Hyperlipidemia in Concert With Hyperglycemia Stimulates the Proliferation of Macrophages in Atherosclerotic Lesions

Potential Role of Glucose-Oxidized LDL

Najib Lamharzi,[1] Catherine B. Renard,[1] Farah Kramer,[1] Subramaniam Pennathur,[2] Jay W. Heinecke,[2] Alan Chait,[2] and Karin E. Bornfeldt[1]

Hyperglycemia and hyperlipidemia are important risk factors for diabetes-accelerated atherosclerosis. Macrophage proliferation has been implicated in the progression of atherosclerosis. We therefore investigated the effects of hyperglycemia and hyperlipidemia on macrophage proliferation in murine atherosclerotic lesions and isolated primary macrophages. Hyperglycemic LDL receptor–deficient mice that were fed a cholesterol-free diet for 12 weeks did not have elevated cholesterol levels compared with nondiabetic mice, and there was no evidence of increased macrophage proliferation in atherosclerotic lesions. Moreover, elevated glucose levels did not increase proliferation of isolated mouse peritoneal macrophages. In contrast, hyperglycemic LDL receptor–deficient mice that were fed a cholesterol-rich diet showed increased cholesterol levels concomitant with macrophage proliferation in atherosclerotic lesions. Glucose promoted lipid and protein oxidation of LDL in vitro. Glucose-oxidized LDL resulted in phosphorylation of extracellular signal–regulated kinase and protein kinase B/Akt and stimulated proliferation of isolated macrophages. The mitogenic effect of glucose-oxidized LDL was mediated by CD36 and by extracellular signal–regulated kinase activation induced by protein kinase C–dependent and phosphatidylinositol 3-kinase–dependent pathways. Thus, hyperglycemia is not sufficient to stimulate macrophage proliferation in lesions of atherosclerosis or in isolated macrophages. A combination of hyperglycemia and hyperlipidemia, however, stimulates macrophage proliferation by a pathway that may involve the glucose-dependent oxidation of LDL. *Diabetes* 53:3217–3225, 2004

From the [1]Department of Pathology, University of Washington, Seattle, Washington; and the [2]Departments of Medicine and Molecular Biology and Pharmacology, University of Washington, Seattle, Washington.

Address correspondence and reprint requests to Dr. Karin Bornfeldt, Department of Pathology, Box 357470, University of Washington School of Medicine, Seattle, WA 98195-7470. E-mail: bornf@u.washington.edu.

Received for publication 1 December 2003 and accepted in revised form 20 August 2004.

C.B.R. is currently affiliated with INSERM Unité 145, UFR Médecine, Nice, France.

BCA, brachiocephalic artery; BHT, butylated hydroxytoluene; BrdU, 5-bromo-2′-deoxyuridine; CSF-1, colony-stimulating factor 1; ERK, extracellular signal–regulated kinase; HPLC, high-performance liquid chromatography; HODE, 13-hydroxyoctadecadienoic acid; LCMV, lymphocytic choriomeningitis virus; LDLR, LDL receptor; PI3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C; RIP-LCMV-GP, rat insulin promoter–lymphocytic choriomeningitis virus glycoprotein.

Diabetes is associated with accelerated atherosclerosis and subsequent cardiovascular disease (1). People with diabetes are at two- to fourfold greater risk of developing cardiovascular disease than people without diabetes (2), and the majority of type 2 diabetic patients die of atherosclerotic disease. The mechanisms underlying diabetes-accelerated atherosclerosis are poorly understood. Hyperglycemia, increased levels of reactive oxygen species, production of advanced glycation end products and glycation of lipoproteins, and lipid abnormalities, such as increases in the levels of VLDL and their remnants, all may play a role in diabetes-accelerated lesion progression.

One of the initiating steps in formation of atherosclerotic lesions is monocyte adhesion to the endothelium and entry into the vessel wall followed by differentiation into macrophages. These macrophages take up excess modified LDL through scavenger receptors and become lipid-laden foam cells. Recent studies have implicated the proliferation of macrophage-derived foam cells as a critical event in the evolution of atherosclerotic lesions (3,4). Furthermore, macrophage-rich lesions are believed to undergo plaque rupture and induce the acute clinical complications of atherosclerosis: myocardial infarction and stroke (5).

It has yet to be established whether diabetes accelerates atherosclerosis by stimulating macrophage proliferation. We used isolated primary mouse macrophages and a newly developed transgenic mouse model of diabetes-accelerated atherosclerosis (6) to address this issue. In this model, diabetes is induced in LDL receptor (LDLR)-deficient mice expressing the lymphocytic choriomeningitis virus (LCMV) glycoprotein under control of the insulin promoter by a single injection of LCMV. LCMV triggers an immune reaction that destroys the glycoprotein-expressing β-cells in the LDLR−/−;GP mice and results in the rapid development of type 1 diabetes (6). The results show that hyperglycemia alone is not sufficient to stimulate macrophage proliferation in lesions of atherosclerosis or in isolated macrophages. Glucose-oxidized LDL, however, stimulates macrophage proliferation, indicating that hyperlipidemia in combination with hyperglycemia may induce macrophage proliferation in vivo.

RESEARCH DESIGN AND METHODS

A transgenic mouse model of diabetes-accelerated atherosclerosis, B6.129-LDL^{tm1hdr} Tg(RIP-LCMV-GP) 1KBUW on the C57BL/6 background (6), was used for in vivo studies. In these LDLR−/−;GP mice, diabetes accelerates atherosclerosis in both the presence and absence of diabetes-induced lipid abnormalities (6). Female mice, which do not develop diabetes when fed high-fat diets (7), were used. For isolation of peritoneal macrophages, adult C57BL/6 mice (The Jackson Laboratories, Bar Harbor, ME) or LDLR−/−;GP mice were used.

All animal protocols were approved by the University of Washington Animal Care Committee. Female mice (7–10 weeks of age) received an intraperitoneal injection of saline solution or LCMV (10^5 pfu). LDLR−/−;GP mice developed diabetes 1–2 weeks after injection. To avoid weight loss and ketonuria, most diabetic mice received slow-release insulin (0.1 units bovine insulin/day) by subcutaneous implantation of pellets (Linshin Canada, Ontario, Canada). Mice were housed in a temperature-controlled facility, which maintained a 12/12-h light/dark cycle, and were given free access to food and water. Mice were fed semipurified diets (TD00241 and TD00242; Harlan Teklad, Madison, WI) for 12 weeks, starting 1 week after injection (6). The diets contained neither cholate nor antioxidants and contained cholesterol levels of 0 and 0.12%, respectively. Body weight, urinary glucose, and ketonuria were monitored weekly; saphenous vein blood glucose was measured at least biweekly; and plasma total cholesterol was measured at the end of the study, as previously described (6). To monitor cell proliferation, mice received intraperitoneal injections of 2.7 mg of 5-bromo-2′-deoxyuridine (BrdU; Roche, Indianapolis, IN) divided into three doses of 0.9 mg (24, 12, and 1 h before they were killed).

