Advanced glycation end products potentiate the stimulatory effect of glucose on macrophage lipoprotein lipase expression

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Abstract **Lipoprotein lipase (LPL) secreted by macrophages in the arterial wall promotes atherosclerosis. We have shown that macrophages of patients with type 2 diabetes overproduce LPL and that metabolic factors, including glucose, stimulate macrophage LPL secretion. In this study, we determined the effect of advanced glycation end products (AGEs) on LPL expression by macrophages cultured in a high-glucose environment and the molecular mechanisms underlying this effect. Our results demonstrate that AGEs potentiate the stimulatory effect of high glucose on murine and human macrophage LPL gene expression and secretion. Induction of macrophage LPL mRNA levels by AGEs was identical to that elicited by physiologically relevant modified albumin and was inhibited by anti-AGE receptor as well as by antioxidants. Treatment of macrophages with AGEs resulted in protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) activation. Inhibition of these kinases abolished the effect of AGEs on LPL mRNA levels. Finally, exposure of macrophages to AGEs increased the binding of nuclear proteins to the activated protein-1 consensus sequence of the LPL promoter. This effect was inhibited by PKC and MAPK inhibitors. These results demonstrate for the first time that AGEs potentiate the stimulatory effect of high glucose on macrophage LPL expression. This effect appears to involve oxidative stress and PKC/MAPK activation.**—Beauchamp, M-C., S-É. Michaud, L. Li, M. R. Sartippour, and G. Renier. **Advanced glycation end products potentiate the stimulatory effect of glucose on macrophage lipoprotein lipase expression.** ***J. Lipid Res.*** **2004.** 45: **1749–1757.**

Supplementary key words oxidative stress • kinases • diabetes • atherosclerosis

Atherosclerosis is the leading cause of death in the Western world and the major complication of type 2 diabetes (1). Despite the well-documented association between diabetes and cardiovascular diseases, the reasons for the increased prevalence of atherosclerosis associated with type 2 diabetes are not well known.

Lipoprotein lipase (LPL) is a key enzyme in lipid metabolism that is secreted by macrophages and smooth muscle cells in the atherosclerotic lesion (2, 3). Evidence indicates that LPL secreted by macrophages in the arterial intima is proatherogenic. Indeed, LPL acts as an atherogenic ligand that associates with lipoproteins, thereby favoring their uptake by vascular cells as well as their retention to the subendothelial matrix (4–6). This enzyme also stimulates the production of the proinflammatory cytokine tumor necrosis factor-α (7, 8), increases monocyte adhesion to endothelial cells (9–11), and enhances the proliferation of vascular smooth muscle cells (12). Finally, recent studies have demonstrated that macrophage LPL promotes foam cell formation and atherosclerosis in vivo (13–16).

We have previously shown that macrophages of type 2 diabetic patients overproduce LPL and that peripheral factors play a key role in this alteration (17). We have also recently proposed a role for glucose, fatty acids, and homocysteine as macrophage LPL-stimulatory factors in human diabetes (18–20). Besides these factors, advanced glycation end products (AGEs) may play a role in the induction of macrophage LPL in human diabetes. Indeed, AGEs accumulate in the plasma and tissues of type 2 diabetic patients (21) and exert proatherogenic effects (22, 23). In the present study, we sought to determine the effect of AGEs on glucose-induced macrophage LPL expression. Our study demonstrates that AGEs potentiate the stimulatory effect of glucose on macrophage LPL expression. This effect appears to involve oxidative stress, protein kinase C (PKC) and mitogen-activated protein kinase (MAPK)-dependent pathways, and activated protein-1 (AP-1) activation.

