Advanced Glycation End Product Precursors Impair ABCA1-Dependent Cholesterol Removal From Cells

Marisa Passarelli,[1] Chongren Tang,[1] Thomas O. McDonald,[1] Kevin D. O'Brien,[1] Ross G. Gerrity,[2] Jay W. Heinecke,[1] and John F. Oram[1]

Abnormal HDL metabolism may contribute to the increased atherosclerosis associated with diabetes. The ATP-binding cassette transporter A1 (ABCA1) is an atheroprotective cell protein that mediates cholesterol transport from cells to apolipoprotein (apo) A-I, the major protein in HDL. Because formation of advanced glycation end products (AGEs) is associated with diabetic vascular complications, we examined the effects of carbonyls implicated in AGE formation on the ABCA1 pathway in cultured fibroblasts and macrophages. Treating cells with glycolaldehyde (GA) and glyoxal (GO) strongly inhibited ABCA1-dependent transport of cholesterol from cells to apoA-I, while methylglyoxal had little effect. This occurred under conditions where other lipoprotein receptors or lipid metabolic pathways were little affected, indicating that ABCA1 was uniquely sensitive to these carbonyls. GA and GO destabilized ABCA1 and nearly abolished its binding of apoA-I, indicating that these carbonyls directly modified ABCA1. Immunohistology of coronary arteries from hyperlipidemic swine revealed that inducing diabetes with streptozotocin increased atherosclerotic lesion area and dramatically reduced the fraction of macrophages that expressed detectable ABCA1. These results raise the possibility that reactive carbonyl-mediated damage to ABCA1 promotes accumulation of cholesterol in arterial macrophages and thus contribute to the increased cardiovascular disease associated with diabetes, insulin resistance, and other inflammatory conditions. *Diabetes* 54:2198–2205, 2005

Atherosclerotic vascular disease is a major complication of diabetes. Certain risk factors for atherosclerosis, such as hyperlipoproteinemia, hypertension, central obesity, and hyperinsulinemia, are frequently present in diabetic patients (1,2). A metabolic hallmark of type 2 diabetes is a low level of HDL (2–4). The strong inverse relationship between HDL levels and cardiovascular risk (5) raises the possibility that altered HDL metabolism contributes to the increased risk of atherosclerosis in diabetes.

One of the atheroprotective factors associated with HDL metabolism is ATP-binding cassette transporter A1 (ABCA1), an integral membrane protein that mediates the transport of cholesterol from cells to lipid-poor apolipoprotein (apo) A-I, the major HDL protein (6–8). This process acts to clear excess cholesterol from cells and initiate HDL particle formation (6). Genetic manipulations of ABCA1 expression in mice affect plasma HDL levels and atherogenesis (9,10). Moreover, human *ABCA1* mutations cause Tangier disease, a severe HDL deficiency syndrome characterized by cholesterol deposition in tissue macrophages and prevalent atherosclerosis (8,11,12).

ABCA1 expression is tightly regulated at both the transcriptional and posttranscriptional level. Cholesterol loading of macrophages dramatically increases ABCA1 mRNA and protein levels (7), consistent with a cholesterol export function. This regulation is mediated by nuclear liver X and retinoid X receptors, which form heterodimers that are activated by oxysterols and retinoic acid, respectively (7). Analogs of cAMP also activate *ABCA1* transcription in cultured murine macrophages by mechanisms distinct from the nuclear receptor system (13).

Animal studies have revealed that there is a major discordance between ABCA1 mRNA and protein levels in tissues (14), implicating posttranscriptional regulation of ABCA1 expression as playing a critical role in the overall activity of this pathway. ABCA1 protein in cultured cells is destabilized by free fatty acids (15,16) and high levels of intracellular free cholesterol (17), indicating that different metabolites can have inhibitory effects on ABCA1 expression. It is therefore possible that impaired ABCA1 activity could contribute to the low HDL levels and increased atherosclerosis that accompany type 2 diabetes and the metabolic syndrome.

Advanced glycation end products (AGEs) have also been implicated in the pathogenesis of diabetic vascular disease (18,19). Carbonyls are an important class of agents that form AGEs, and they can be generated in the body by a variety of enzymatic and nonenzymatic mechanisms (20). One important pathway involves glucose, an α-hydroxy carbonyl (18–21). Glycolaldehyde (GA) may be an intermediate in the reactions that convert glucose into AGEs (22). Oxidation of amino acids and lipids may also contribute to GA formation and AGE production during inflammation (23–25). Glycolysis produces the highly re-

From the [1]Department of Medicine, University of Washington, Seattle, Washington; and the [2]Department of Pathology, Medical College of Georgia, Augusta, Georgia.

Address correspondence and reprint requests to John F. Oram, Department of Medicine, Box 356426, University of Washington, Seattle, WA 98195-6426. E-mail: joram@u.washington.edu.

Received for publication 19 November 2004 and accepted in revised form 14 April 2005.

ABCA1, ATP-binding cassette transporter A1; AGE, advanced glycation end product; apo, apolipoprotein; DMEM, Dulbecco's modified Eagle's medium; DSS, 2,2-dimethyl-2-silapentanesulfonic acid; GA, glycolaldehyde; GO, glyoxal; MGO, methylglyoxal.

active dicarbonyl compounds glyoxal (GO) and methylglyoxal (MGO) (18,26). Tissue and plasma levels of GO and MGO and their protein adducts are elevated in diabetes (18,19,26,27). Thus, reactive carbonyls derived from metabolic and oxidative pathways may contribute to AGE formation in diabetes.

AGEs have been detected on lipoproteins isolated from diabetic subjects (28,29), raising the possibility that carbonyl modifications of apoA-I contribute to abnormal HDL metabolism. However, little is known about the impact of reactive carbonyls on cholesterol removal from cells. We therefore examined the effects of carbonyls implicated in AGE formation on the ABCA1 pathway in cultured human skin fibroblasts and murine macrophages. We found that GO or GA, but not MGO, markedly impaired ABCA1 cholesterol transport activity and destabilized ABCA1 protein. In contrast, plasma membrane receptors for other lipoproteins were little affected. Thus, ABCA1 is sensitive to damage by specific carbonyl species. These findings raise the possibility that carbonyl stress–impaired ABCA1 activity may contribute to macrophage foam cell formation and increased cardiovascular disease in diabetes and other inflammatory states.

RESEARCH DESIGN AND METHODS

LDL, HDL, and apoA-I. LDL and HDL were isolated by sequential density ultracentrifugation from fresh plasma obtained from a pool of plasma from healthy donors, and apoE was removed from HDL by heparin-Sepharose column chromatography (30). ApoA-I was purified from HDL, LDL was acetylated, and apos were labeled with ^{125}I, as previously described (30,31).

