Hyperlipidemia and hyperglycemia 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

D iabetes 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<sup>−/−</sup> R+RIP-LCMV-GP<sup>1KBUW</sup> on the C57BL/6 background (6), was used for in vivo studies. In these LDLR<sup>−/−</sup>-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<sup>−/−</sup>-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<sup>6</sup> pfu). LDLR<sup>−/−</sup>-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 i.p. injections of 2.7 mg of 5-bromo-2′-deoxyuri- nine (BrdU) 1 h prior to death. BrdU was detected using an anti-mouse BrdU antibody, as previously described (6). BrdU 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 = 1.010–1.063 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 Modified Eagle’s Medium (DMEM) without calf serum or antioxidants and containing 20 μmol/l dihydroxyacetone phosphate (DHAP), 25 μmol/l 3-O-methylglucose, 25 μmol/l sodium pyruvate, 25 μmol/l ascorbic acid, 10 μmol/l catalase, and 100 μmol/l EDTA. 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 <sup>14</sup>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 heptfluorobutyric acid derivatives and quantified using isotope dilution negative-ion chemical ionization mass chromatography/mass spectrometry (11).

1,3-Dihydroxyacetaldenedioic 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 cultured from male C57BL/6 (25–30 g) or male and female LDLR<sup>−/−</sup>-GP mice (6) and female mice 5 days after intraperitoneal injection of 2 ml of thiglycollate (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 650g 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 consistency of >98% Mac-2-positive cells were seen (6). The cells were then washed with PBS, scraped from the surface of the plate using a cell scraper and were counted using a cell counter (Coulter, Hialeah, FL).

Measurement of DNA synthesis and cell proliferation. DNA synthesis was measured as [3H]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<sup>2+</sup>-oxidized LDL, or control LDL (30 μg/ml) for 15 min in the presence or absence of Wortmannin (100 nmol/l). All inhibitors were purchased from Calbiochem (La Jolla, 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<sup>−/−</sup>-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. 1A versus C and 2A and B). No BrdU-positive macrophages were
detected in the BCA of nondiabetic or diabetic mice that were fed the cholesterol-free diet (Figs. 1B and D and 2C).

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. 1G and H and 2C) compared with nondiabetic mice (Figs. 1E and F and 2C). 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-
listerolemic (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 vitro 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 mmol/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.

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 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 Cu²⁺-oxidized LDL, stimulates proliferation of mouse peritoneal macrophages.
Cu²⁺-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. 3A). Glucose-oxidized LDL at 10–30 µg/ml, however, stimulated DNA synthesis in a dose-dependent manner compared with native LDL incubated for up to 6 days. 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

| Table 1 Protein and lipid oxidation products in LDL incubated with glucose |
|-------------------|-------------------|-------------------|
|                   | o-Tyrosine (mmol/mol) | m-Tyrosine (mmol/mol) | HODE (mmol/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   |

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. Copper oxidation increased in LDL exposed to glucose (Table 1). Thus, high glucose levels alone have no effect on proliferation of primary peritoneal macrophages.
findings on DNA synthesis, both glucose-oxidized LDL and peritoneal macrophages after 5 days of incubation (Fig. 3).

Glucose-oxidized LDL (GOD-ox.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 Cu²⁺-oxidized LDL or Cu²⁺-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. Statistical analysis was performed using one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001).

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. 4B). 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. 4C). 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. 4C).

Glucose-oxidized LDL is a weak stimulator of cholesteryl ester accumulation compared with Cu²⁺-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 Cu²⁺-oxidized LDL, which induced a 65.2-fold increase in \[^{3}H\]cholesterol incorporation into cholesteryl esters (data not shown). Cu²⁺-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 Cu²⁺-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. 6A) (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. 6B). A 2.3-fold increase 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 Cu²⁺-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. 4A). Thus, the mitogenic effect of glucose-oxidized LDL is primarily mediated by CD36.
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 hyperglycemia may primarily accelerate macrophage accumulation in the arterial wall by stimulating recruitment.

![FIG. 4](image-url)

**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 [3H]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 [3H]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), Cu²⁺-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 antibodies or total ERK antibodies 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.

![FIG. 5](image-url)

**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 Cu²⁺-oxidized LDL, glucose-oxidized LDL (30 μg/ml), or control native LDL (30 μg/ml) in the presence of trace amounts of [3H]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 (\(^*P < 0.05\)).
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 Cu²⁺-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. Cu²⁺-oxidized LDL contained higher levels of protein and lipid oxidation products than glucose-oxidized LDL. Cu²⁺-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 Cu²⁺, the quantitative differences between glucose-oxidized LDL and Cu²⁺-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-tyroine and m-tyroine were found in aortic proteins compared with nondiabetic monkeys (11). In vitro studies have shown that proteins that are exposed to glucose (or reactive carboxyls 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 Cu²⁺-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 Cu²⁺-oxidized LDL has been
reported to lead to activation of the ERK pathway (40, 41), PKCβ and PKCζ (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 royally 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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