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METHODS

Reagents

FBS was purchased from Wisent (St. Bruno, Quebec, Canada). RPMI 1640 medium, DMEM, and Trizol reagent were purchased from Life Technologies (Burlington, Ontario, Canada). Calphostin C, *N*-acetyl-L-cysteine (NAC), GF109203X, U0126, and PD98059 were obtained from Calbiochem (La Jolla, CA). Affinity-purified polyclonal antibody against AGE receptor (RAGE) (M-20), CD36 (24), and extracellular signal-regulated protein kinase (ERK) 1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-scavenger receptor-A (SR-A) (25) and anti-phospho-ERK1/2 antibodies were obtained from Serotec (Raleigh, NC) and Cell Signaling Technology (Pickering, Ontario, Canada), respectively. Irrelevant mouse IgG_1 was purchased from R&D Systems (Minneapolis, MN). The anti-FC-γ receptor II antibody was kindly provided by Dr. M. Sarfati (University of Montreal, Montreal, Quebec, Canada). Immunoglobulin- and fatty acid-free BSA was obtained from Sigma (St. Louis, MO). LY379196 was kindly provided by Eli Lilly (Indianapolis, IN). U0126, LY379196, PD98059, and GF109203X were dissolved in 0.02, 0.03, 0.3, and 0.08% DMSO, respectively. DMSO used at these concentrations had no impact on LPL mRNA expression (data not shown).

Murine macrophages

The J774 murine macrophage cell line was obtained from the American Type Culture Collection (ATCC; Rockville, MD). J774 cells were cultured in DMEM containing 10% FBS and 100 μg/ml penicillin-streptomycin (Life Technologies).

Human macrophages

Human monocytes were isolated as previously described (26). Differentiation of monocytes into monocyte-derived macrophages was achieved by culturing monocytes in RPMI 1640 medium supplemented with 1% (v/v) penicillin-streptomycin and 20% (v/v) autologous serum. The cells were incubated for 8 days at 37°C in a humidified 5% CO_2, 95% air atmosphere. The culture medium was changed at days 4 and 8.

Preparation of AGEs

Immunoglobulin- and fatty acid-free BSA (low endotoxin) was subjected to nonenzymatic glycation by incubation with 0.5 mol/l (AGEs) or 50 mmol/l glucose (GlyBSA) in 0.4 mol/l sodium phosphate buffer containing 0.5 mmol/l EDTA. The solutions were sterile filtered by passage through a 0.2 μm filter and then incubated at 37°C for 6 weeks under aerobic conditions. Nonglycated albumin was obtained by incubating BSA in the same reaction mixture in the absence of glucose. At the end of the incubation period, samples were extensively dialyzed against 10 mmol/l PBS, pH 7.4, at 4°C to remove unreacted glucose. The presence of AGEs was confirmed by the typical absorption and fluorescent spectra patterns of these proteins (27, 28). The intensity of fluorescence of AGEs at excitation of 370 nm and emission of 440 nm was increased by ~3- and 6-fold in comparison with GlyBSA and nonglycated BSA, respectively. The endotoxin content of the BSA and AGE preparations (200 μg/ml) was determined by the Limulus Amebocyte Lysate Assay (Sigma) and was consistently found to be lower than 0.003 ng/ml. Cell viability as assessed by Trypan blue exclusion was unaffected by BSA or AGEs (200 μg/ml) [cell viability (% of control values): BSA, 91.7 ± 1.7; AGEs, 93.2 ± 1.5].

Analysis of LPL mRNA expression

RT-PCR. Total RNA for use in the PCR was extracted from human macrophages by an improvement of the acid-phenol technique of Chomczynski and Sacchi (29), precipitated, and resuspended in diethyl pyrocarbonate water. cDNA was synthesized from RNA by incubating 1 μg of total cellular RNA with 0.1 μg of oligo(dT) (Pharmacia, Piscataway, NJ) for 5 min at 98°C and then incubating the mixture with reverse transcription buffer for 60 min at 37°C. The cDNA obtained was amplified and analyzed as described previously (17).

Northern blot. Six million J774 macrophages were plated on plastic petri dishes (100 × 20 mm; Falcon, Lincoln Park, NJ). After treatment, cells were lysed with Trizol reagent. Total RNA was isolated and separated on a 1.2% agarose gel. The blots were prehybridized, then mRNA expression was analyzed by hybridization with [^{32}P]dCTP-labeled LPL and S28 cDNA probes. Hybridization was detected by autoradiography with Kodak X-Omat-AR film (Rochester, NY). mRNA expression was quantified by high-resolution optical densitometry (Alpha Imager 2000; Packard Instruments, Meriden, CT).

