Glutathione Preconditioning Attenuates Ac-LDL–Induced Macrophage Apoptosis *via* Protein Kinase C–Dependent Ac-LDL Trafficking

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Oxidized low-density lipoprotein (ox-LDL) incorporation into intimally resident vascular cells *via* scavenger receptors marks one of the early steps in atherosclerosis. Cellular apoptotic damage results from two major serial intracellular events: the binding and scavenger receptor-mediated uptake of oxidizable lipoproteins and the intracellular oxidative responses of accumulated lipoproteins. Most molecular approaches to prevent apoptotic damage have focused on singular events within the cascade of lipoprotein trafficking. To identify a multifocal strategy against LDL-induced apoptosis, we evaluated the role of cellular preconditioning by glutathione-ethyl ester (GSH-Et), a native redox regulator, in the prevention of the uptake and apoptotic effects of an oxidizable scavenger receptor-specific ligand, acetylated low-density lipoprotein (Ac-LDL). Our results indicate that GSH-Et–mediated protein kinase C (PKC) pathway modulation regulates Ac-LDL binding and incorporation into GSH-Et preconditioned cells and subsequently delays reactive oxygen intermediate generation and apoptotic conversion. The GSH-Et protective effects on apoptosis and Ac-LDL binding were reversed by calphostin C, a PKC inhibitor, and were accompanied by an increase in PKC phosphorylation. However, the rate of reactive oxygen intermediate accumulation was not increased following calphostin C treatment, suggesting that GSH-Et may play an important nonreactive oxygen-intermediate–based protective role in regulating apoptotic dynamics. Overall, we report on the novel role for GSH-Et preconditioning as a molecular strategy to limit lipoprotein entry into the cells, which presents a proactive modality to prevent cellular apoptosis in contrast with the prevalent antioxidant approaches that treat damage retroactively. Exp Biol Med 230:40–48, 2005

Key words: glutathione; Ac-LDL; macrophage; PKC; apoptosis; scavenger receptor

Introduction

Atherosclerosis, an occlusive arterial disease, can be escalated through the early recruitment of monocyte/macrophage leukocytes to nascent plaque sites (1). This is followed by macrophage ingestion of oxidized forms of low-density lipid (LDL) *via* scavenger receptors and their differentiation into foam cells. Unlike native LDL receptors, scavenger receptor expression is not downregulated by intracellular oxidized low-density lipoprotein (ox-LDL) accumulation, resulting in uncontrolled ox-LDL accumulation and ultimately irreversible apoptotic damage and cell death (2).

Many therapeutic approaches to mitigate the damaging consequences of intracellular oxidized liproprotein accumulation and apoptotic transitional events have been investigated. Several studies have identified key apoptotic or stress pathway intermediates as potential therapeutic targets (3–7). Others have assessed the role of antioxidant supplementation to neutralize and prevent the accumulation of damaging reactive oxygen intermediates. The effects of ascorbic acid, α-tocopherol, N-acetylcysteine, glutathione, lycopene, and many other chemical compounds on reactive oxygen intermediate accumulation and apoptosis reduction have been extensively studied (8–14).

Among these, the tripeptide, γ-Glu-Cys-Gly, glutathione (GSH), containing a reactive thiol group, is one of the key physiologic antioxidants in mammalian cells. GSH maintains the redox equilibrium in the cell (15) and engages in native stress-neutralizing pathways. In quiescent macro-

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phages, it has been shown to prevent apoptosis mediated by reactive oxygen intermediates (16), and in the absence of cellular defenses, the decomposition of potentially cytotoxic lipid peroxidation products within macrophages can result in severe oxidative stress and accelerate the early progress of atheroma (9). Thus, cellular conditioning with GSH may play key roles in limiting either the oxidation of LDL or the accumulation of intracellular reactive oxygen intermediates (17). Further, GSH can regulate a number of complex intracellular pathways (18–21). The antioxidant functions of GSH in rescuing cells from ox-LDL effects have been widely reported (15, 18, 22, 23). However, given the multifunctional role of GSH in intracellular regulation, the mechanism of GSH protection against LDL-induced damage remains to be clarified.

Here, we assessed the role of glutathione-ethyl ester (hereon referred to as GSH-ethyl ester, and abbreviated as GSH-Et) preconditioning on the interactions of IC21 macrophages with an analog of oxidized LDL, acetylated low-density lipoprotein (Ac-LDL). Ac-LDL was selected as a model lipoprotein in these studies because both Ac-LDL and ox-LDL cross-react with several specific scavenger receptors and induce macrophage foam cell evolution. However, unlike ox-LDL oxidation, Ac-LDL oxidation is predominantly controlled by intracellular reactivity (24, 25). Our results indicate that both Ac-LDL–mediated apoptosis and reactive oxygen intermediate accumulation is mitigated by preconditioning macrophages with GSH-Et. More importantly, our studies indicate that GSH preconditioning can modulate Ac-LDL binding and uptake *via* a protein kinase C (PKC)-dependant pathway. The antioxidant function of GSH-Et may actually play a secondary protective role in preconditioned macrophages, limited in effect, by the rate of Ac-LDL binding to cell surface receptors. Thus, GSH-Et may regulate the rate-limiting step of macrophage apoptotic conversion by effectively reducing Ac-LDL entry into cells.