Cell culture, cholesterol efflux, and carbonyl treatments. Human skin fibroblasts and murine J774 macrophages were grown in Dulbecco's modified Eagle's medium (DMEM) (Bio-Whittaker, Waskersville, MD) supplemented with 10% fetal bovine serum. ABCA1 was induced in fibroblasts by 48-h incubations with DMEM containing 1 mg/ml BSA plus 30 μg/ml cholesterol followed by 24-h incubations with the same medium lacking cholesterol (31) and in macrophages by 24-h incubations with DMEM/BSA containing 50 μg/ml acetylated LDL followed by 24-h incubations with DMEM/BSA containing 0.5 mmol/l 8-Br-cAMP (15).

To radiolabel cellular cholesterol, 1 μCi/ml [^{3}H]cholesterol (NEN Life Science Products) was added to the growth medium 72 h before the cholesterol-loading incubations (fibroblasts) or to the acetylated LDL medium (macrophages) (15,31). To measure cholesterol efflux, cells were incubated with DMEM/BSA with or without 10 μg/ml apoA-I for 6 h (fibroblasts) or 2 h (macrophages) at 37°C and chilled on ice, and the medium was collected and centrifuged to remove detached cells. The medium was counted for ^{3}H, and the cells were assayed for free and esterified [^{3}H]cholesterol after isolation by thin-layer chromatography (31). ApoA-I–mediated cholesterol efflux is expressed as the fraction of [^{3}H]cholesterol released into the medium after subtraction of values obtained in the absence of apoA-I. Free and esterified cholesterol mass and phospholipid mass were measured as described previously (30,32) and normalized to the cell protein content of the wells. For the treatments, carbonyls (Sigma) were added during the 24-h incubations (fibroblasts) or for 6 h (macrophages) immediately preceding the efflux incubations. For the fibroblast time courses, the times exposed to carbonyl and carbonyl-free medium were adjusted for a total time of 24 h.

Lipoprotein receptors and fatty acid esterification. To induce LDL receptors in fibroblasts, cells were incubated in DMEM containing 10% lipoprotein-deficient serum for 48 h (33). Presumably, because they are a transformed line, J774 macrophages have constitutively active LDL receptors (data not shown) and thus were maintained in normal growth medium. To measure lipoprotein receptor activities, fibroblasts and macrophages were incubated for 6 h with 10 μg ^{125}I-LDL (LDL receptor) or macrophages with 10 μg ^{125}I-acetylated LDL (scavenger receptors) after the carbonyl treatment incubations, chilled cells were washed four times with PBS, and cell-associated radiolabel and cell protein were measured after digestion in 0.1 N NaOH (33). Results were calculated as nanograms lipoprotein protein per milligram cell protein.

To measure the incorporation of oleate into lipids, cells were washed and incubated with ^{14}C-oleic acid bound to BSA for 2 h after the treatments (32). Washed cells were extracted in hexane:isopropanol (3:2, vol:vol). After solvent evaporation and addition of lipid standards, samples were extracted in chloroform/methanol, and esterified cholesterol, triglycerides, and phospholipids were isolated by thin-layer chromatography. Bands were scraped into vials and the radioactivity counted. Results were calculated as cpm/mg cell protein.

ApoA-I binding to cells and to ABCA1. For the cell-binding assay, cells were incubated for 2 h at 37°C with 1 μg/ml ^{125}I–apoA-I ± 200 μg/ml unlabeled apoA-I, and cell-associated radioactivity and cell protein were measured in 0.2 N NaOH digests of washed cells (13). Results are expressed as nanograms apoA-I per milligram of cell protein after subtraction of values in the presence of unlabeled apoA-I. For the ABCA1 binding assay, cells were incubated with 5 μg/ml ^{125}I–apoA-I for 2 h, treated for 30 min at room temperature with PBS containing 1 mg/ml 2,2-dimethyl-2-silapentanesulfonic acid (DSS) (cross-linking agent), and washed twice with cold PBS containing 20 mmol/l glycine (13,15). ABCA1 was isolated from detergent extracts by immunoprecipitation and SDS-PAGE, and ^{125}I-apoA-I cross-linked to ABCA1 was visualized by phosphorimaging.

ABCA1 protein levels and translation. To measure ABCA1 protein levels, microsomal membranes were isolated from homogenized cells by ultracentrifugation, membrane proteins were solubilized in SDS buffer and resolved by SDS-PAGE, and ABCA1 was detected by immunoblot analysis (15,16). Equal amounts of membrane protein were added per gel lane. For the translation efficiency assay, macrophages were metabolically labeled by incubation for 15 min at 37°C with DMEM/BSA containing 100 μCi/ml [^{35}S] methionine (Amersham Pharmacia Biotech), and ABCA1 was isolated from 1% Triton X-100 cell digests by immunoprecipitation and SDS-PAGE, as described (15,16). Each gel lane received immunoprecipitated protein corresponding to equal amounts of cells. [^{35}S]methionine-labeled ABCA1 was detected on gels by PhosporImaging (Cyclone; Packard Instrument) and quantitated using an OptiQuant computer program. Similar results were obtained using two peptide-specific antibodies corresponding to the human C-termini (13) and a segment of the first cytosolic loop (Novus Biological).

Quantitation of ABCA1 mRNA. The one-step real-time RT-PCR was achieved by using the Mx4000 Muliplex Quantitative PCR System (Stratagene, La Jolla, CA) and the Brilliant Single-step Quantitative RT-PCR Core Reagent Kit (Stratagene), according to their instructions. The amplification of ABCA1 and glyceraldehyde-3-phosphate dehydrogenase RNA were run using 100 ng of total RNA retrieved from samples using Stratagene Absolutely RNA miniprep kits (Stratagene). Reactions were prepared in a 96-well optical grade PCR plate in a total of 50 μl containing the following components: 100 ng of total RNA, 25 μl of 2 × Master Mix, 2 units of the reverse transcriptase SuperScript II, 250 nmol/l of probe, and 600 nmol/l of each primer. Thermal cycling conditions consisted of an initial reverse transcription step at 45°C for 30 mintues, followed by 10 min at 95°C to stop reverse transcription and activate SureStart *Taq*, then followed by 40 cycles of amplification. Each cycle of amplification consisted of a denaturation step at 95°C for 30 s and an annealing/extension step at 60°C for 1 min. The primers and probe for ABCA1 used for the RT-PCR are forward: 5′-GGACATGCACAAGGTCCTGA-3′; reverse: 5′-CAGAAAATCCTGGAGCTTCAAA-3′; and probe: 5′-FAM-AATGTT ACGGCAGATCAAGCATCCCAAC-BHQ1–3′. The primers and probe for glyceraldehyde-3-phosphate dehydrogenase used for the RT-PCR are forward: 5′-AGCCTCGTCCCGTAGACAAA-3′; reverse: 5′-ACCAGGCGCCCAATACG-3′; and probe: 5′hEX-AAATCCGTTCACACCGACCTTCACCA-BHQ1–3′.

Diabetic swine. Coronary arteries were obtained as previously described (34) from two groups of male Yorkshire swine (16 nondiabetic and 15 coronary arteries) made hyperlipidemic through feeding a high-fat, high-cholesterol diet containing 15% lard and 1.5% cholesterol. Diabetic animals were generated by daily injections of streptozotocin at 50 mg · kg^{-1} · dose^{-1} for 3 days. Proximal coronary arteries were fixed in formalin or methacarn (60% methanol/30% chloroform/10% glacial acetic acid) and embedded in paraffin wax.

