content of untreated and CE-enriched SMC exposed to plasma cholesterol acceptors. Cells were exposed to DMEM alone or to DMEM containing cLDL for 7 days, followed by exposure to serum-free DMEM or to DMEM containing HDL or BSA for 24 h in the presence and absence of aspirin. Cell lipids were then extracted into hexane/2-propanol and analyzed for free (panel A) and esterified (panel B) cholesterol by GLC. The extracted cell monolayer was digested in 0.2 N NaOH; aliquots were taken for cell protein. Data are expressed as micrograms of cholesterol (or cholesteryl ester) per milligram of cell protein (mean ± SEM, each treatment performed in triplicate). This is a representative of two such experiments. Statistical comparisons are made by using the legend of Table I.

Hajjar, 1989). These data are intriguing since HDL contains significant amounts of AA in CE and phospholipid. Because CE-enriched SMC cannot utilize this abundant source of AA as substrate for eicosanoid biosynthesis, HDL (and presumably other sources of substrate) cannot offset the deficit in eicosanoid synthetic capacity following CE enrichment.

The stimulation of ACEH by HDL was in part eicosanoid-dependent, since stimulation of ACEH activity was suppressed 78% by aspirin treatment (Figure 4A). In addition, CE enrichment decreased basal ACEH activity as well as attenuating HDL-induced PGI_2 production and ACEH activity relative to untreated cells. The observation that the initial reduction in CE hydrolase activity precedes actual CE accumulation in the cell (which requires 5–7 days) (Figure 2) suggests that the metabolism of cLDL CE prior to CE enrichment results in down-regulation of these enzymes (Figure 4A); thus, it is not CE enrichment per se that results in reductions in the activities of CE hydrolytic enzymes. We conclude that eicosanoid-dependent HDL-induced elevation in lysosomal CE hydrolytic activity is abrogated by CE enrichment. Importantly, albumin significantly reduced PGI_2 production by SMC, without affecting ACEH activities. Albumin can either inhibit or stimulate eicosanoid generation (by scavenging or providing arachidonate, respectively) (Spector et al., 1983; Mann et al., 1986; Badway et al., 1984; Isakson et al., 1977). These data suggest that augmented eicosanoid synthesis appears to be a prerequisite for stimulation of ACEH activity, as was observed with HDL.

The mechanisms by which CE enrichment decreases ACEH activities are multifactorial. ACEH activity is decreased by CE enrichment concomitant with an increase in CE whose fatty acid composition resembles that of the cLDL (Pomerantz & Hajjar, 1989). Since CE accumulates in phagolysosomes under conditions of hypercholesterolemia (Shio et al., 1979; Falcone & Salisbury, 1988; Stein et al., 1979), the accretion of unhydrolyzed lipoprotein-derived CE in lysosomal compartments of the cell may reduce ACEH activity, and subsequently proceed into the development of foam cells. However, an important implication of this observation is that ACEH activity may be reduced by dilution with endogenous (nonradioactive) lipid (Nakao et al., 1981; Brecher et al., 1977). Indeed, LDL CE inhibits ACEH in a dose-dependent manner (Figure 3). However, amounts of CE from CE-enriched cells that are introduced into the ACEH assay contribute only to a 30% reduction of apparent ACEH activity. Therefore, other mechanisms contributing to reduced ACEH activity in CE-enriched cells may be operative. ACEH activity may be regulated through end-product inhibition by free cholesterol (Sakurada et al., 1976); in fact, CE-enriched SMC exhibit elevated free cholesterol levels as well (Figure 7; Pomerantz & Hajjar, 1989).

The mediation of NCEH activity by eicosanoids is different than what was observed on ACEH activity. Untreated SMC possess approximately 15-fold less NCEH activity than ACEH activity; however, NCEH activity in untreated cells is refractory to stimulation by HDL. Thus, NCEH activity may be functioning at maximal levels. Both aspirin and CE enrichment reduced NCEH activity (17% and 50%, respectively) (Figure 4B). We interpret these data to indicate that endogenous eicosanoids maintain NCEH activity, while inhibition of eicosanoid production reduces NCEH activities.

Since the data presented in Figures 1, 2, and 4 support the hypothesis that eicosanoid biosynthesis, CE hydrolysis, and CE efflux may be linked in arterial smooth muscle derived foam cells, we therefore examined the possibility that eicosanoid-mediated alterations in CE hydrolytic activity were requisite for alterations in promoting cholesterol efflux. Cholesteryl esters of lipid-enriched cells were initially labeled by exposure either to [^{3}H]oleate (which labels the NCEH-sensitive CE pool) or to cLDL containing [^{3}H]cholesteryl oleate (which is ACEH-sensitive). The rate of disappearance of radioactivity in CE ([^{3}H]oleate) or total cholesterol ([^{3}H]cholesteryl oleate labeled cells) from cells exposed to media containing HDL and BSA in the presence and absence of aspirin was then evaluated. Results of these experiments demonstrated that CE hydrolysis proceeded independently of eicosanoid biosynthesis, since (i) HDL-induced CE hydrolysis occurs independently of reduced eicosanoid synthetic capacity and aspirin treatment and (ii) BSA, which does not stimulate eicosanoid production, also promotes cholesterol efflux, in the presence or absence of aspirin (Figures 5 and 6).[2] Furthermore, aspirin had no effect in altering the reduction in free cholesterol and CE content of either *untreated* or CE-enriched SMC after exposure to HDL or BSA (Figure 7), suggesting a dissociation between eicosanoid-mediated cholesterol efflux and eicosanoid-independent mediation of cellular cholesterol content. Thus, net cholesterol efflux by plasma cholesterol