Materials and Methods

Tissue Culture. IC21 macrophage cells (ATCC, Rockville, MD) were propagated by culture with RPMI media containing 10% fetal bovine serum and penicillin/streptomycin, in a humidified CO_2 atmosphere as previously described (26). All media components were purchased from Biowhittaker (Walkersville, MD).

Cell Preconditioning. Cells (5×10^3) were plated in eight-well Nunc chamber slides with 2 m*M* reduced GSH-Et (Sigma, St. Louis, MO) for 15–24 hours. Increased intracellular GSH-Et levels generated by preconditioning were confirmed using a glutathione detection kit (Molecular Probes, Eugene, OR) followed by thin-layer chromatography. Fluorescent spots were analyzed using densitometry with Image-Pro image analysis software (Media Cybernetics, Silver Spring, MD) and quantified by comparing image intensities with those generated using a glutathione standard curve. Control cells contained approximately 0.75 pg/cell reduced GSH. Following buthionine sulfoximine (BSO) exposure, GSH levels were reduced to 50% of that measured in control cells, while GSH-Et preconditioning increased intracellular reduced GSH to levels greater than 230% compared with those in control cells. Preconditioning media was aspirated and replaced with 25 μg/ml fluorescently labeled or unlabeled Ac-LDL (Molecular Probes). Normal tissue culture media was used as a control for both GSH-Et and Ac-LDL conditions.

Fluorescence Microscopy and Assessment of Ac-LDL Binding and Incorporation. Cells were cultured overnight in either 2 m*M* GSH-Et or control media conditions, the preconditioning media aspirated, and exposed to 25 μg/ml Ac-LDL-Bodipy for 30–60 minutes at 4°C to assess Ac-LDL binding or for 15–30 minutes at 37°C to assess Ac-LDL incorporation. Cells were washed with cold PBS and images were captured using a computer-interfaced inverted Zeiss confocal microscope. Specimens were excited at a peak wavelength of 488 nm, and fluorescent images (520-nm peak emission) were acquired and analyzed using densitometry with Image-Pro image analysis software.

Reactive Oxygen Intermediate Detection. Cells were precultured with either 2 m*M* GSH-Et (Sigma) or in control media conditions overnight. Preculturing media was gently aspirated and 25 μg/ml Ac-LDL was added to the cells. Intracellular reactive oxygen intermediate levels were assessed by measuring the conversion of DH_2DCDFA (Molecular Probes) into a fluorescent product following exposure to cellular peroxide and oxygen free radicals. Images were captured using a computer-interfaced inverted Zeiss epifluorescence microscope imaging system. Specimens were excited at a peak wavelength of 488 nm and fluorescent images (520-nm peak emission) were acquired and analyzed using densitometry with Image-Pro image analysis software.

Ac-LDL–Induced Apoptosis. Cells were precultured with either 2 m*M* GSH-Et (Sigma), 50 μ*M* BSO, a glutathione synthetic inhibitor (Sigma), or in control media conditions overnight. 5×10^3 cells were incubated in wells for a maximum of 7 hours with 25 μg/ml of an oxidized LDL analog, Ac-LDL (Molecular Probes) in a humidified 37°C incubator. Apoptotic and necrotic cells were distinguished by double labeling, using green fluorescent fluorescein (FITC)-annexinV, which binds to externalized phosphatidyl serine in apoptotic cells, and red fluorescent propidium iodide, which binds to nuclear DNA in necrotic cells. Images were captured with a computer-interfaced inverted Zeiss epifluorescence microscope and percentage apoptotic cells were enumerated for each condition.

PKC Inhibition. Experiments were performed as above except that, after GSH-Et or control preconditioning, cells were incubated with calphostin C (Cal C; 50 n*M*), a broad PKC inhibitor (27–29), purchased from Sigma, or SB202474, a negative inhibitor control (Calbiochem, San

Diego, CA), for 30–45 minutes at 37°C. Enzyme inhibitors were removed and cells were exposed to Ac-LDL for 30–60 minutes at 4°C. Bound Ac-LDL was assessed as described above. In the case of apoptosis or reactive oxygen intermediate detection experiments, Cal C or control enzymes were added prior to Ac-LDL exposure and remained throughout the incubation period.