Tissue preparation, quantification of lesion area, and immunohistochemistry. Diabetic and nondiabetic LDLR−/−;GP mice were perfusion-fixed with 4% paraformaldehyde. Brachiocephalic arteries (BCAs) were paraffin-embedded and then serial-sectioned into 5-μm sections. Every 20 μm, two sections were stained using the Movat's pentachrome method (6), which allowed detection of the maximal lesion site. Cross-sectional lesion area at the maximal site was quantified using Image Pro-Plus (Media-Cybernetics, Silver Spring, MD). Macrophages were detected using a rat monoclonal anti-mouse Mac-2 antibody, as previously described (6). BrdU was detected using an anti-BrdU antibody (Roche) according to the manufacturer's instructions. Macrophage proliferation in lesions was determined as the number of Mac-2–positive cells that had incorporated BrdU. Three sections, 40 μm apart, from the maximal lesion site were analyzed per animal.

Oxidation of LDL. Human LDL (d = 1.019–1.063 g/ml) was isolated by sequential ultracentrifugation from the plasma of normolipidemic human subjects after overnight fasting, as previously described (8). Glucose-oxidized LDL was prepared by incubating 1 mg/ml LDL for 10 days at 37°C in Dulbecco's PBS (pH 7.4; without calcium or magnesium) that contained 100 μmol/l EDTA and 25 mmol/l D-glucose. EDTA was included in the buffer to inhibit metal catalyzed oxidation reactions. Glycated, nonoxidized LDL was prepared in parallel reactions with the addition of the antioxidant butylated hydroxytoluene (BHT; 25 μmol/l). Control LDL was prepared in reactions without glucose.

Cu^{2+}-oxidized LDL was prepared and characterized according to Sakai et al. (9). The levels of endotoxin associated with these LDL preparations were <0.1 units/ml as measured using the Limulus Amebocyte lysate assay (Whittaker M.A. Bioproducts, Walkersville, MD). For the determination of amino acid oxidation and lipid peroxidation products, all reactions were terminated by addition of 300 nmol/l catalase, 100 μmol/l BHT, and 200 μmol/l diethylenetriamine pentaacetic acid. The proteins were immediately precipitated at 4°C with ice-cold trichloroacetic acid (10% vol/vol). The protein pellet was washed with 0.5 ml of 10% trichloroacetic acid and delipidated twice with 10 ml of a mixture of water, methanol, and water-washed diethyl ether (1:3:7 vol/vol/vol). After addition of ^{13}C-labeled internal standards, the resultant protein pellet was hydrolyzed as described (10). Amino acids were isolated from the acid hydrolysate using a solid-phase C-18 column (3 ml; Supelclean SPE, Supelco, Bellefonte, PA), as previously described (11). Amino acids were converted to n-propyl, heptafluorobutyric anhydride derivatives and quantified using isotope dilution negative-ion chemical ionization gas chromatography/mass spectrometry (11).

13-Hydroxyoctadecadienoic acid (HODE) was determined by reverse-phase high-performance liquid chromatography (HPLC) of triphenylphosphine-reduced lipid extracts after base hydrolysis to determine the lipid peroxidation products (12). Authentic HODE was prepared from linoleic acid with soybean lipoxygenase (13).

Cell culture. Peritoneal macrophages were collected from male C57BL/6 (25–30 g) or male and female LDLR−/−;GP mice 5 days after intraperitoneal injection of 2 ml of thioglycollate (40 mg/ml). Briefly, the cells were harvested by peritoneal lavage using 5 ml of RPMI-1640 that contained 10% heat-inactivated FBS (GIBCO/BRL Life Technologies, Rockville, MD) and antibiotics. These cells were centrifuged at 650*g* for 5 min, and the pellet was resuspended in fresh medium. The cells were plated on tissue culture plastic for 24 h, then washed three times with PBS to remove unattached cells and incubated in RPMI-1640 that contained 1% FBS. The cell population was characterized using a Mac-2 antibody and consisted of >98% Mac-2–positive cells. The cells then were treated with increasing concentrations of D-glucose, human recombinant colony-stimulating factor 1 (CSF-1/macrophage-CSF; Pepro Tech, Rocky Hill, NJ), or different forms of LDL with or without inhibitors for 3–6 days. Conditioned media were harvested at different time points and assayed for glucose and lactate levels using chromogenic assays (Sigma, St. Louis, MO). CD36 was inhibited by using a blocking anti-mouse CD36 monoclonal antibody (clone 63; Cascade Bioscience, Winchester, MA) at 2.5 μg/ml. Mouse IgA (Sigma) was used as a control. Phosphatidylinositol 3-kinase (PI3-K) was inhibited by wortmannin (100 nmol/l). The extracellular signal–regulated kinase (ERK) pathway was inhibited by PD98059 (2 μmol/l) or by U0126 (500 nmol/l). Diacylglycerol-activated protein kinase C (PKC) isoforms were inhibited by calphostin C (100 nmol/l). All inhibitors were purchased from Calbiochem (La Jolla, CA).

Measurement of DNA synthesis and cell proliferation. DNA synthesis was measured as [^{3}H]thymidine incorporation into DNA during 24 h before termination of the experiment, as described previously (14). For cell number determinations, macrophages were incubated for 5 days, then washed three times with PBS. The cells were gently scraped from the surface of the plate using a cell scraper and were counted using a cell counter (Coulter, Hialeah, FL).

Analysis of phosphorylation of p42/p44 ERK and PKB/Akt. Peritoneal macrophages were stimulated with CSF-1 (5 ng/ml), Cu^{2+}-oxidized LDL, or glucose-oxidized LDL (30 μg/ml) for 15 min in the presence or absence of wortmannin, calphostin C, PD98059, or U0126. Phosphorylation of ERK and PKB/Akt was evaluated by Western blot analysis, as previously described (15).