Histochemistry and immunohistochemistry. Serial 6-μm-thick tissue sections were mounted on glass slides, deparaffinized, and stained with the Movat's pentachrome stain for morphology (35). Single-label immunohistochemistry was performed using well-established techniques. Briefly, 6-μm-thick tissue sections were mounted on glass slides, deparaffinized with xylene, and then rehydrated in graded alcohols. The sections were treated with 3% H_2O_2 (Sigma) to block endogenous peroxidases. Sections then were washed with PBS and then incubated with the primary antibody or antiserum for 1 h at room temperature. A biotin-labeled anti-mouse or anti-rabbit secondary antibody was applied for 30 min, followed by an avidin-biotin-peroxidase conjugate (ABC Elite; Vector) for 30 min. The peroxidase substrate, Nova Red (Vector), was then added to yield a red-brown reaction product. Slides were counterstained with hematoxylin and a coverslip applied.

Primary antibodies used were a macrophage-specific monoclonal antibody (ATCC HB 142.1; American Type Culture Collection, Rockville, MD; titer =

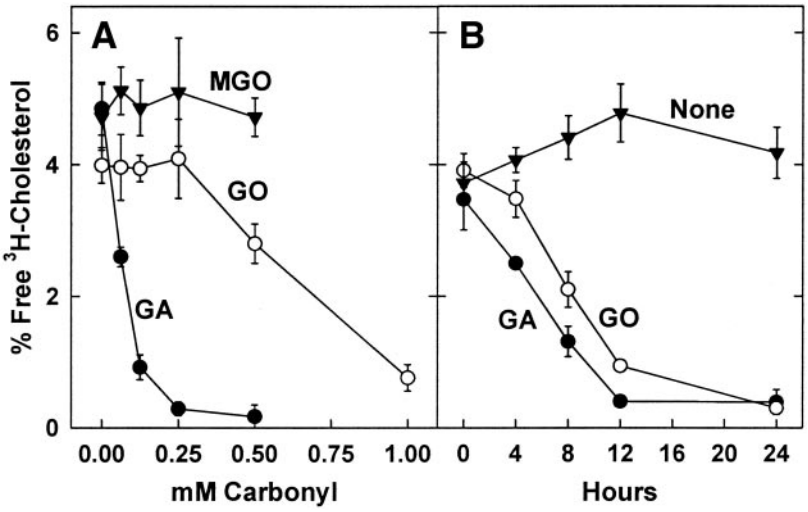

FIG. 1. GA and GO but not MGO inhibit ABCA1-dependent cholesterol efflux from fibroblasts. Fibroblasts were radiolabeled with [^{3}H]cholesterol, loaded with cholesterol to induce ABCA1, and treated for 24 h with the indicated concentrations of carbonyls (*A*) or for the indicated times with no carbonyls (None), 0.5 mmol/l GA, or 1.0 mmol/l GO (*B*). Washed cells were then incubated for 6 h in the absence of carbonyls without or with 10 μg/ml apoA-I, and efflux of [^{3}H]cholesterol into the medium was measured as described in RESEARCH DESIGN AND METHODS. Results are means ± SD of three incubations and are representative of those observed in three independent experiments.

neat) (36) and rabbit polyclonal antiserum raised against a human ABCA1 peptide sequence in the second extracellular loop (CV Therapeutics; titer = 1:1,000). Immunohistologic reactivity of the ABCA1 antibody was verified using cultured mono-layers of nontransfected and ABCA1-transfected baby hamster kidney cells (37).

Morphometric analyses. Digital images were captured using a Nikon CoolPix 990 camera mounted on an Olympus microscope and then transferred to a 2-GHz Pentium IV computer. Using the ImagePro Plus (Version 4, MediaCybernetics, Silver Spring, MD) morphometric analysis program, total intimal area was determined from Movat's–stained sections. From macrophage-stained sections, macrophage foam cells were identified as those cells with lipid inclusions. Percent foam-cell macrophages negative for ABCA1 was determined for each coronary artery segment by manual counting, using side-by-side comparison of high-power photomicrographs of the macrophage- and ABCA1-immunostained sections.

Statistical analyses. Statistical analyses were performed using GraphPad Prism 3.0 software (GraphPad Prism, San Diego, CA). Student's *t* test was used to compare groups. Results are reported as means ± SD (cell culture) or ± SE (tissue). A P value <0.05 was considered significant.

RESULTS

Reactive carbonyls severely impair ABCA1 activity in cultured fibroblasts and macrophages. We examined the activity of the ABCA1 cholesterol efflux pathway in cultured cells exposed to the reactive carbonyls GO, MGO, and GA. After inducing ABCA1 in human skin fibroblasts by loading them with cholesterol, we incubated cells for 20 h with increasing concentrations of GO, MGO, or GA and then measured apoA-I–mediated cholesterol efflux during subsequent 6-h incubations in the absence of carbonyls. Under these conditions, cholesterol efflux to lipid-free apoA-I depends exclusively on ABCA1 activity (6).

Both GA and GO markedly inhibited apoA-I–mediated cholesterol efflux in a concentration-dependent manner (Fig. 1*A*). Similar results were found for apoA-I–mediated phospholipid efflux (not shown). GA was consistently more efficacious than GO, having a maximum effect at ~0.25 mmol/l compared with 1.0 mmol/l for GO. At concentrations up to 0.5 mmol/l, MGO had no significant effect on ABCA1 activity. Time course studies revealed that both GA and GO significantly inhibited cholesterol efflux after only 4 h of exposure, with near maximal effects occurring at 12 h (Fig. 1*B*). The inhibitory effects of GA and GO were reversed by including the carbonyl scavenger aminoguanadine (5 mmol/l) in the medium (not shown), implicating protein modification as a potential mechanism. These observations demonstrate that GA and GO, but not MGO, severely impair ABCA1-dependent cholesterol transport in cultured fibroblasts.

We next examined the effects of carbonyls on ABCA1 activity in cholesterol-loaded murine J774 macrophages, a model for foam-cell macrophages in atherosclerotic lesions. Treating these cells with a cAMP analog robustly induces ABCA1 transcription and cholesterol efflux to lipid-poor apoA-I (15). Incubating ABCA1-induced macrophages with increasing concentrations of GA and GO for only 6 h almost completely suppressed apoA-I–mediated cholesterol efflux (Fig. 2*A*). As with fibroblasts, GA was more efficacious than GO, MGO had only a small effect (Fig. 2*A*), and GA and GO had no effect in the presence of aminoguanidine (not shown). Treating macrophages for 18 h with 8-Br-cAMP caused a concentration-dependent increase in apoA-I–mediated cholesterol efflux, which was almost completely reversed by subsequent 6-h incubations with GA or GO (Fig. 2*B*). In contrast, treating cells for 6 h with GA and GO had little effect on apoA-I–mediated cholesterol efflux when ABCA1 was subsequently induced for 18 h with 8-Br-cAMP (Fig. 2*B*, *insert*), indicating that expression of ABCA1 before carbonyl exposure was required for their inhibitory effects. These results show that the ABCA1 activity in these macrophages is highly sensitive to exposure to GA or GO.