Gel Electrophoresis and Western Blotting. GSH-Et preconditioned or control IC21 cells were detached and washed with ice-cold phosphate-buffered saline (PBS), and then lysed in 5 m*M* $MgCl_2$, 0.1% sodium deoxycholate (Fisher Scientific, Fair Lawn, NJ), 1% Triton X-100 (Bio-Rad Laboratories, Hercules, CA), 50 m*M* Tris-HCl, and 100 m*M* NaCl, containing protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) (lysis buffer). Protein concentrations in the lysates were measured using the Coomassie Plus Protein Assay Kit (Pierce Biotechnology, Rockford, IL) and bovine serum albumin (BSA; Sigma) standards prepared in the lysis buffer described above. Equal amounts of protein were separated under reducing conditions on 4%–15% Tris-HCl Criterion precast sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene difluoride membranes (BioRad). Non-specific protein adsorption was blocked by incubating the membranes in 20 m*M* Tris-HCl, pH 7.6, 137 m*M* NaCl, and 0.1% Tween 20 (TBS-T; Fisher Scientific) containing 10% nonfat dry milk (Krasdale Foods, White Plains, NY). Membranes were probed overnight at 4°C with rabbit polyclonal antibodies to phosphorylated PKC (pan), (Cell Signaling Technology, Beverly, MA) diluted 1:1000 or to non–phospho-specific PKC (pan) (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200. Antibodies that do not discriminate between PKC isoforms were deliberately chosen to parallel our PKC inhibition studies, which involve general PKC isoform inhibition (27–29). To ensure proper loading, membranes were probed with a mouse monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon International, Temecula, CA) diluted 1:1000. Development of chemiluminescence was performed using the SuperSignal West Dura Kit (Pierce Biotechnology). Appropriate secondary antibodies were used as supplied in the kit, diluted 1:1500, except on blots for phosphorylated PKC, which was probed with secondary antibodies diluted 1:800. All primary and secondary antibodies were diluted in TBS-T containing 5% nonfat dry milk, except for primary antibodies to phosphorylated PKC, which was diluted in TBS-T containing 5% BSA (Sigma). Chemiluminescent signals were captured on autoradiography films and processed using photofinishing chemicals (Eastman Kodak Company, New Haven, CT). The molecular weight of detected proteins was verified using molecular-weight markers (Pierce Biotechnology). The two protein products were probed on a single membrane, stripped between blots using a commercially available stripping buffer (Pierce Biotechnology) for 15 minutes at 37°C, and verified for signal absence by redeveloping the blot with secondary antibodies and chemiluminescent substrates.

Indirect Immunofluorescence and Flow Cytometry. Subconfluent preconditioned or control IC21 macrophages were lightly fixed for 20 minutes with 0.25% formalin in PBS at 4°C in T25 tissue-culture flasks. Nonspecific binding was blocked by preincubation with 3% BSA, 1% normal goat serum in PBS for 1 hour at 4°C. Cells were incubated with Ac-LDL specific anti-MARCO (Serotec, Raleigh, NC) or 2F8 (Accurate, Westbury, NY) antibodies or with control rat immunoglobulin, each at a concentration of 10 μg/ml and subsequently with FITC goat anti-rat IgG, diluted 1:200 (Jackson Immunoresearch, West Grove, PA). Anti-MARCO (clone ED31) recognizes the murine surface antigen, macrophage receptor with collagenous structure, a member of the Class A scavenger receptor family. 2F8 (clone 2f8) recognizes the murine scavenger receptor Types I and II and has been found to inhibit the uptake of Ac-LDL. The Ac-LDL binding within IC21 cell populations, in the presence or absence of blocking antibody, was assessed by incubating control or preconditioned cells with Bodipy-Ac-LDL, 10 μg/ml (Molecular Probes) (instead of secondary antibody) for 1 hour at 4°C. Cells were then treated with 1.0% formalin in PBS to immobilize the scavenger receptor/ligand or receptor/antibody complexes, scraped from the flask, and analyzed using a Becton Dickinson FACScan. All reagents were diluted in PBS/blocking buffer.

Kinetics of Apoptosis and Reactive Oxygen Intermediate Accumulation. The kinetics of apoptosis and reactive oxygen intermediate changes were quantified from experimental data as the rate of their change between 3 and 5 hours and was normalized to the data at 3 hours. Thus, the kinetic index was indicated by $\Delta F_{3\text{–}5\ \text{hour}}/(\Delta t)(F_{3\ \text{hour}})$, where F is the relative level of either reactive oxygen intermediate generation or apoptosis. Early events (3–5 hours) were studied, prior to severe necrotic damage onset, which was escalated by Cal C treatment, skewing the results of our studies. Data values were normalized to control cells, exposed to neither Ac-LDL nor GSH-Et, at each time point.

Statistical Analysis. Data were expressed as the mean of three or more replicate experiments ± the standard error (SE) of the mean. Statistical significance was determined using either a two-tailed Student's t test, assuming unequal variances, or the analysis of variance test, ANOVA, for a P value of <0.05.

Results

Effect of GSH-Et Preconditioning on Ac-LDL Binding and Incorporation. The effect of GSH-Et preconditioning in modulating the earliest Ac-LDL/cell interactions was evaluated by quantifying the extent of Ac-LDL binding to the membranes of individual IC21 cells. GSH-Et–preconditioned or control IC21 cells were incubated with Ac-LDL at 4°C to minimize cellular internalization and

the binding of Ac-LDL was assessed with a fluorescently labeled derivative, Ac-LDL-Bodipy. These results are presented in Figure 1. A dramatic decrease in binding was apparent in preconditioned cells 1 hour following Ac-LDL exposure. In fact, GSH-Et preconditioning lowered Ac-LDL binding as early as 15 minutes postexposure (data not shown). In addition, incubation at 37°C showed that GSH-Et preconditioning similarly led to a reduction in the levels of intracellular incorporation of Ac-LDL.