Cholesteryl ester synthesis and foam-cell formation. Mouse peritoneal macrophages were plated in 100-mm dishes and incubated for 48 h in RPMI-1640 that contained 1% FBS and 30 μg/ml glucose-oxidized LDL, Cu^{2+}-oxidized LDL, or control LDL in the presence of [^{3}H]cholesterol (37 kBq/ml, 1.85 kBq/μmol). The cells then were washed with PBS, scraped from the dishes, and subjected to lipid extraction according to Bligh and Dyer (16) and protein quantification using the BCA kit (Pearce, Rockford, IL). Lipids were separated by thin-layer chromatography on silica gel G plates with hexane:diethylether:glacial acetic acid (105:45:3; vol/vol/vol) as the solvent system. Cholesteryl esters were visualized by iodine vapor and scraped off, and the radioactivity of the cholesteryl ester spots was measured in a liquid scintillation counter.

For investigating whether glucose-oxidized LDL can induce foam-cell formation, peritoneal macrophages were plated on four-well chamber slides and incubated for 48 h in RPMI-1640 that contained 1% FBS and 30 μg/ml of glucose-oxidized LDL or Cu^{2+}-oxidized LDL with or without a blocking anti-CD36 antibody, or mouse IgA control. The cells then were washed with PBS, fixed with 4% paraformaldehyde, stained with Oil Red O in 60% isopropanol, then counterstained with hematoxylin. The cells were visualized using light microscopy and photographed using a Nikon Coolpix 4500 digital camera.

Statistical analyses. Comparisons between two groups were performed by two-tailed unpaired student's *t* test (GraphPad Prism 3.0; GraphPad Software, San Diego, CA). Comparisons between multiple groups were performed using one-way ANOVA. Simultaneous multiple comparisons were based on post hoc comparison tests using a Student-Newman-Keuls test. Statistical significance was established at $P < 0.05$.

RESULTS

Diabetes combined with hyperlipidemia causes macrophage accumulation and proliferation in the BCA. We have previously reported glucose and cholesterol levels in these diabetic and nondiabetic LDLR−/−;GP mice (6). Diabetic mice that were fed the cholesterol-free diet for 12 weeks had blood glucose levels 2.6-fold those of nondiabetic mice but no significant changes in cholesterol levels or lipoprotein profiles (6). These diabetic mice demonstrated a 9.4-fold increase in cross-sectional lesion area and a 5-fold increase in macrophage accumulation in the BCA compared with nondiabetic mice (Figs. 1*A* versus *C* and 2*A* and *B*). No BrdU-positive macrophages were