ABCA1 is uniquely sensitive to reactive carbonyls. We characterized the relative specificity of the carbonyl effects by measuring the activities of a wide range of other pathways for lipoprotein and lipid metabolism by cells. These included receptor-mediated uptake of ^{125}I-labeled LDL and acetylated LDL; cellular levels of free cholesterol, esterified cholesterol, and phospholipid mass; and [^{14}C] oleate incorporation into cholesteryl esters, triglycerides, and phospholipids. These processes were unaffected or modestly altered by incubating fibroblasts for 24 h (Fig. 3*A*) or macrophages for 6 h (Fig. 3*B*) with concentrations of GA or GO (0.25 mmol/l or 0.5 mmol/l) that markedly affected ABCA1 activity (compare Fig. 3 with Figs. 1 and 2). Thus, the ABCA1 pathway was much more sensitive to carbonyl exposure than were other pathways for lipoprotein uptake and lipid metabolism.

We also found that treating fibroblasts for 24 h and

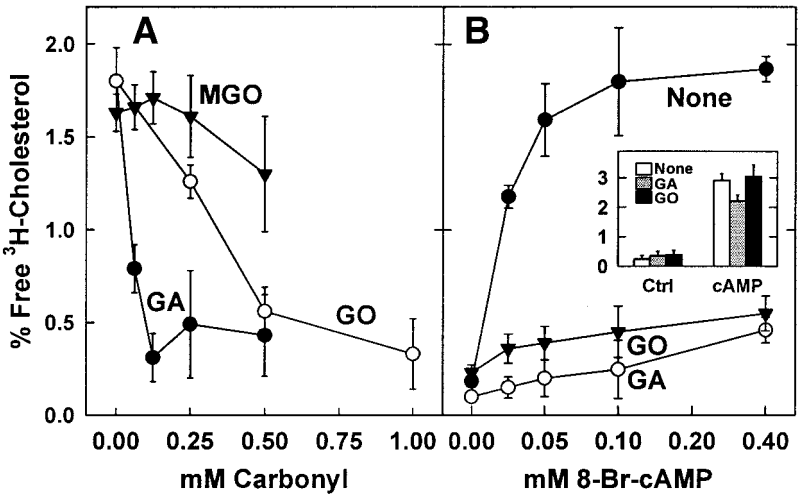

FIG. 2. GA and GO inhibit ABCA1-mediated cholesterol efflux from macrophages. J774 macrophages were radiolabeled with [^{3}H]cholesterol, loaded with acetylated LDL-derived cholesterol, and treated for 20 h with 0.5 mmol/l (*A*) or the indicated concentration (*B*) of 8-Br-cAMP. Cells were then incubated for 6 h with the indicated concentrations of carbonyls (*A*) or with no carbonyls (None), 0.25 mmol/l GA, or 0.5 mmol/l GO (*B*). *B* (*insert*): Cells were treated with no carbonyl (None), 0.25 mmol/l GA, or 0.5 mmol/l GO for 6 h before the 20-h incubations without (Ctrl) or with (cAMP) 0.5 mmol/l 8-Br-cAMP. Washed cells were then incubated for 2 h in the absence of carbonyls without or with 10 μg/ml apoA-I, and efflux of [^{3}H]cholesterol into the medium was measured as described in RESEARCH DESIGN AND METHODS. Results are means ± SD of three incubations and are representative of those observed in four independent experiments.

macrophages for 6 h with GA or GO had no effect on total cellular protein or on the pattern of membrane proteins resolved by gradient SDS-PAGE and visualized with silver staining (not shown). These observations, in concert with the studies of lipid metabolism, provide strong evidence that carbonyls were not cytotoxic or exerting global effects on the overall composition of proteins in cell membranes.

Carbonyls reduce apoA-I binding to ABCA1-expressing cells and to ABCA1. Previous studies (13,38,39) have shown that cAMP treatment of J774 macrophages markedly increases ^{125}I–apoA-I binding to cells and covalent cross-linking of ^{125}I–apoA-I to ABCA1. The cAMP-induced increase in cell-surface binding of ^{125}I–apoA-I was reduced by >60% when cells were preincubated for 6 h with GA or GO (Fig. 4), indicating that these carbonyls selectively inhibited apoA-I binding to ABCA1-expressing cells. When cAMP-induced cells were treated with the cross-linking agent DSS after binding ^{125}I–apoA-I, preincubations with either GA or GO dramatically reduced the amount of ^{125}I–apoA-I cross-linked to detergent-solubilized, immunoprecipitated ABCA1 (SDS-PAGE image; Fig. 4*B*, *insert*). Thus, these carbonyls either severely impaired the apo binding activity of ABCA1 or modified key lysine residues in ABCA1 that are cross-linked to apoA-I.

Carbonyls destabilize ABCA1 protein. Immunoblot analysis revealed that GA and GO greatly reduced the ABCA1 protein content of both fibroblast and macrophage membranes (Fig. 5*A*). GA or GO had no significant effect on ABCA1 mRNA levels (Fig. 5*B*) or the initial rate of

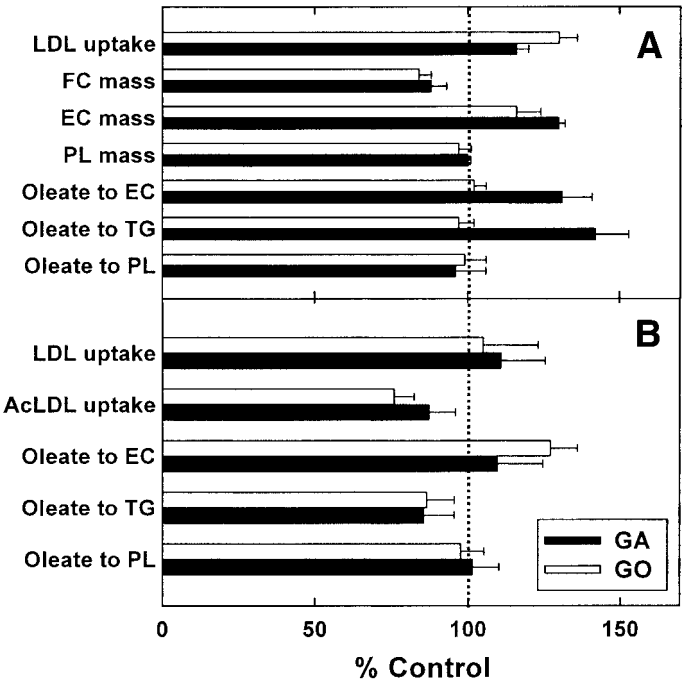

FIG. 3. GA and GO have modest effects on other parameters of cellular lipoprotein and lipid metabolism. Fibrobasts (*A*) or J774 macrophages (*B*) were treated for 24 or 6 h, respectively, with either no carbonyls (Control) or with 0.25 mmol/l GA or 0.5 mmol/l GO. ^{125}I-LDL and acetylated ^{125}I-LDL (AcLDL) uptake; free cholesterol (FC), esterified cholesterol (EC), and phospholipid (PL) mass; and [^{14}C]oleate incorporation into esterified cholesterol, triglycerides (TG), and phospholipids were measured as described in RESEARCH DESIGN AND METHODS. Results were normalized to cell protein and then normalized to the mean values for control cells (100%). Each value is the means ± SD of three to nine incubations combined from three independent experiments.