GSH-Et Preconditioning, Reactive Oxygen Intermediate Accumulation, and Apoptosis. The physiological relevance of GSH-Et–mediated Ac-LDL binding regulation was assessed. Modulation of the intracellular oxidative state was evaluated by measuring the intracellular generation of reactive oxygen intermediates in IC21 macrophage cells, following exposure to Ac-LDL. As indicated in Figure 2, Ac-LDL exposure increased reactive oxygen intermediate levels, while preconditioning with GSH-Et dramatically reduced Ac-LDL–induced reactive oxygen intermediate generation. The reactive oxygen intermediate levels remained uniformly low in GSH-Et preconditioned/Ac-LDL–exposed cells but reached maximum values at 3 hours in control/Ac-LDL–exposed cells. Thus, GSH-Et preconditioning reduced not only cell Ac-LDL binding and incorporation but also the generation of intracellular oxidative intermediates.

The effect of GSH-Et preconditioning on cell death induction by Ac-LDL was evaluated by measuring the induction kinetics of cell apoptosis. Cellular labeling of relative number of apoptotic cells by an early apoptotic marker, phosphatidyl serine externalization, was assessed. Pre-exposure to the GSH synthesis inhibitor, BSO, and subsequently to Ac-LDL, resulted in a 3-fold increase in apoptotic cells by 3 hours post–AC-LDL exposure and

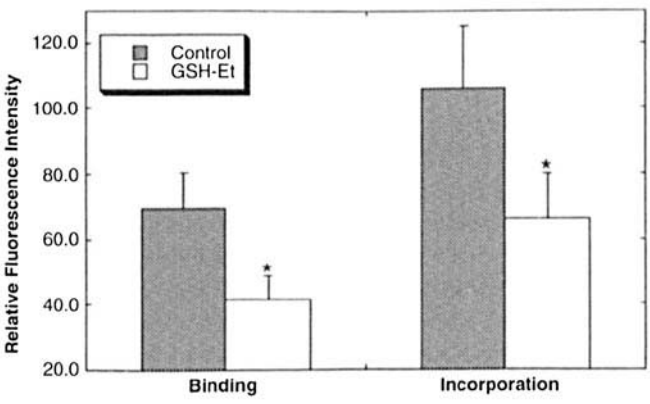

Figure 1. Effect of glutathione-ethyl ester (GSH-Et) preconditioning on acetylated low-density lipoprotein (Ac-LDL) association: Preconditioned or control IC21 cells were cultured as above, subsequently incubated with Ac-LDL–Bodipy for 1 hour and levels of bound Ac-LDL or incorporated Ac-LDL were quantified on a per cell basis. At least 25 cells were imaged per experimental group for each experiment. Results represent average levels of Ac-LDL binding ± standard error. Asterisks reflect a statistically significant change in Ac-LDL binding or incorporation relative to the respective control (no GSH-Et preconditioning).

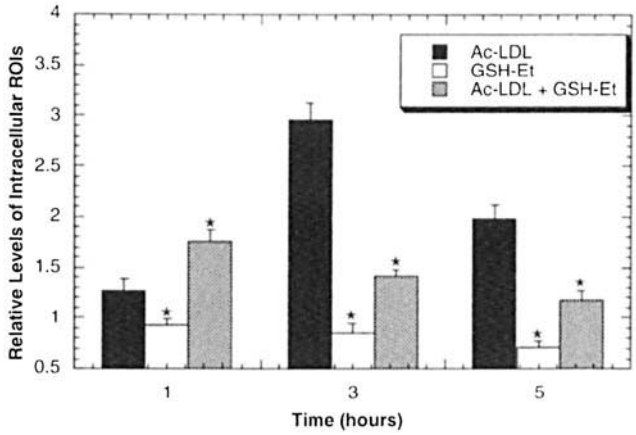

Figure 2. Effect of glutathione-ethyl ester (GSH-Et) preconditioning on oxygen radical accumulation: intracellular reactive oxygen intermediate (ROI) levels were assessed by measuring the conversion of DH_2DCDFA into a fluorescent product. Cells were precultured with either 2 m*M* GSH-Et or in control media conditions and subsequently acetylated low-density lipoprotein (Ac-LDL) for up to 5 hours. Data are normalized to cells incubated in the absence of both Ac-LDL and GSH-Et and reflect an average of three experiments. Asterisks reflect a statistically significant change in reactive oxygen intermediate induction relative to that observed for the respective control (no GSH-Et preconditioning).

maximum apoptosis was realized by 5 hours (Fig. 3). In addition, preconditioning with GSH-Et resulted in significantly lower apoptosis than either BSO-treated or control Ac-LDL–exposed cells. Thus, the GSH-Et binding effects were accompanied by inhibition of reactive oxygen intermediates and reduction of apoptosis.