Determination of human LPL immunoreactive mass and activity

One hour before the end of the incubation period, 50 U/ml heparin was added to the medium. The amount of LPL immunoreactive mass in the supernatants was measured by enzyme-linked immunosorbent assay using the Markit-F LPL kit (Dainippon Pharmaceutical, Osaka, Japan) (30). Extracellular LPL activity was determined using the Confluolip kit (Progen, American Research Products) (31). LPL mass and activity values were normalized to the levels of total cell proteins.

Measurement of PKC activity

PKC activity was measured in cytosolic and particulate fractions of the cells using myelin basic protein as kinase substrate as previously described (20). PKC activity was normalized to the levels of total cell proteins. Data were expressed as percentages considering the control as 100% activity.

Western blot analysis

Protein extracts (10 μg) were applied to 10% SDS-PAGE and transferred to a nitrocellulose membrane. Nonspecific binding was blocked with 5% nonfat dry milk for 1 h at room temperature. After washing with PBS-Tween 0.1%, blots were incubated overnight with either an anti-ERK1/2 or an anti-phospho-ERK1/2 antibody. After further washing, membranes were incubated for 1 h at room temperature with a horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:5,000). Antigen detection was performed with an enhanced chemiluminescence detection system (Amersham). Protein expression was quantified by high-resolution optical densitometry (Alpha Imager 2000; Packard Instruments).

Electrophoretic mobility shift assay

The isolation of the nuclei and the DNA binding assay were performed as described previously (20).

DNA probes

The cDNA probe for the detection of murine LPL was prepared by the PCR technique. cDNA was obtained from total RNA using a reverse transcription reaction. Two synthetic primers spanning bases 255–287 and 1117–1149 of the LPL cDNA were used to enzymatically amplify a 894 bp region of the LPL probe. The cDNA probe for murine S28 was purchased from the ATCC. A 20-mer double-stranded oligonucleotide (5′-GGGCACCTGACTAAGGCCAG-3′; 5′-TGTGCTGGCCTTAGTCAGGT-3′) containing the consensus sequence for the AP-1-responsive element of the murine LPL gene promoter (32) was synthesized with the aid of an automated DNA synthesizer. After annealing, the double-stranded oligonucleotide was labeled with [γ-^{32}P]ATP using the Boehringer Mannheim 5′ end-labeling kit (Indianapolis, IN).

Determination of total protein concentrations

Total protein content was measured according to the Bradford method (33) using a colorimetric assay (Bio-Rad, Mississauga, Ontario, Canada).

Statistical analysis

All values are expressed as means ± SEM. For single comparisons, data were analyzed by Student's *t*-test. For multiple comparisons, data were analyzed by one-way ANOVA followed by the Student-Newman-Keuls test or Dunn's method. $P < 0.05$ was considered significant.

RESULTS

Effect of AGEs on LPL mRNA levels and secretion by J774 macrophages

J774 murine macrophages were incubated for 24 or 72 h in culture medium containing low (5.6 mmol/l) or high (20 mmol/l) glucose concentrations in the presence or absence of BSA or AGEs. At the end of the incubation period, LPL mRNA levels and secretion were measured. Treatment of J774 macrophages for 24 h with BSA did not alter LPL mRNA levels regardless of the experimental conditions used (**Fig. 1A**). AGEs did not affect the levels of LPL mRNA in J774 macrophages cultured in a low-glucose medium, whereas both AGEs and GlyBSA (200 μg/ml) increased LPL mRNA levels in macrophages cultured in a high-glucose environment (Fig. 1A). The effect of AGEs on LPL mRNA levels was dose dependent, with maximal effect at a concentration of 200 μg/ml [LPL mRNA levels (% of control values): AGE (50 μg/ml), 108.6 ± 9.9; AGE (100 μg/ml), 137.5 ± 10.6 ($P < 0.05$); AGE (200 μg/ml), 162.1 ± 10.1 ($P < 0.01$)]. No modulation of LPL mRNA levels was observed when cells were treated with AGEs in the presence of 20 mmol/l D-mannitol (data not shown). Recovery of enhanced amounts of LPL immu-