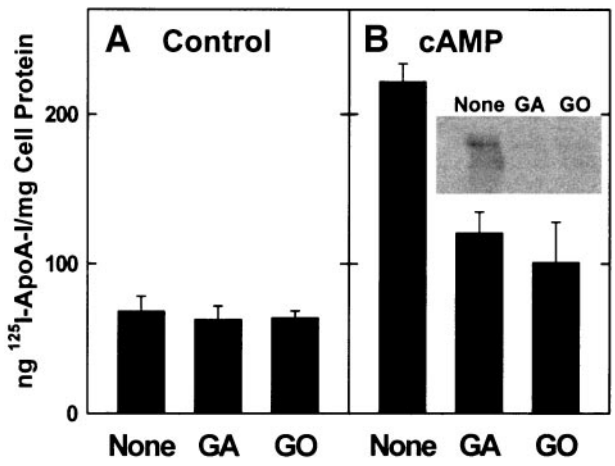

FIG. 4. GA and GO inhibit apoA-I binding to ABCA1-expressing macrophages and to ABCA1. Cholesterol-loaded macrophages were incubated for 20 h without (*A*) or with (*B*) 8-Br-cAMP and then incubated for 6 h without or with 0.25 mmol/l GA or 0.5 mmol/l GO, and cellular ^{125}I–apoA-I binding was assayed after subsequent 2-h incubations. Each value is the means ± SD of quadruplicates. *B* (*insert*): 8-Br-cAMP–induced macrophages were treated for 6 h without or with carbonyls, incubated for 2 h with 10 μg/ml ^{125}I–apoA-I, and treated with the covalent cross-linker DSS. ABCA1 was isolated by immunoprecipitation and SDS-PAGE, and ^{125}I–apoA-I–labeled ABCA1 was detected by PhosphorImaging. Data are representative of at least three experiments.

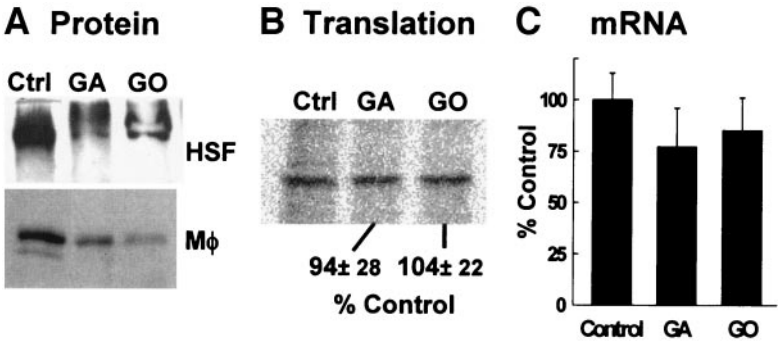

FIG. 5. GA and GO destabilize ABCA1 protein. Cholesterol-loaded and 8-Br-cAMP–treated macrophages (*A* [Mϕ] and *B* and *C*) were incubated for 6 h or cholesterol-loaded fibroblasts (*A* [HSF]) were incubated for 24 h without (Control) or with 0.25 mmol/l GA or 0.5 mmol/l GO. *A*: Equal amounts of cell membrane were subjected to SDS-PAGE, and ABCA1 was identified by immunoblot analysis. *B*: Macrophage proteins were radiolabeled with [^{35}S]methionine (100 μCi/ml) for 15 min, ABCA1 was isolated by immunoprecipitation and SDS-PAGE, and ^{35}S-labeled ABCA1 was detected by PhosphorImaging, and band intensities were quantitated by computer. Numbers represent means ± SD of values from three experiments expressed as percent controls. *C*: ABCA1 mRNA from macrophages was quantitated by real-time RT-PCR (means ± SD, $n = 3$). Each panel is representative of at least three separate experiments.

incorporation of [^{35}S]methionine into cell-associated ABCA1 protein detected by immunoprecipitation and SDS-PAGE (Fig. 5*C*). Thus, the reduced ABCA1 levels in carbonyl-treated cells could not be attributed to inhibition of ABCA1 transcription or translation. These results imply that GA and GO reduce ABCA1 levels in macrophages by selectively destabilizing the protein.

ABCA1 is impaired in macrophage foam cells in atherosclerotic lesions of diabetic swine. We used an animal model approach to investigate the possibility that impaired ABCA1 contributes to the increased atherosclerosis associated with diabetes. For these studies, we compared intimal lesion sizes and macrophage ABCA1 levels in cross-sections of coronary arteries harvested from nondiabetic and streptozotocin-induced diabetic swine fed a high-fat, high-cholesterol diet. Previous studies (34) have shown that diet-induced hyperlipidemia in these animals promotes atherosclerosis that is further enhanced by diabetes. In our study, mean intimal lesion area increased fourfold when hyperlipidemic swine were made diabetic (Fig. 6*C*).

We examined the relative level of ABCA1 protein in macrophages localized in nondiabetic and diabetic atherosclerotic lesions by immunohistochemically staining cross-sections of intimal lesions with antibodies specific for ABCA1 and macrophages. ABCA1 was uniformly detected in nearly all foam-cell macrophages in lesions from the nondiabetic group (Fig. 6*A*, *red stain*). Large areas of