Protein Kinase C Regulation of Ac-LDL/Cell Interaction. Because protein kinase C regulates many

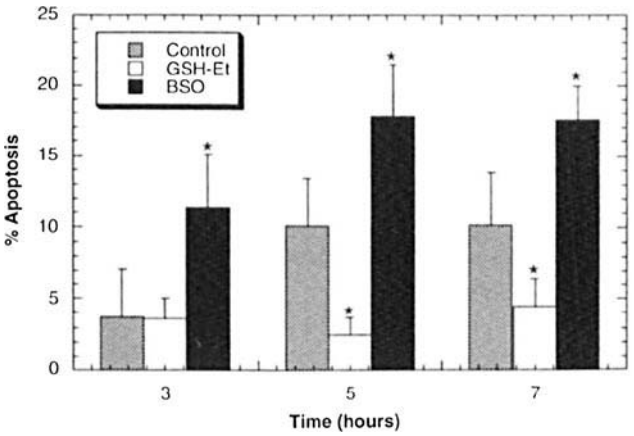

Figure 3. Effect of preconditioning on acetylated low-density lipoprotein (Ac-LDL)–mediated cell death: cells were precultured with either 2 mM glutathione-ethyl ester (GSH-Et), 50 μ*M* butathione sulfoximine (BSO; a GSH synthetic inhibitor) or in control medial conditions and subsequently Ac-LDL. The percentage apoptotic cells were quantified as cells with membrane-bound annexin after exposure to Ac-LDL. Results are normalized against non–Ac-LDL control cell values and reflect the average of three experiments. A minimum of 100 cells was evaluated per group/experiment. Asterisks reflect a statistically significant change in fractional apoptosis relative to that observed for the respective control (no GSH-Et preconditioning).

intracellular functions, including monocyte mediated lipid oxidation and apoptosis (30), we examined the effect of PKC inhibition on GSH-Et–mediated regulation of Ac-LDL binding, reactive oxygen intermediate (ROI) generation, and apoptotic conversion. The results of these experiments are presented in the panel in Figure 4. Incubation with Cal C, an isoform nonspecific PKC inhibitor (27–29), increased Ac-LDL binding in GSH-Et–treated cells (Fig. 4A). In fact, as a result of Cal C exposure, both GSH-Et–mediated Ac-LDL binding and Ac-LDL incorporation (data not shown) into cells were restored to control levels.

The role of PKC pathways in regulating ROI levels was examined next. These results, summarized in Figure 4B, indicate that PKC inhibition also resulted in increased intracellular ROI accumulation compared with control levels as early as 1 hour post–Ac-LDL exposure and remained high through the 5-hour experimental time period. Thus, despite the presence of preconditioning levels of intracellular antioxidant GSH-Et, ROI accumulation continued in Cal C–treated cells following Ac-LDL exposure.

Finally, the effect of PKC inhibition on cell apoptosis was assessed. As indicated in Figure 4C, Cal C treatment increased relative percent of apoptotic cells within GSH-Et–preconditioned cultures. This change occurred at the earliest time point assessed (3 hours) and continued through 7 hours post–Ac-LDL exposure. It should be noted that, because Cal C increased cell damage over time, there was an apparent decrease in apoptosis in Cal C/GSH-Et–treated cells due to cell necrosis (data not shown). Nevertheless, PKC inhibition reversed the antiapoptotic preconditioning effect. In addition, Cal C treatment increased apoptosis in nonpreconditioned cells (Fig. 4D). In this case, percentage of cells exhibiting apoptosis was greater than preconditioned cells at 3 hours post–Ac-LDL exposure, but was equivalent to preconditioned cells thereafter.

GSH-Et Preconditioning and PKC Phosphorylation. Because our studies indicated that PKC could mediate the GSH-Et preconditioning effect, we investigated the effect of GSH-Et preconditioning on the activation of PKC, a key signaling intermediate. The relative levels of phosphorylated and total PKC were examined *via* Western blotting. We chose to examine PKC levels with non–isoform-specific antibodies to produce results that could be integrated with our non–isoform-specific functional inhibition studies. GSH-Et–preconditioned IC21 cells exhibited a higher level of phosphorylated PKC than control, untreated cells. In contrast, total PKC and levels were relatively invariant in response to GSH-Et preconditioning (Fig. 5).

GSH-Et Preconditioning and Scavenger Receptor Expression. The mechanism of GSH-Et protection was probed by assessing the modulation of Ac-LDL–specific scavenger receptor expression within preconditioned or control IC21 cell populations. Cytofluorometric