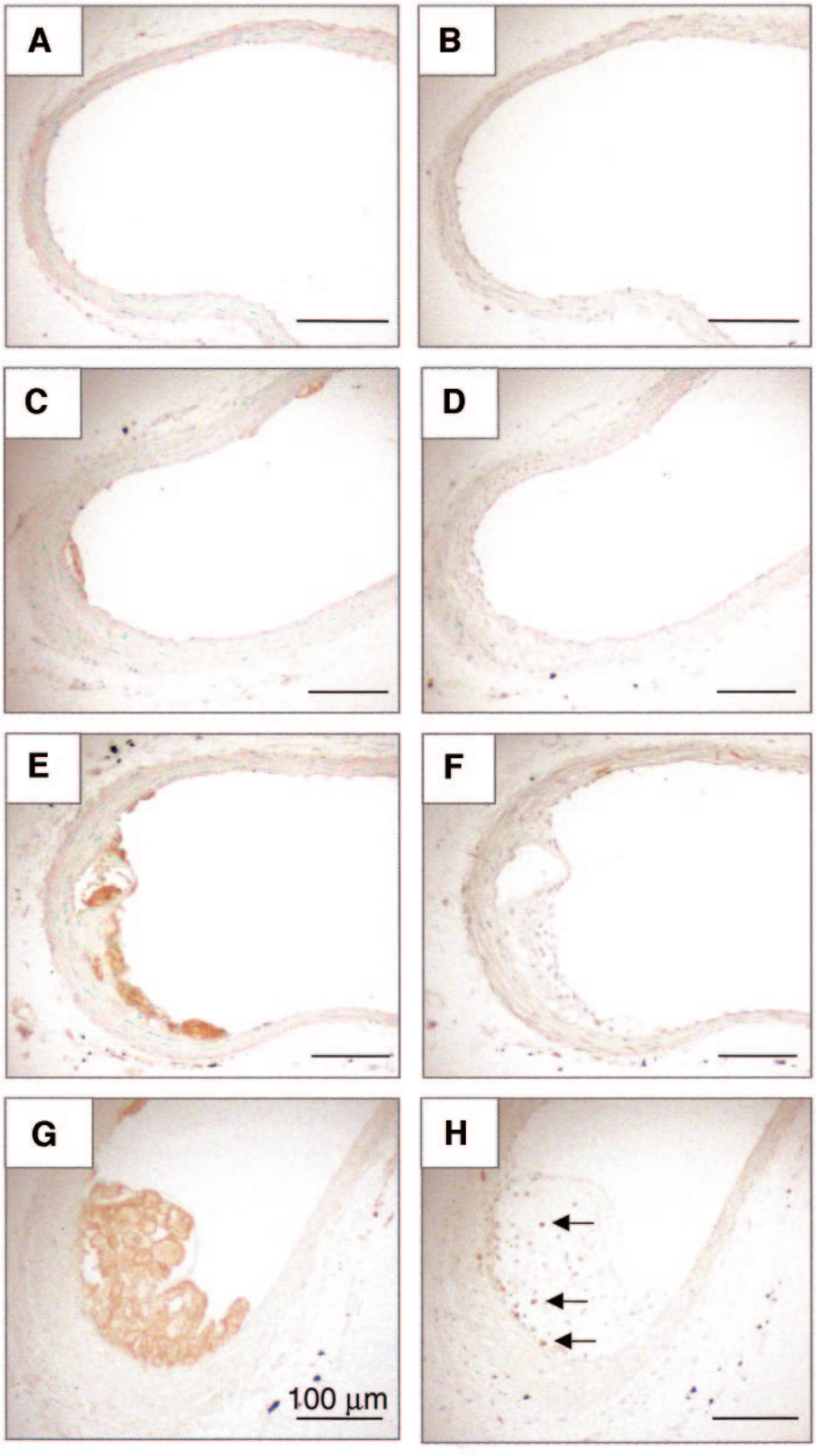

FIG. 1. BrdU-positive macrophages are primarily found in lesions from diabetic mice that were fed a cholesterol-rich diet. Nondiabetic and diabetic LDLR−/−;GP mice were perfusion-fixed after 12 weeks on a 0% cholesterol diet or a 0.12% cholesterol diet, as described in RESEARCH DESIGN AND METHODS. The BCA was serial-sectioned, and every 20 μm, sections were stained using a Movat's pentachrome procedure. Adjacent sections obtained from the site at which the lesion was maximal were used to detect macrophages using a rat anti-mouse monoclonal Mac-2 antibody (*A*, *C*, *E*, and *G*) or to detect proliferating cells using an anti-BrdU antibody (*B*, *D*, *F*, and *H*). Representative sections from nondiabetic mice that were fed a cholesterol-free diet (*A* and *B*), diabetic mice that were fed a cholesterol-free diet (*C* and *D*), nondiabetic mice that were fed a 0.12% cholesterol diet (*E* and *F*), and diabetic mice that were fed a 0.12% cholesterol diet (*G* and *H*) are shown. BrdU-positive macrophages are indicated by arrows.

detected in the BCA of nondiabetic or diabetic mice that were fed the cholesterol-free diet (Figs. 1*B* and *D* and 2*C*).

Diabetic mice that were fed a 0.12% cholesterol diet for 12 weeks showed a 2.2-fold increase in blood glucose levels compared with nondiabetic mice and exhibited an approximate 3-fold increase in total cholesterol levels (6). These diabetic mice demonstrated increased macrophage accumulation and more BrdU-positive macrophages in the BCA (Figs. 1*G* and *H* and 2*C*) compared with nondiabetic mice (Figs. 1*E* and *F* and 2*C*). There were no significant differences in mean blood glucose levels or insulin levels between diabetic mice that were fed the cholesterol-free diet and the 0.12% cholesterol diet ($P > 0.05$, ANOVA). Conversely, cholesterol-fed diabetic mice were hypercho-

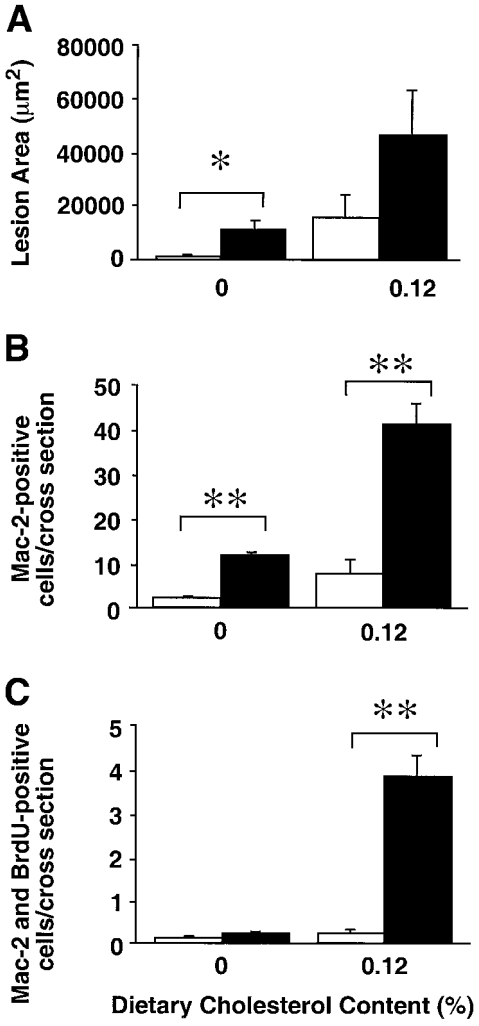

FIG. 2. Diabetic hyperlipidemic mice show increased macrophage accumulation and proliferation. BCAs from nondiabetic and diabetic mice were prepared and fixed as described in Fig. 1. *A*: The cross-sectional lesion area at the maximal lesion site was quantified using Image Pro-Plus (Media-Cybernetics). *B*: The number of Mac-2–positive cells per lesional cross-section was analyzed as Mac-2–positive cells that contained a detectable nucleus. *C*: The number of cells that were positive for both Mac-2 and BrdU immunostaining was determined. Lesions from 6–13 animals per group were analyzed. Data are means + SE. Statistical analysis was performed using two-tailed unpaired Student's *t* test (*$P < 0.05$, **$P < 0.01$). □, nondiabetic mice; ■, diabetic mice.

lesterolemic ($P < 0.001$) compared with diabetic mice that were fed the cholesterol-free diet. These results show that hyperglycemia is not sufficient to stimulate macrophage proliferation in vivo, at least at the time point studied, and that diabetes-induced hyperlipidemia is required for macrophage proliferation to occur in this model.

Elevated glucose levels do not increase proliferation of isolated mouse peritoneal macrophages. The in vivo studies suggested that hyperglycemia alone, without hyperlipidemia secondary to diabetes, does not stimulate macrophage proliferation in lesions of atherosclerosis. To confirm these findings, we evaluated the ability of glucose to stimulate proliferation of isolated mouse peritoneal macrophages. Macrophages were incubated in the presence of D-glucose or mannitol for up to 6 days. The medium was changed every 2 days to avoid glucose depletion. Neither elevated glucose at 10, 20, or 30 mmol/l nor mannitol (up to 25 mmol/l) stimulated DNA synthesis (data not shown). However, glucose was metabolized by the macrophages. After a 72-h incubation in the presence of 11.2 mmol/l glucose, the cells had consumed ~0.01 nmol/cell of glucose and, because 1 mol of glucose results in 2 mol of lactate, had converted ~60% of it to lactate (4.8 ± 0.2 mmol/l glucose had been consumed and 6.8 ± 0.5 mmol/l lactate had been released; $n = 3$). Thus, high glucose levels alone have no effect on proliferation of primary peritoneal macrophages.