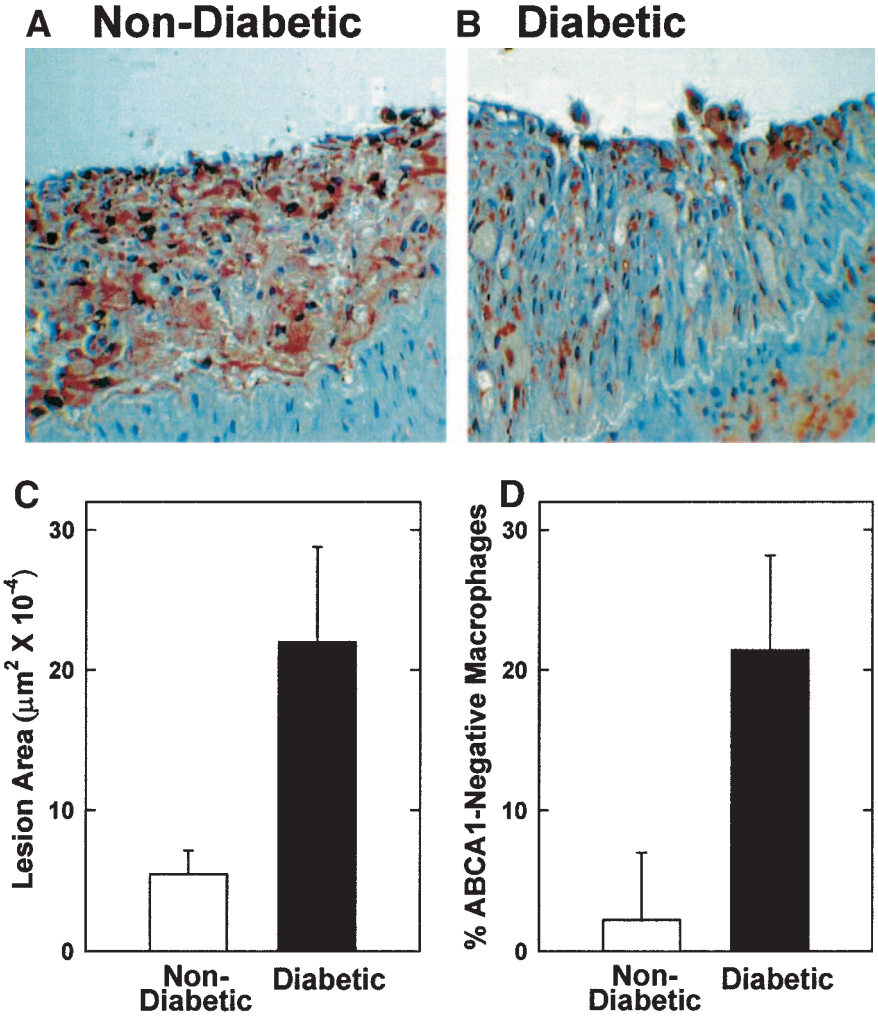

FIG. 6. Streptozotocin-induced diabetes in hyperlipidemic swine increases atherosclerotic lesion area and the fraction of ABCA1-negative macrophage foam cells. *A* and *B*: Atherosclerotic lesions in the proximal coronary arteries of diabetic and nondiabetic swine were analyzed for ABCA1 protein by immunohistological staining (red) *C*: Means (±SE) atherosclerotic lesion area of sections from 16 nondiabetic and 15 diabetic animals was measured by morphometric analysis ($P = 0.023$). *D*: The percent foam-cell macrophages that lacked immunodectable ABCA1 was quantified by manual counting of cells that stained positively for a macrophage-specific antibody but negatively for an ABCA1 antibody (means ± SD, $P = 0.0001$, eight animals per group).

lesions from the diabetic animals, however, contained no immunodectable ABCA1 (Fig. 6*B*). Quantification by cell counting revealed that the percentage of foam-cell macrophages that lacked immunodetectable ABCA1 was 10-fold greater in the diabetic lesions (2.2 ± 4.8 vs. 21.4 ± 6.8%, $P = 0.0001$). Thus, impaired expression of ABCA1 in foam-cell macrophages is associated with increased atherosclerotic lesion area in diabetic swine.

DISCUSSION

We found that the reactive carbonyls GA and GO severely impaired ABCA1-dependent cholesterol efflux from cultured human fibroblasts and murine macrophages. ABCA1-dependent transport of cholesterol and phospholipids was progressively and almost completely inhibited in cells exposed to increasing concentrations of GA or GO. This inhibition was reversed by aminoguanidine, implying that the carbonyl moieties of these compounds were responsible for the inhibitory effects (22,40). GA and GO appeared to damage and destabilize ABCA1 protein. Thus, the ABCA1 pathway is highly sensitive to carbonyl stress. The possible pathological relevance of these findings was supported by our studies with swine showing a markedly reduced ABCA1 content of lipid-laden macrophages in diabetic atherosclerotic lesions.

GA and GO maximally reduced ABCA1 lipid transport activity under conditions where the activities of other plasma membrane lipoprotein receptors and intracellular lipid metabolic pathways were unaffected or only modestly altered. Nor did we observe any alterations in the pattern of proteins detectable in membrane proteins separated by SDS-PAGE and visualized with silver staining. Moreover, treating cells with GA or GO before induction of ABCA1 had no significant effect on ABCA1 function. Thus, the carbonyls had no global effects on cell viability and metabolic activity. These results demonstrate that the ABCA1 pathway is uniquely responsive to the damaging effects of reactive carbonyls that are increased in the diabetic milieu.

Our observations suggest that derivatization of ABCA1 protein by reactive carbonyls is responsible for the impaired lipid transport activity. Exposure of macrophages to GA and GO greatly decreased apoA-I binding to ABCA1-expressing cells and nearly abolished chemical cross-linking of apoA-I to cell-surface ABCA1, consistent with a reduced apo binding activity of ABCA1. Cells incubated with GA or GO also had markedly reduced ABCA1 protein levels, but there was little effect on ABCA1 mRNA levels or translation efficiency. Thus, ABCA1 in carbonyl-treated cells appears to undergo an enhanced rate of proteolysis. Taken together, these results suggest that GA and GO directly damage ABCA1, severely impairing its activity and destabilizing the protein.

ABCA1 is an integral membrane protein that contains two large extracellular loops. Naturally occurring single amino acid substitutions in these loops can nearly abolish the lipid transport activity of ABCA1 (8). These carbonyls are also lipid soluble, allowing access to amino acids located within the plasma membrane. It is therefore possible that the formation of carbonyl adducts on one or more key amino acids in these protein regions could account for the loss of function induced by GA and GO. More extensive modifications of ABCA1 structure could destabilize the protein and promote its degradation. Previous studies have shown that ABCA1 is a highly unstable protein (13) and is rapidly degraded by a calpain protease (7). We found that the calpain inhibitor calpeptin did not prevent the carbonyl-induced reduction in ABCA1 (data not shown), indicating that carbonyls destabilize ABCA1 by a calpain-independent mechanism.

Inhibition of ABCA1 activity by GA, GO, and MGO occurred over different concentration ranges, suggesting that they had selective effects on this protein. Although they impaired ABCA1 activity to similar extents, GA was more efficacious than GO, having maximum effects at 0.125–0.25 mmol/l compared with 0.5–1.0 mmol/l for GO. In contrast, MGO was the least efficacious of the three carbonyls and only slightly inhibited ABCA1 activity at concentrations up to 0.5 mmol/l. It is likely that these differences reflect the unique chemistry of the compounds. GA reacts with lysine residues to form poorly characterized immine cross-links and carboxymethyllysine (22), one of the major AGEs detected in tissues of diabetic subjects (41). GO and MGO react with arginine residues to form imidazolium cross-links as well as adducts and cross-links with lysine residues (18,26). It is also possible that differences in membrane solubility and metabolism contribute to the selective effects of the carbonyls on ABCA1 activity in cells. It will be of interest to determine whether the carbonyls react with different sites on ABCA1 and to investigate the nature of these adducts.