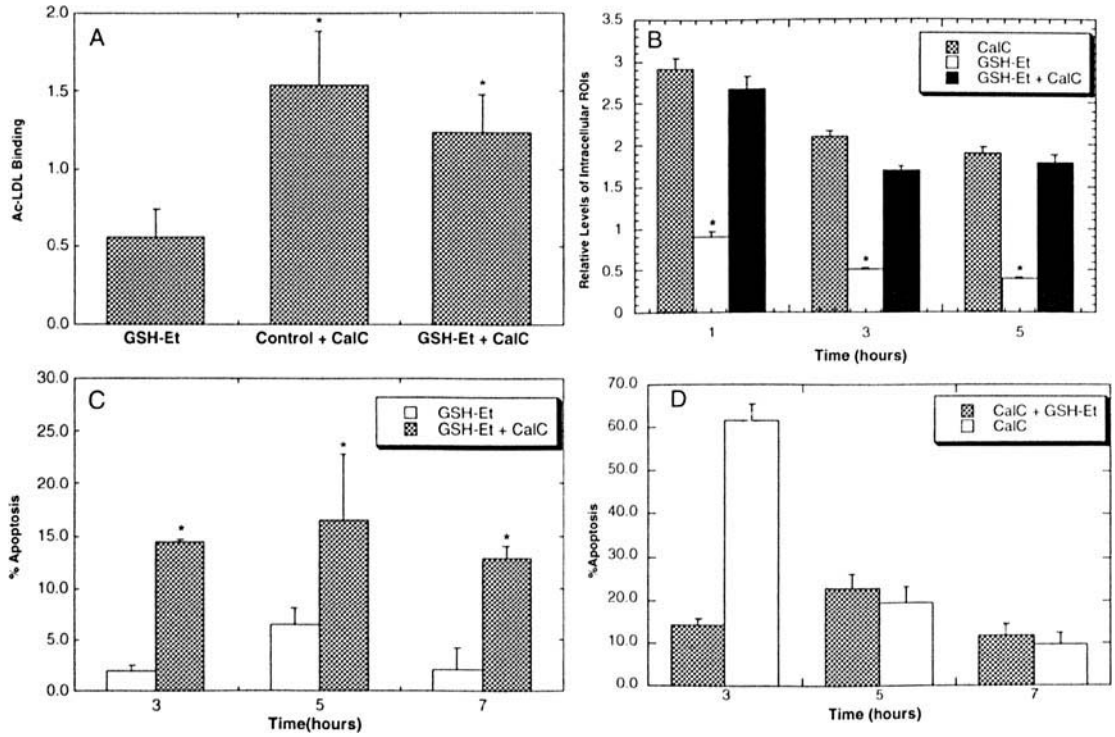

Figure 4. Effect of protein kinase C (PKC) in glutathione-ethyl ester (GSH-Et)-preconditioned cells. Acetylated low-density lipoprotein (Ac-LDL) binding: cells were cultured as described in Figure 1. Following preconditioning culture, cells were incubated with calphostin C or control enzyme SB202474 and, subsequently, Ac-LDL for either 1 hour at 4°C to evaluate Ac-LDL binding (A) or at 37°C for up to 5 hours to assess reactive oxygen intermediate accumulation (B), and up to 7 hours to measure apoptosis (C and D). Results represent average Ac-LDL binding for three experiments ± standard error and are normalized against control cells that were incubated in the absence of GSH-Et or calphostin C. Asterisks (A–C) reflect deviations from calphostin C–pretreated control.

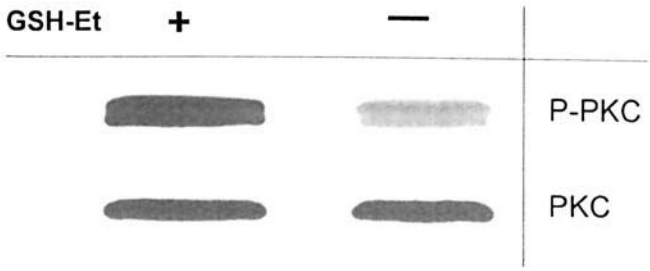

Figure 5. Effect of glutathione (GSH)-reconditioning on protein kinase C (PKC) phosphorylation: IC21 cells were cultured in GSH-Et preconditioning media or control media overnight. Proteins were extracted and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western Blotting with nonisoform specific antibodies to phosphorylated PKC (p-PKC) and total PKC (PKC). Left lane, GSH-treated cells. Right lane, control cells. Results are representative of duplicate experiments.

analyses indicate that, while GSH-Et reduced Ac-LDL binding, Class A scavenger receptor-specific anti-MARCO or 2F8 antibody binding was unchanged in GSH-Et–treated cells (Fig. 6). In fact, relative receptor expression appeared to be unchanged despite the inhibitory effect of 2F8 antibody on Ac-LDL binding in both GSH-Et and untreated cells (data not shown).

Evaluation of Rate Limiting Apoptotic Events. Because GSH-Et preconditioning was not mediated by regulating the number of scavenger receptors, we investigated the role of ROI accumulation. With a view to decoupling the intracellular consequences of Ac-LDL binding events from those due to ROI accumulation, the rates of ROI generation and apoptotic conversion were compared in the presence and absence of Cal C. While the PKC inhibitor, Cal-C, can theoretically affect many downstream targets within the PKC biochemical pathway, including Ac-LDL binding, it has no effect on the direct neutralization of ROI. Therefore, the kinetics of apoptosis and ROI generation in GSH-Et–preconditioned or unconditioned cells were evaluated for the period 3–5 hours, following treatment with or without Cal C. The results of these analyses are presented in Figure 7. In the presence of Cal C, the rate of GSH-Et preconditioned cell apoptosis increased and approached that of control cells. However, Cal C treatment did not increase the rate of ROI generation in GSH-Et–treated cells. Similarly, changes in reactive oxygen intermediate accumulation rates even at earlier time points (data not shown) did not correlate with Cal C–mediated apoptosis effects. These trends indicate a non-ROI-based component to be the rate-limiting event in GSH-Et–mediated regulation of apoptotic events.