TABLE 1
Protein and lipid oxidation products in LDL incubated with glucose

	o-Tyrosine (mmol/mol)	*m*-Tyrosine (mmol/mol)	HODE (nmol/mg)
LDL	0.15 ± 0.01	0.11 ± 0.01	0.94 ± 0.02
LDL + glucose	0.25 ± 0.01*	0.18 ± 0.01†	1.42 ± 0.09*
LDL + BHT	0.15 ± 0.01	0.11 ± 0.01	0.97 ± 0.06
LDL + glucose + BHT	0.15 ± 0.01	0.12 ± 0.01	0.94 ± 0.03
LDL + copper	0.42 ± 0.05*	0.28 ± 0.06*	1.85 ± 0.22*

Data are means ± SE of 12 independent determinations. LDL (1 mg/ml) in PBS (pH 7.4) supplemented with 0.1 mmol/l EDTA was incubated for 10 days at 37°C. Where indicated, the reaction mixture was supplemented with 25 mmol/l glucose and/or 25 μmol/l BHT. For copper oxidation, LDL was incubated for 24 h in the presence of 20 μmol/l copper sulfate without EDTA. Reactions were terminated by adding 0.2 mmol/l DTPA, 300 nmol/l catalase, and 0.1 mmol/l BHT. Amino acids composition and HODE content of LDL were determined as described in RESEARCH DESIGN AND METHODS. LDL + copper was used as a positive control, and this value is based on six independent determinations. Levels of oxidized amino acids are normalized to levels of precursor amino acid (mmol/mol phenylalanine). Levels of HODE are normalized to LDL protein (nmol/mg). *$P < 0.001$ and †$P < 0.01$, by one-way ANOVA when compared with LDL alone.

Glucose promotes lipid and protein oxidation of LDL. To determine whether pathologically relevant concentrations of glucose promote LDL oxidation, we exposed LDL to 25 mmol/l D-glucose in vitro for 10 days. *o*-Tyrosine and *m*-tyrosine, two stable markers of protein oxidation, and the lipid peroxidation product HODE then were measured in hydrolysates of LDL. Levels of both protein and lipid oxidation increased in LDL exposed to glucose (Table 1). These results suggest that glucose promotes both lipid and protein oxidation in LDL by a pathway that does not require redox active transition metal ions. When the lipid-soluble antioxidant BHT (25 μmol/l) was included in the reaction mixture, both protein oxidation and lipid peroxidation were inhibited (Table 1). Because the reaction of glucose with nucleophilic groups (e.g., the ε amino group of lysine) on the lipoprotein should not be affected by BHT, these results show that glycation alone is insufficient to promote LDL oxidation.

Glucose-oxidized LDL, like Cu^{2+}-oxidized LDL, stimulates proliferation of mouse peritoneal macrophages. Cu^{2+}-oxidized LDL promotes macrophage proliferation in vitro (9,17–19). We therefore investigated the effect of glucose-oxidized LDL on DNA synthesis in isolated mouse peritoneal macrophages. Incubation with native LDL in the presence of BHT for 3 days did not stimulate DNA synthesis (Fig. 3*A*). Glucose-oxidized LDL at 10–30 μg/ml, however, stimulated DNA synthesis in a dose-dependent manner compared with native LDL incu-

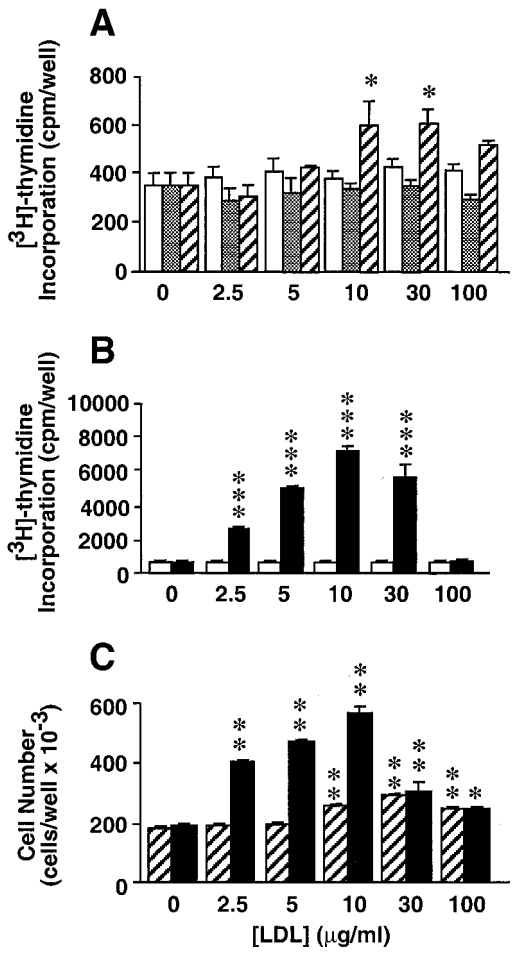

FIG. 3. Glucose-oxidized LDL stimulates proliferation of peritoneal macrophages. Effects of glucose-oxidized LDL (*A*) and Cu^{2+}-oxidized LDL (*B*) on DNA synthesis by peritoneal macrophages. Mouse peritoneal macrophages from C57BL/6 mice were treated with increasing concentrations of native LDL, glycated LDL, glucose-oxidized LDL, or Cu^{2+}-oxidized LDL for 3 days. Thymidine incorporation into DNA was determined after an incubation in the presence of [^{3}H]thymidine during the final 24 h. Values are means + SE from triplicate cultures of a representative experiment that was repeated three times. *C*: Mouse peritoneal macrophages were treated with increasing concentrations of Cu^{2+}-oxidized LDL or glucose-oxidized LDL for 5 days. Subsequently, the macrophages were washed three times with PBS and scraped gently from the surface of the wells and counted using a Coulter Counter. Data are means + SE of triplicate samples of a representative experiment performed three times. Statistical analysis was performed using one-way ANOVA (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). □, native LDL; ▨, glycated LDL; ▨, glucose-ox.LDL; ■, Cu^{2+}-ox.LDL.

bated in the absence of glucose or with glycated LDL incubated in the presence of glucose plus BHT for 10 days (Fig. 3*A*). Cu^{2+}-oxidized LDL exerted a biphasic effect on DNA synthesis with a dramatic stimulatory effect at lower concentrations (2.5–30 μg/ml) and a decline at higher concentrations (Fig. 3*B*). This decline seems to be present also for glucose-oxidized LDL at concentrations >30 μg/ml. The maximal increase in DNA synthesis induced by Cu^{2+}-oxidized LDL was 20-fold compared with 1.7-fold for glucose-oxidized LDL (Fig. 3*A* and *B*). Consistent with the findings on DNA synthesis, both glucose-oxidized LDL and Cu^{2+}-oxidized LDL resulted in an increased number of peritoneal macrophages after 5 days of incubation (Fig. 3*C*). The ability of Cu^{2+}-oxidized LDL and glucose-oxidized LDL to induce DNA synthesis was also observed in peritoneal macrophage from male and female LDLR−/−;GP mice (data not shown), indicating that the LDLR is not required for the mitogenic effects of Cu^{2+}-oxidized LDL or glucose-oxidized LDL.