A key question is whether the concentrations of reactive carbonyls used in our studies are physiologically relevant. The steady-state concentrations of glyoxal and methylglyoxal in plasma of humans are estimated to be ~100 nmol/l (42). However, the extent of accumulation of carbonyls will depend on both their rate of formation and their reactivity with cellular components. It is noteworthy that methylglyoxal formation from human erythrocytes was estimated to be ~100 nmol · ml packed cells^{-1} · day^{-1}, which represents a cumulative concentration of ~100 mmol/l (43). A similar concentration of glycolaldehyde had near maximal inhibitory effects on ABCA1 in cultured cells. These observations raise the possibility that our cell culture conditions may reflect the environment of tissues like atherosclerotic arteries, where oxidative processes are highly active and ABCA1 is expressed to high levels.

As a direct test of this possibility, we measured ABCA1 levels in atherosclerotic lesions isolated from a porcine animal model. When fed a high-fat and high-cholesterol diet, these swine became hypercholesterolemic and developed macrophage-rich atherosclerotic lesions. Inducing diabetes in these fat-fed animals by streptozotocin treatment had no further effect on cholesterol levels but increased plasma glucose and triglycerides (34). The diabetic animals had dramatically larger atherosclerotic lesions than those in the nondiabetic animals. Quantitative immunohistology showed that ABCA1 protein was detectable in nearly all of the foam-cell macrophages in the nondiabetic animals. When the animals were made diabetic, however, the fraction of macrophages having undetectable levels of ABCA1 increased from 2 to 20%. This is a conservative estimate of ABCA1 damage because it does not take into account cells with low but detectable ABCA1

levels or those with dysfunctional ABCA1. These findings provide the first in vivo evidence that an impairment of the ABCA1 cholesterol secretion pathway in arterial macrophages may play a role in the increased atherosclerotic cardiovascular disease that occurs in diabetes.

These studies have important pathological implications for aging, diabetes, and inflammatory diseases in general. Chronic exposure of ABCA1 to GA and GO is likely to be increased under hyperglycemic conditions associated with diabetes (18,19,22). Phagocytes can also generate GA from amino acids using myeloperoxidase (23,24), and lipid peroxidation produces GO and MGO (25). Many lines of evidence suggest that oxidative stress is enhanced in diabetes and other inflammatory states (18). Subjects with type 2 diabetes and insulin resistance also have higher than normal levels of plasma triglycerides and fatty acid (3,44,45). We have shown that free fatty acids impair the ABCA1 pathway by destabilizing ABCA1 protein (15,16), and others have shown that elevated fatty acids (46) and hyperglycemia (47) can suppress ABCA1 transcription. The diverse inhibitory effects of diabetes-associated metabolic factors on the ABCA1 pathway could act in concert to suppress cholesterol transport from arterial macrophages, contributing to the increased risk for cardiovascular disease.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants DK-02456, HL-18645, HL-55362, and HL-55798 and by the state of Sao Paulo Research Foundation grant 99/01039-9, Brazil (to M.P.).

REFERENCES

1. Kannel WB, McGee DL: Diabetes and cardiovascular risk factors: the Framingham Study. *Circulation* 59:8–13, 1979
2. Bierman EL: George Lyman Duff Memorial Lecture: Atherogenesis in diabetes. *Arterioscler Thromb* 12:647–656, 1992
3. Ginsberg HN: Lipoprotein physiology in nondiabetic and diabetic states: relationship to atherogenesis. *Diabetes Care* 14:839–855, 1991
4. Quintao EC, Medina WL, Passarelli M: Reverse cholesterol transport in diabetes mellitus. *Diabetes Metab Res Rev* 16:237–250, 2000
5. Gordon DJ, Knoke J, Probstfield JL, Superko R, Tyroler HA: High-density lipoprotein cholesterol and coronary heart disease in hypercholesterolemic men: the Lipid Research Clinics Coronary Primary Prevention Trial. *Circulation* 74:1217–1225, 1986
6. Oram JF: HDL apolipoproteins and ABCA1: partners in the removal of excess cellular cholesterol. *Arterioscler Thromb Vasc Biol* 23:720–727, 2003
7. Wang N, Tall AR: Regulation and mechanisms of ATP-binding cassette transporter A1-mediated cellular cholesterol efflux. *Arterioscler Thromb Vasc Biol* 23:1178–1184, 2003
8. Singaraja RR, Brunham LR, Visscher H, Kastelein JJ, Hayden MR: Efflux and atherosclerosis: the clinical and biochemical impact of variations in the ABCA1 gene. *Arterioscler Thromb Vasc Biol* 23:1322–1332, 2003
9. Joyce C, Freeman L, Brewer HB Jr, Santamarina-Fojo S: Study of ABCA1 function in transgenic mice. *Arterioscler Thromb Vasc Biol* 23:965–971, 2003
10. Aiello RJ, Brees D, Francone OL: ABCA1-deficient mice: insights into the role of monocyte lipid efflux in HDL formation and inflammation. *Arterioscler Thromb Vasc Biol* 23:972–980, 2003
11. Assman G, von Eckardstein A, Brewer HB: Familial high density lipoprotein deficiency: Tangier disease. In *The Metabolic and Molecular Bases of Inherited Disease*. Scriver CR, Beaudet AL, Sly WS, Valle D, Eds. New York, McGraw-Hill, 1995, p. 2053–2072
12. Serfaty-Lacrosniere C, Civeira F, Lanzberg A, Isaia P, Berg J, Janus ED, Smith MP Jr, Pritchard PH, Frohlich J, Lees RS, Barnard GF, Ordovas JM, Schaefer EJ: Homozygous Tangier disease and cardiovascular disease. *Atherosclerosis* 107:85–98, 1994
13. Oram JF, Lawn RM, Garvin MR, Wade DP: ABCA1 is the cAMP-inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages. *J Biol Chem* 275:34508–34511, 2000
14. Wellington CL, Walker EK, Suarez A, Kwok A, Bissada N, Singaraja R, Yang YZ, Zhang LH, James E, Wilson JE, Francone O, McManus BM, Hayden MR: ABCA1 mRNA and protein distribution patterns predict multiple different roles and levels of regulation. *Lab Invest* 82:273–283, 2002
15. Wang Y, Oram JF: Unsaturated fatty acids inhibit cholesterol efflux from macrophages by increasing degradation of ATP-binding cassette transporter A1. *J Biol Chem* 277:5692–5697, 2002
16. Wang Y, Kurdi-Haidar B, Oram JF: LXR-mediated activation of macrophage stearoyl-CoA desaturase generates unsaturated fatty acids that destabilize ABCA1. *J Lipid Res* 45:972–980, 2004
17. Feng B, Tabas I: ABCA1-mediated cholesterol efflux is defective in free cholesterol-loaded macrophages mechanism involves enhanced ABCA1 degradation in a process requiring full NPC1 activity. *J Biol Chem* 277:43271–43280, 2002
18. Baynes JW, Thorpe SR: Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. *Diabetes* 48:1–9, 1999
19. Brownlee M, Cerami A, Vlassara H: Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N Engl J Med* 318:1315–1321, 1988
20. Thornalley PJ, Langborg A, Minhas HS: Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. *Biochem J* 344:109–116, 1999
21. Wolff SP, Dean RT: Glucose autoxidation and protein modification. The potential role of 'autoxidative glycosylation' in diabetes. *Biochem J* 245:243–250, 1987
22. Glomb MA, Monnier VM: Mechanism of protein modification by glyoxal and glycolaldehyde, reactive intermediates of the Maillard reaction. *J Biol Chem* 270:10017–10026, 1995
23. Anderson MM, Hazen SL, Hsu FF, Heinecke JW: Human neutrophils employ the myeloperoxidase-hydrogen peroxide-chloride system to convert hydroxy-amino acids into glycolaldehyde, 2-hydroxypropanal, and acrolein: a mechanism for the generation of highly reactive alpha-hydroxy and alpha,beta-unsaturated aldehydes by phagocytes at sites of inflammation. *J Clin Invest* 99:424–432, 1997
24. Anderson MM, Requena JR, Crowley JR, Thorpe SR, Heinecke JW: The myeloperoxidase system of human phagocytes generates Nepsilon-(carboxymethyl)lysine on proteins: a mechanism for producing advanced glycation end products at sites of inflammation. *J Clin Invest* 104:103–113, 1999
25. Fu MX, Requena JR, Jenkins AJ, Lyons TJ, Baynes JW, Thorpe SR: The advanced glycation end product, Nepsilon-(carboxymethyl)lysine, is a product of both lipid peroxidation and glycoxidation reactions. *J Biol Chem* 271:9982–9986, 1996
26. Thornalley PJ: Pharmacology of methylglyoxal: formation, modification of proteins and nucleic acids, and enzymatic detoxification: a role in pathogenesis and antiproliferative chemotherapy. *Gen Pharmacol* 27:565–573, 1996
27. Sady C, Jiang CL, Chellan P, Madhun Z, Duve Y, Glomb MA, Nagaraj RH: Maillard reactions by alpha-oxoaldehydes: detection of glyoxal-modified proteins. *Biochim Biophys Acta* 1481:255–264, 2000
28. Bucala R, Makita Z, Koschinsky T, Cerami A, Vlassara H: Lipid advanced glycosylation: pathway for lipid oxidation in vivo. *Proc Natl Acad Sci U S A* 90:6434–6438, 1993
29. Requena JR, Ahmed MU, Fountain CW, Degenhardt TP, Reddy S, Perez C, Lyons TJ, Jenkins AJ, Baynes JW, Thorpe SR: Carboxymethylethanolamine, a biomarker of phospholipid modification during the maillard reaction in vivo. *J Biol Chem* 272:17473–17479, 1997
30. Mendez AJ, Oram JF, Bierman EL: Protein kinase C as a mediator of high density lipoprotein receptor-dependent efflux of intracellular cholesterol. *J Biol Chem* 266:10104–10111, 1991
31. Oram JF, Mendez AJ, Lymp J, Kavanagh TJ, Halbert CL: Reduction in apolipoprotein-mediated removal of cellular lipids by immortalization of human fibroblasts and its reversion by cAMP: lack of effect with Tangier disease cells. *J Lipid Res* 40:1769–1781, 1999
32. Mendez AJ, Anantharamaiah GM, Segrest JP, Oram JF: Synthetic amphipathic helical peptides that mimic apolipoprotein A-I in clearing cellular cholesterol. *J Clin Invest* 94:1698–1705, 1994
33. Oram JF, Albers JJ, Bierman EL: Rapid regulation of the activity of the low density lipoprotein receptor of cultured human fibroblasts. *J Biol Chem* 255:475–485, 1980
34. Gerrity RG, Natarajan R, Nadler JL, Kimsey T: Diabetes-induced accelerated atherosclerosis in swine. *Diabetes* 50:1654–1665, 2001
35. O'Brien KD, Olin KL, Alpers CE, Chiu W, Ferguson M, Hudkins K, Wight TN, Chait A: Comparison of apolipoprotein and proteoglycan deposits in