Discussion

We have investigated a preconditioning regimen to protect cells against Ac-LDL–mediated cell damage. The role of reduced glutathione as an antioxidant has been examined previously by several investigators (15, 18). The current investigations uncovered a new GSH function, the regulation of Ac-LDL entry into macrophage cells *via* a

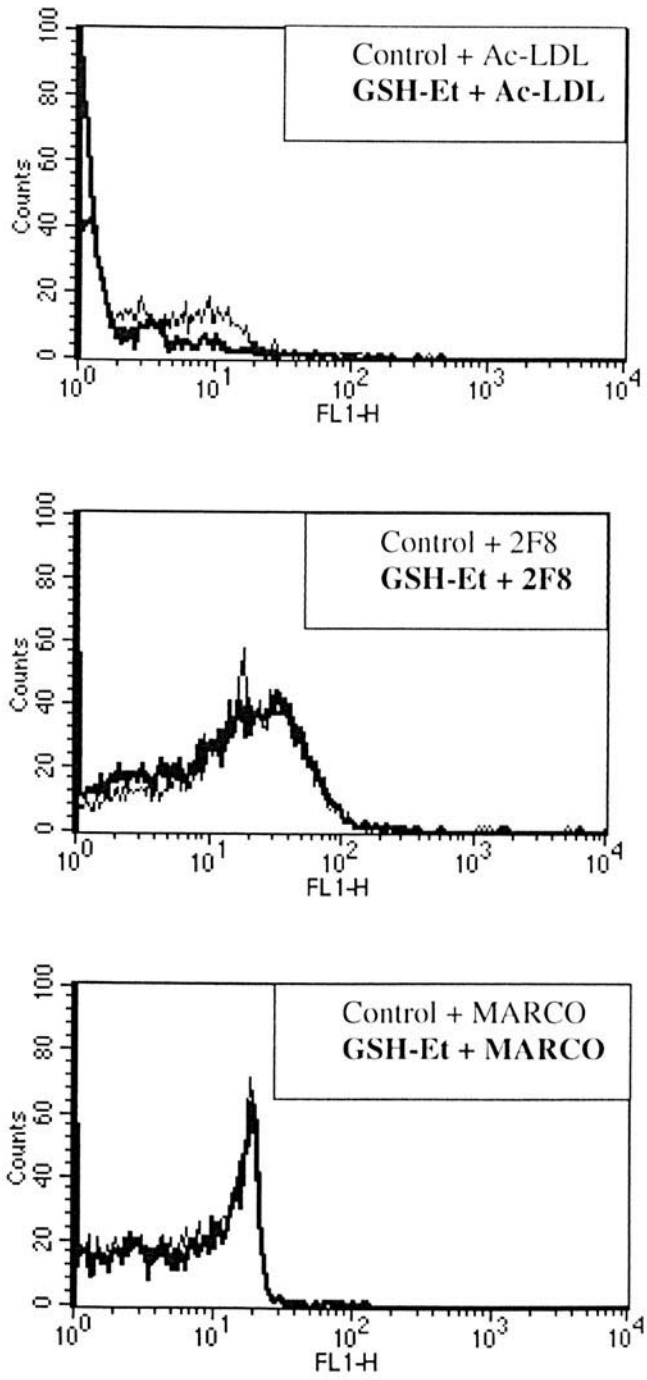

Figure 6. Immunofluorescence analysis and acetylated low-density lipoprotein (Ac-LDL) binding: Cells were cultured in preconditioning or control media overnight, lightly formalin fixed (20 minutes, 0.1%) to inhibit receptor mobility, treated with anti-MARCO, 2F8, or control immunoglobulin. Following incubation with secondary fluorescently labeled Ig, cells were fixed with 1% formalin and 10,000 cells were analyzed per group. Fluorescence intensity is graphed as a function of cell number. In order to assess Ac-LDL binding, Bodipy–Ac-LDL was added instead of fluorescently labeled secondary antibody. A representative experiment, repeated three times, is presented.

PKC-specific pathway. Specifically, our data indicates that GSH-Et regulates Ac-LDL–induced apoptosis without altering scavenger receptor number, but instead through the activation *via* a PKC pathway intermediate, which

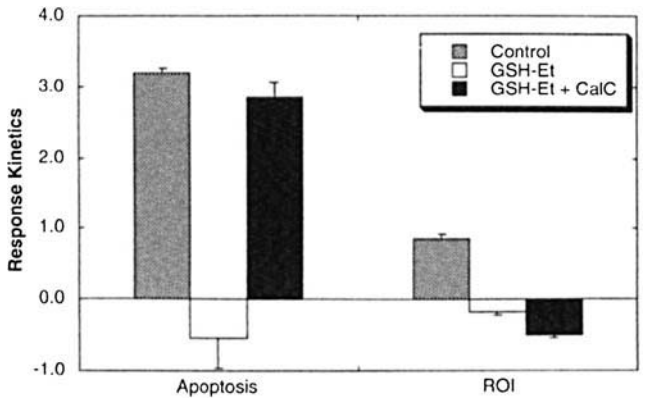

Figure 7. The rates of change in levels of intracellular reactive oxygen intermediates (ROI) and the apoptotic cell conversion between 3 and 5 hours were calculated as $\Delta F_{3-5\ hours}/(\Delta t)(F_{3\ hour})$, using data from Figure 4B (reactive oxygen intermediate level) and 4C (apoptosis), where F is the relative extent of either ROI generation or apoptosis.

notably does not correlate with the kinetics of ROI generation.The lipoprotein trafficking role for an antioxidant is particularly significant because the intracellular accumulation of oxidized LDL cannot typically cause the downregulation of the expression of ox-LDL/Ac-LDL receptors. In fact, ox-LDL, the natural scavenger receptor ligand, can actually increase scavenger receptor expression (31), leading to damage escalation and ultimately increased cell death. The GSH-Et downregulation of Ac-LDL binding and incorporation described in our experimental studies may involve quantitative changes in receptor number, conformational changes, or receptor redistribution on the cell membrane (32), all of which may affect ligand-receptor interaction. Our cytofluorometric analyses of anti-MARCO and 2F8-labeled cells indicate that GSH-Et does not likely downregulate the major scavenger receptors on IC21 macrophages known for Ac-LDL. Instead, our studies suggest that Ac-LDL receptor-mediated regulation may proceed not *via* direct ligand control but rather through secondary messengers, specifically, utilizing targets regulated by PKC.