A blocking anti-CD36 antibody abolishes the stimulatory effect of glucose-oxidized LDL on DNA synthesis. To determine whether the mitogenic effects of glucose-oxidized LDL are mediated through a specific scavenger receptor, we used a blocking anti-mouse CD36 monoclonal antibody. The anti-CD36 antibody inhibited the effect of glucose-oxidized LDL on DNA synthesis in a dose-dependent manner (data not shown). A 92% inhibition of DNA synthesis induced by glucose-oxidized LDL was obtained using 2.5 μg/ml anti-CD36, whereas mouse IgA (2.5 μg/ml) had no effect (Fig. 4*A*). Thus, the mitogenic effect of glucose-oxidized LDL is primarily mediated by CD36.

The mitogenic effect of glucose-oxidized LDL is mediated by ERK through activation of PKC and PI3-K. Inhibitors selective for MEK (ERK kinase; PD98059 and U0126), diacylglycerol-activated PKC isoforms (calphostin C), and PI3-K (wortmannin) abolished the stimulatory effect of glucose-oxidized LDL on DNA synthesis (Fig. 4*B*). Accordingly, glucose-oxidized LDL stimulated phosphorylation of p42/p44 ERK, and this effect was inhibited by PD98059, calphostin C, and wortmannin, suggesting that PKC and PI3-K act upstream of ERK (Fig. 4*C*). Glucose-oxidized LDL also stimulated phosphorylation of PKB/Akt (a protein kinase downstream of PI3-K), and this effect was abolished by wortmannin, partially inhibited by calphostin C, and not inhibited by PD98059 (Fig. 4*C*).

Glucose-oxidized LDL is a weak stimulator of cholesteryl ester accumulation compared with Cu^{2+}-oxidized LDL. Next, we investigated the ability of glucose-oxidized LDL to stimulate cholesteryl ester accumulation and foam-cell formation. After 48 h, glucose-oxidized LDL caused a 12.7-fold increase in [^{3}H]cholesterol incorporation into cholesteryl esters compared with control LDL (Fig. 5). The effect of glucose-oxidized LDL was weak compared with that of Cu^{2+}-oxidized LDL, which induced a 65.2-fold increase in [^{3}H]cholesterol incorporation into cholesteryl esters (data not shown). Cu^{2+}-oxidized LDL but not glucose-oxidized LDL induced a demonstrable increase in foam-cell formation measured as Oil Red O–positive lipid droplets, and this effect was inhibited by the blocking anti-CD36 antibody, whereas control IgA had no effect (data not shown). Thus, glucose-oxidized LDL stimulates cholesteryl ester accumulation, but the effect is weaker than that of Cu^{2+}-oxidized LDL and not pronounced enough to lead to demonstrable lipid droplet formation during a 48-h stimulation.

Synergistic effects of glucose-oxidized LDL and cerebrospinal fluid-1 on DNA synthesis in mouse peritoneal macrophages. CSF-1 is present in atherosclerotic lesions (20) and is a mitogen for mature macrophages (Fig. 6*A*) (21). To determine whether CSF-1 and glucose-oxidized LDL have synergistic effects on DNA synthesis, we co-incubated increasing concentrations of glucose-oxidized LDL and a suboptimal concentration of CSF-1 (1 ng/ml). Glucose-oxidized LDL potentiated the effect of CSF-1 on DNA synthesis (Fig. 6*B*). A 2.3-fold increase in