human coronary atherosclerotic plaques: colocalization of biglycan with apolipoproteins. *Circulation* 98:519–527, 1998
36. Suzuki LA, Poot M, Gerrity RG, Bornfeldt KE: Diabetes accelerates smooth muscle accumulation in lesions of atherosclerosis: lack of direct growth-promoting effects of high glucose levels. *Diabetes* 50:851–860, 2001
37. Vaughan AM, Oram JF: ABCA1 redistributes membrane cholesterol independent of apolipoprotein interactions. *J Lipid Res* 44:1373–1380, 2003
38. Wang N, Silver DL, Costet P, Tall AR: Specific binding of ApoA-I, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing ABC1. *J Biol Chem* 275:33053–33058, 2000
39. Fitzgerald ML, Morris AL, Rhee JS, Andersson LP, Mendez AJ, Freeman MW: Naturally occurring mutations in the largest extracellular loops of ABCA1 can disrupt its direct interaction with apolipoprotein A-I. *J Biol Chem* 277:33178–33187, 2002
40. Hirsch J, Petrakova E, Feather MS: The reaction of some dicarbonyl sugars with aminoguanidine. *Carbohydr Res* 232:125–130, 1992
41. Reddy S, Bichler J, Wells-Knecht KJ, Thorpe SR, Baynes JW: N epsilon-(carboxymethyl)lysine is a dominant advanced glycation end product (AGE) antigen in tissue proteins. *Biochemistry* 34:10872–10878, 1995
42. Thornalley PJ, Yurek-George A, Argirov OK: Kinetics and mechanism of the reaction of aminoguanidine with the alpha-oxoaldehydes glyoxal, methylglyoxal, and 3-deoxyglucosone under physiological conditions. *Biochem Pharmacol* 60:55–65, 2000
43. Thornalley PJ: Modification of the glyoxalase system in human red blood cells by glucose in vitro. *Biochem J* 254:751–755, 1988
44. Seigneur M, Freyburger G, Gin H, Claverie M, Lardeau D, Lacape G, Le Moigne F, Crockett R, Boisseau MR: Serum fatty acid profiles in type I and type II diabetes: metabolic alterations of fatty acids of the main serum lipids. *Diabetes Res Clin Pract* 23:169–177, 1994
45. Hayden JM, Reaven PD: Cardiovascular disease in diabetes mellitus type 2: a potential role for novel cardiovascular risk factors. *Curr Opin Lipidol* 11:519–528, 2000
46. Uehara Y, Engel T, Li Z, Goepfert C, Rust S, Zhou X, Langer C, Schachtrup C, Wiekowski J, Lorkowski S, Assmann G, Von Eckardstein A: Polyunsaturated fatty acids and acetoacetate downregulate the expression of the ATP-binding cassette transporter A1. *Diabetes* 51:2922–2928, 2002
47. Albrecht C, Simon-Vermot I, Elliott JI, Higgins CF, Johnston DG, Valabhji J: Leukocyte ABCA1 gene expression is associated with fasting glucose concentration in normoglycemic men. *Metabolism* 53:17–21, 2004