[2] The [^{3}H]oleate experiments were not conducted in the presence of an ACAT inhibitor. Given the possibility that some reesterification might have occurred during the efflux portion of these studies, the actual amount of CE hydrolysis may be underestimated.

acceptors may ultimately be independent of eicosanoids. The observation that BSA also promoted cholesterol efflux is provocative, since there are reports indicating that albumin alone does not promote net cholesterol efflux, but rather facilitates cholesterol *transfer* between cell–lipoprotein and lipoprotein–lipoprotein systems (Fielding & Moser, 1981; Stein et al., 1986). However, albumin does promote cholesterol efflux (St. Clair & Leight, 1983; Mendel & Kunitake, 1988; Chau & Geyer, 1978) and synergizes with phospholipid in this effect (Bartholow & Geyer, 1981; Chau & Geyer, 1978). Thus, our observation that albumin promotes cholesterol efflux from CE-enriched cells may be due to its intrinsic ability to promote cholesterol efflux and/or the presence of phospholipid in the commercial albumin preparation used in these studies. Although albumin is not the most efficacious cholesterol acceptor protein, the fact that albumin promoted cholesterol efflux without stimulating eicosanoid generation establishes the validity of its use to explore eicosanoid-independent cholesterol efflux.

Our data are supported by recent observations that the eicosanoid-dependent portion of HDL-induced cholesterol efflux in CE-enriched macrophages represented only 10–15% of total efflux (Van Sickle & Nasjletti, 1987). In other studies, HDL stimulated cholesterol efflux from cells derived from human atherosclerotic lesions (Orekhov et al., 1987; Gabrielyan et al., 1988), but the eicosanoid-dependent component of HDL-induced cholesterol efflux in these studies was not determined.

In summary, cholesterol metabolism in SMC can be divided into two functional compartments—an eicosanoid-dependent compartment consisting of lysosomal and cytoplasmic CE hydrolytic enzymes and an eicosanoid-independent compartment consisting of pathways leading to translocation of cholesterol to the cell membrane and ultimate removal by extracellular cholesterol acceptors. It has been suggested that the delivery of PGI_2 to SMC derived from an "activated" adjacent endothelium (Hajjar et al., 1987) or in the form of a pharmacologic regimen (Gabrielyan et al., 1988; Willis et al., 1986) may facilitate mobilization of cholesterol in atherosclerosis. However, mechanisms by which cholesterol is translocated from the cytoplasm to the cell membrane and subsequent desorption by plasma cholesterol acceptors may represent a critical level of intracellular regulation of cellular cholesterol content.

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Registry No. PGI_2, 35121-78-9; CE-hydrolase, 9026-00-0; 6-keto-$PGF_{1\alpha}$, 58962-34-8.

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Nucleotide and Deduced Amino Acid Sequence of a Human cDNA (NQO_2) Corresponding to a Second Member of the NAD(P)H:Quinone Oxidoreductase Gene Family. Extensive Polymorphism at the NQO_2 Gene Locus on Chromosome 6[†,‡]

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ABSTRACT: NAD(P)H:quinone oxidoreductases (NQOs) are flavoproteins that catalyze the oxidation of NADH or NADPH by various quinones and oxidation–reduction dyes. We have previously described a complementary DNA that encodes a dioxin-inducible cytosolic form of human NAD(P)H:quinone oxidoreductase (NQO_1). In the present report we describe the nucleotide sequence and deduced amino acid sequence for a cDNA clone that is likely to encode a second form of NAD(P)H:quinone oxidoreductase (NQO_2) which was isolated by screening a human liver cDNA library by hybridization with a NQO_1 cDNA probe. The NQO_2 cDNA is 976 nucleotides long and encodes a protein of 231 amino acids (M_r = 25 956). The human NQO_2 cDNA and protein are 54% and 49% similar to human liver cytosolic NQO_1 cDNA and protein, respectively. COS1 cells transfected with NQO_2 cDNA showed a 5–7-fold increase in NAD(P)H:quinone oxidoreductase activity as compared to nontransfected cells when either 2,6-dichlorophenolindophenol or menadione was used as substrate. Western blot analysis of the expressed NQO_1 and NQO_2 cDNA proteins showed cross-reactivity with rat NQO_1 antiserum, indicating that NQO_1 and NQO_2 proteins are immunologically related. Northern blot analysis shows the presence of one NQO_2 mRNA of 1.2 kb in control and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) treated human hepatoblastoma Hep-G2 cells and that TCDD treatment does not lead to enhanced levels of NQO_2 mRNA as it does for NQO_1 mRNA. Southern blot analysis of human genomic DNA suggests the presence of a single gene approximately 14–17 kb in length. The NQO_2 gene locus is highly polymorphic as indicated by several restriction fragment length polymorphisms detected with five different restriction enzymes. The NQO_2 gene was localized to human chromosome 6 by Southern analysis of human–rodent somatic cell hybrids. Further analysis of several hybrids containing breaks or translocations involving chromosome 6 allowed regional localization of the NQO_2 gene to chromosome 6pter-q12.

NAD(P)H:quinone oxidoreductases, formerly known as DT-diaphorases (EC 1.6.99.2), are flavoproteins that catalyze the oxidation of NADH or NADPH by various quinones and oxidation–reduction dyes (Lind et al., 1982; Thor et al., 1982; Morrison et al., 1984; Di Monte et al., 1984a; 1984b; Ernster et al., 1960). The physiological functions of these enzymes are not yet understood though they seem to be involved both in the detoxification of nonphysiological quinones (Ernster et al., 1982) and in the bioactivation of vitamin K (Stenflo et al., 1974). In rat liver the oxidoreductase activity is found mainly (approximately 95%) in the cytosolic fraction, but 5–10% of the total cellular activity is recovered in the mi-

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