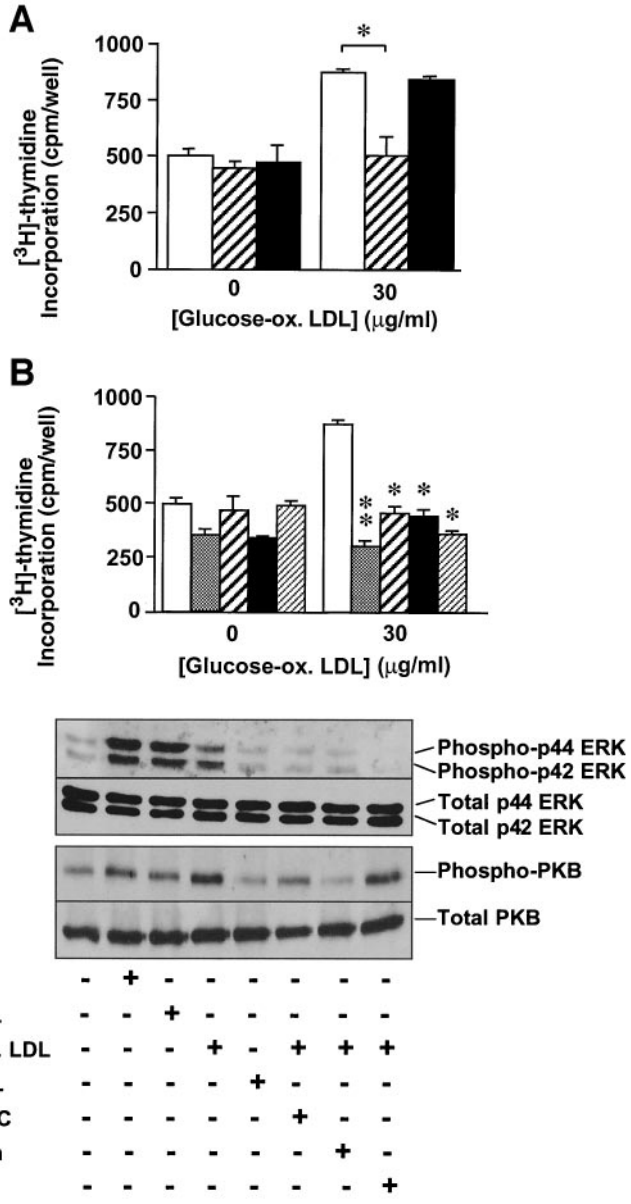

FIG. 4. The mitogenic effect of glucose-oxidized LDL is mediated by CD36 and subsequent activation of the ERK pathway. *A*: Mouse peritoneal macrophages from C57BL/6 mice were treated with 30 μg/ml glucose-oxidized LDL with or without 2.5 μg/ml anti-mouse CD36 or mouse IgA for 3 days. The cells were pulsed with [^{3}H]thymidine during the last 24 h. Data are means + SE from triplicate cultures from a representative experiment that was repeated three times. Statistical analysis was performed using one-way ANOVA (*P < 0.05). □, no inhibitor; ▨, anti-CD36; ■, IgA. *B*: Cells were pretreated with calphostin C (100 nmol/l), wortmannin (100 nmol/l), U0126 (500 nmol/l), or PD98059 (2 μmol/l) for 1 h before addition of 30 μg/ml glucose-oxidized LDL. The cells were pulsed with [^{3}H]thymidine during the last 24 h. Data are means + SE from triplicate cultures from a representative experiment that was repeated three times. Statistical analysis was performed using one-way ANOVA (*P < 0.05, **P < 0.01). □, no inhibitor; ▨, calphostin C; ▨, wortmannin; ■, U0126; ▨, PD98059. *C*: Macrophages were stimulated with CSF-1 (5 ng/ml, positive control), Cu^{2+}-oxidized LDL (30 μg/ml), glucose-oxidized LDL (30 μg/ml), or control native LDL (30 μg/ml) for 15 min. Some cells were pretreated with calphostin C (100 nmol/l), wortmannin (100 nmol/l), or PD98059 (2 μmol/l) for 1 h before stimulation with glucose-oxidized LDL. Total cell lysates (60 μg/lane) were separated using 10% SDS-PAGE, transferred to membranes, and incubated with rabbit anti-mouse phospho-(T202/Y204) ERK antibody or total ERK antibodies (15) at a final dilution of 1:1,000 and 1:15,000, respectively (*top*). Rabbit anti-mouse phospho-(S473) PKB/Akt or total PKB/Akt polyclonal antibodies were used at 1:1,000 dilutions (*bottom*). After incubation with a horseradish peroxidase–labeled donkey anti-rabbit secondary antibody, the signal was detected using enhanced chemiluminescence.

CSF-1–induced DNA synthesis was obtained using 30 μg/ml glucose-oxidized LDL.

DISCUSSION

Elevated glucose is not sufficient to stimulate macrophage proliferation in vivo or in vitro. The results of this study demonstrate that hyperglycemia alone, without diabetes-associated dyslipidemia, is associated with accumulation of macrophages in the BCA of LDLR−/−;GP mice but fails to promote macrophage proliferation. These results suggest that hyperglycemia may primarily accelerate macrophage accumulation in the arterial wall by stimulating recruitment. Consistently, hyperglycemia has been shown to induce monocyte adhesion molecule expression on the endothelium, and increased accumulation of white blood cells on the endothelium has been observed in models of streptozotocin- or alloxan-induced diabetes and hyperglycemia (22–24). We recently showed that diabetes-associated lipid abnormalities are not required for atherosclerotic lesion initiation, indicating that hyper-

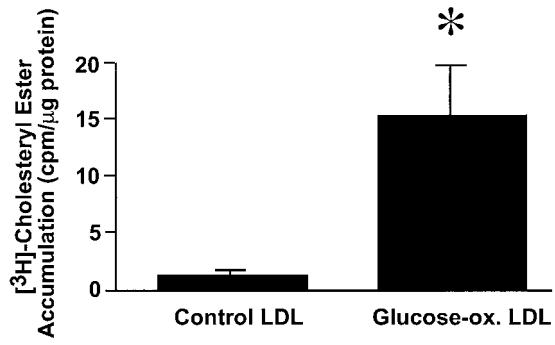

FIG. 5. Glucose-oxidized LDL induces cholesteryl ester accumulation. Mouse peritoneal macrophages from C57BL/6 mice were incubated in RPMI-1640 that contained 1% FBS and Cu^{2+}-oxidized LDL, glucose-oxidized LDL, or control native LDL (30 μg/ml) in the presence of trace amounts of [^{3}H]cholesterol for 48 h. The cells were harvested and subjected to lipid and protein extraction. Lipids were separated by thin-layer chromatography on silica gel G plates. Radioactivity incorporated into cholesteryl esters was analyzed by liquid scintillation counting. Data are means + SE of four independent experiments. Statistical analysis was performed by two-tailed unpaired Student's t test (*P < 0.05).

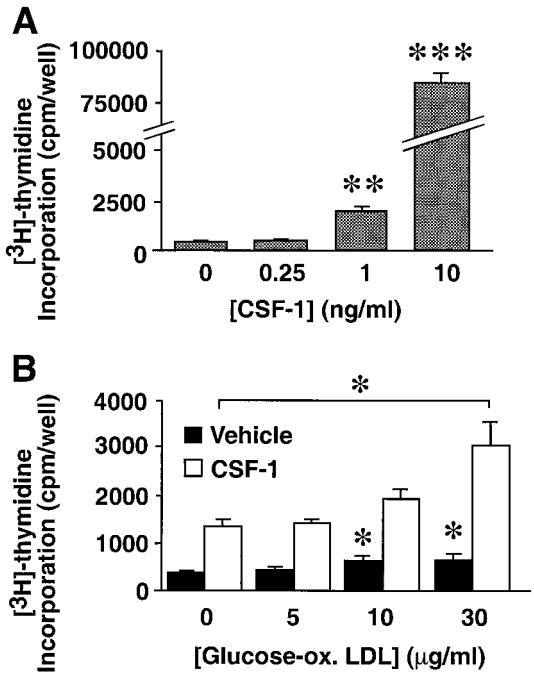

FIG. 6. Glucose-oxidized LDL enhances the mitogenic effects of CSF-1. Mouse peritoneal macrophages from C57BL/6 mice were treated with increasing concentrations of CSF-1 (*A*) or increasing concentrations of glucose-oxidized LDL with or without 1 ng/ml of CSF-1 for 3 days (*B*). The cells were pulsed with [^{3}H]thymidine in the last 24 h. Data are means + SE of triplicate cultures from a representative experiment that was repeated three times. Statistical analysis was performed using one-way ANOVA (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

glycemia is sufficient for initiation of macrophage-rich lesions (6). In addition, monocytes isolated from normolipidemic hyperglycemic patients with type 1 diabetes have an increased binding ability to endothelial cells in vitro (25).

The lack of effects of hyperglycemia on lesion macrophage proliferation in vivo is consistent with the inability of elevated glucose levels to induce proliferation of isolated peritoneal macrophages (this study) and splenic macrophages (26). In the present study, elevated glucose also did not enhance the mitogenic effect of CSF-1 (data not shown), in contrast to findings on splenic macrophages (26), suggesting that different macrophage populations may respond differently to elevated glucose levels. Together, our results indicate that glucose alone has no stimulatory effect on mouse macrophage proliferation in vitro or in vivo.