The interaction between Ac-LDL–binding/internalization pathways and cellular kinases has been previously described. Specifically, lipid peroxidation was found to activate tyrosine kinases, phospholipases, PKC, and DNA fragmentation, and several of these studies examined the regulatory role of GSH (33–35). In addition, lipoprotein binding to scavenger receptors or scavenger receptor upregulation induced protein tyrosine phosphorylation and increased PKC activity (36, 37). In accordance with the known functional multiplicity of PKC-mediated pathways (38), including cell differentiation, apoptosis, adhesion, and migration, our results also suggest that PKC is regulated by GSH-Et preconditioning and may secondarily control Ac-LDL binding to macrophages.

Several investigators have described nonredox regulatory GSH functions, including thiol reduction, stabilization of protein conformation, and metabolic pathway regulation (18, 19, 23, 39). In our studies, we have demonstrated that GSH-Et preconditioning activates PKC phosphorylation, an important step in achieving a high level of PKC activity (40–42). Others have shown that changes in the intracellular redox state can both upregulate and downregulate PKC activity in a cell-specific manner (43–45). It is unclear whether the PKC phosphorylation that we have observed is due to the nonredox GSH function or to the alteration of the intracellular redox state by increased intracellular GSH concentration. However, Ward *et al.* described a nonredox GSH-mediated reduction of PKCα activity in N-acetylcysteine-treated cells (44) and our kinetic studies support a GSH effect occurring independently of ROI accumulation. This observation is particularly important because reduced PKC activity has been shown to concomitantly decrease nicotinamide adenine dinucleotide phosphate (NADPH) complex formation and hence ROI formation, yet PKC inhibition by Cal C did not diminish ROI generation in our system. However, NADPH complex independent mechanisms, including electron transfer, have also been described as having pathways whereby NADPH may be activated with non-PKC enzymatic mediators (46–48). In addition, increases in ROI generation following scavenger receptor activation have been documented (49). Potential targets for molecular interactions with GSH-Et include messengers in the cascade leading to PKC phosphorylation comprised of phosphatidylinositol (PtdIns) 3-kinase, PtdIns (3,4,5) trisphosphate, and phosphoinositide-dependent kinase (PDK), as well as PKC autophosphorylation itself (41, 50–53). Further studies are necessary to elucidate the role of these messengers as well as phosphatases that can attenuate the PKC phosphorylation cascade (42, 54) in the control of PKC phosphorylation by GSH-Et.

While the mechanism of GSH/PKC regulation of Ac-LDL binding and uptake in our system is presently unknown, several studies have linked PKC activation with increased membrane activity, including cell secretion, adhesion, and receptor mobility, which may occur concomitantly with cytoskeletal rearrangement (43, 45, 55, 56). Regardless of the mechanism, it is likely that scavenger receptor membrane redistribution contributes to decreased Ac-LDL binding in our studies because our preliminary data indicate the presence of cytoskeletal clustering following GSH-Et preconditioning. In an effort to explore the intracellular responses to GSH-Et preconditioning, future studies will include quantitative assessment of phosphorylated PKC enzymatic activity as well as the targets of phosphorylated PKC enzymes in GSH-Et–preconditioned cells. Such studies will serve to determine whether the molecular activation that we have observed *via* biochemical characterization, can be correlated to functional PKC activation. Further, these studies would determine the extent of GSH-Et–induced shifting between multiple PKC effector pathways, leading to a more detailed mechanistic under-

standing of changes in apoptosis and Ac-LDL binding, as well as the lack of change in total PKC expression, resulting from PKC activation. It is important to note that, although GSH-Et and PKC pathways can interact to ultimately regulate protein expression *via de novo* protein synthesis, the short enzyme inhibition protocol used in our studies precludes transcriptional regulation of Ac-LDL binding in our experiments.

In summary, we have demonstrated that a preconditioning regimen, using the antioxidant GSH-Et, can reduce the accumulation of ROIs and delay apoptotic induction engendered by exposure to intracellularly oxidizable lipoprotein, Ac-LDL. These phenomena may be mediated not only by the GSH free radical scavenger role but also by regulation of Ac-LDL binding and uptake into macrophages *via* a PKC-dependent pathway. Further study is necessary to identify additional regulatory intermediates of GSH-Et–mediated chemical macrophage preconditioning. However, regardless of the mechanism, the results of our studies may be used as a basis to identify multifocal molecular therapies against atherogenesis precipitated through the interaction of scavenger receptors on adherent macrophage with ox-LDL or ox-LDL analog ligands.

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