A combination of glucose and lipids results in increased macrophage proliferation in vivo and in vitro. Glucose-oxidized LDL may have proatherogenic effects. Thus, glucose-oxidized LDL stimulated proliferation of isolated mouse peritoneal macrophages and enhanced the mitogenic effect of CSF-1. Furthermore, there was increased macrophage proliferation in atherosclerotic lesions of hyperlipidemic hyperglycemic diabetic mice. The increased BrdU incorporation most likely reflects macrophage proliferation within the lesion, consistent with findings from a study analyzing host-derived versus donor-derived white blood cells in macrophage-rich carotid lesions (27). However, we cannot rule out the possibility that part of the BrdU-positive macrophages may have incorporated BrdU during a 24-h period before entering the lesion.

Oxidatively modified LDL is recognized as a critical factor in atherogenesis (28). Macrophage proliferation also seems to be of central importance in lesion formation and progression (29). There is now ample evidence that Cu^{2+}-oxidized LDL stimulates proliferation of isolated macrophages (9,17–19,30). However, the role of metal ions in promoting atherogenesis is uncertain (31,32). Perhaps more relevant to the diabetic state is our finding that levels of glucose present in the diabetic milieu promote protein oxidation and lipid peroxidation of LDL in vitro. Thus glucose, in the absence of metal ions, caused an increase in LDL lipid peroxidation protein oxidation. Cu^{2+}-oxidized LDL contained higher levels of protein and lipid oxidation products than glucose-oxidized LDL. Cu^{2+}-oxidized LDL also was a much stronger inducer of DNA synthesis and cholesteryl ester accumulation compared with glucose-oxidized LDL. In addition to the fact that 25 mmol/l glucose is a weaker oxidant than Cu^{2+}, the quantitative differences between glucose-oxidized LDL and Cu^{2+}-oxidized LDL may be due to a lower uptake of glucose-oxidized LDL by macrophages (33–35). In a recent study on streptozotocin-induced diabetic *Cynomolgus* monkeys, increased levels of *o*-tyrosine and *m*-tyrosine were found in aortic proteins compared with nondiabetic monkeys (11). In vitro studies have shown that proteins that are exposed to glucose (or reactive carbonyls derived from glucose) and polyunsaturated fatty acids exhibit a similar pattern of oxidation products (S.P., Y. Ido, J.I. Heller, J.R. Williamson, J.W.H.; unpublished observations). Thus, there is increasing evidence of elevated oxidation in the arterial wall in diabetes, part of which may be mediated by hyperglycemia.

How does glucose-oxidized LDL stimulate macrophage proliferation? The results of this study show that the mitogenic effects of glucose-oxidized LDL are mediated primarily through CD36. Similarly, the ability of Cu^{2+}-oxidized LDL to induce foam-cell formation was mainly dependent on CD36, consistent with a previous study that used macrophages from CD36- and SR-A–deficient mice (36). Recognition of oxidized LDL by macrophage scavenger receptors seems to be mediated by both the modified protein and lipid moieties (37,38). It is unclear whether the mitogenic effects are due to oxidized apolipoprotein B100 or to lipid oxidation products (9,18,19,39). In preliminary experiments, we found that neither the protein fraction nor the lipid fraction isolated from glucose-oxidized LDL increased DNA synthesis by macrophages (data not shown). These results suggest that combination of the lipid and the protein components may be essential to induce the mitogenic effect of glucose-oxidized LDL. One potential mechanism would involve binding of the protein component of glucose-oxidized LDL to CD36, followed by internalization and degradation of the modified lipoprotein. Delivery of lipids by this pathway to specific cellular compartments might then trigger cellular proliferation. Such lipids generated during LDL oxidation may include 7-ketocholesterol (39) and/or lysophosphatidylcholine (18).

Exposure of transformed macrophage cell lines and primary macrophages to Cu^{2+}-oxidized LDL has been

reported to lead to activation of the ERK pathway (40,41), PKCβ and PKCδ (42), and PI3-K (43,44). Consistent with these observations, stimulation of macrophage DNA synthesis by glucose-oxidized LDL, like Cu^{2+}-oxidized LDL (data not shown) (45), was abolished using PD98059 and U0126, calphostin C, and wortmannin. Because the ability of glucose-oxidized LDL to induce ERK phosphorylation was abolished by wortmannin and calphostin C, PI3-K and PKC are likely to act upstream of ERK. Consistently, the ability of glucose-oxidized LDL to induce PKB/Akt phosphorylation at 15 min was not inhibited by ERK inhibition and was partially inhibited by PKC inhibition, in agreement with previous studies (44). These results suggest that PI3-K may act upstream of ERK and also suggest that a diacylglycerol-activated PKC isoform may act upstream of ERK in a PKB/Akt-independent manner. The role of PKC in PI3-K activation and ERK activation induced by Cu^{2+}-oxidized LDL is controversial, perhaps because of lack of specificity of available inhibitors (42), as is the role of CSF-1 release (45,46). Taken together, the results of the present study suggest that a CD36-dependent activation of PI3-K and PKC isoforms and subsequent activation of the ERK pathway mediate the mitogenic effect of glucose-oxidized LDL on macrophages.

In conclusion, hyperglycemia alone is not sufficient to stimulate macrophage proliferation in lesions of atherosclerosis or in isolated macrophages. In contrast, hyperlipidemia in concert with hyperglycemia induces accumulation of proliferating lesion macrophages. Moreover, glucose-oxidized LDL significantly stimulates macrophage proliferation. Therefore, a combination of hyperglycemia and hyperlipidemia may contribute to macrophage proliferation in diabetes, perhaps by a pathway that involves the promotion of LDL oxidation by glucose.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (NIH; HL-62887 and HL-076719), a grant from the Juvenile Diabetes Research Foundation (JDRF; 1-2003-33), and a grant from the royalty Research Fund at the University of Washington (to K.E.B.), and NIH grants AG-02119-1 (to J.W.H.) and DK-02456 (to A.C. and J.W.H.). N.L. is supported by the cardiovascular training grant 32HL07820. S.P. is supported by a Career Development Award (2-2003-149) from the JDRF.

We thank Ashley Sherrid for excellent technical assistance.

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