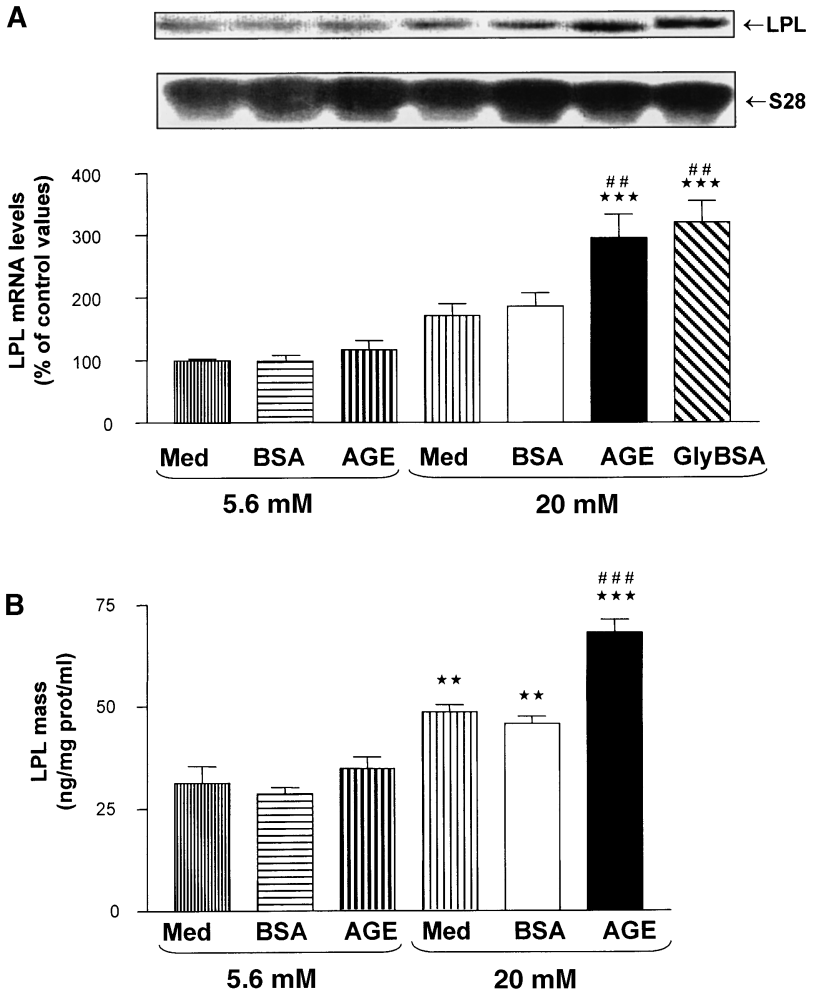

Fig. 1. Effect of advanced glycation end products (AGEs) on LPL mRNA levels and secretion in J774 macrophages. J774 cells were cultured for 24 h (LPL mRNA) or 72 h (LPL mass) in 5.6 or 20 mmol/l glucose in the presence of BSA, AGEs, or GlyBSA (200 μg/ml). At the end of these incubation periods, cells were lysed and total RNA was extracted and analyzed by Northern blot for LPL and S28 mRNA expression. A: LPL mRNA levels were normalized to the levels of S28 mRNA. *** $P < 0.001$ versus 5.6 mM medium (Med); ## $P < 0.01$ versus 20 mM medium. B: LPL mass was measured in supernatants and normalized to the levels of total cell proteins. ** $P < 0.01$, *** $P < 0.001$ versus 5.6 mM medium; ### $P < 0.001$ versus 20 mM medium.

noreactive mass in the supernatants of cells treated with AGEs for 72 h reflected the increase in macrophage LPL mRNA expression in response to AGEs (Fig. 1B). In contrast, no effect of AGEs on extracellular LPL activity was observed (data not shown).

Effect of AGEs on LPL mRNA levels and secretion by human macrophages

To assess the physiological relevance of our findings in J774 macrophages, we next determined the effect of AGEs on LPL gene expression and secretion by human macrophages cultured for 24 to 72 h in the presence of 5.6 or 20 mmol/l glucose. As documented in J774 macrophages, AGEs did not change LPL mRNA expression in human macrophages exposed for 24 h to a low-glucose environment (data not shown). In contrast, an increase in LPL mRNA expression was found when human macrophages were incubated for 24 h with AGEs or GlyBSA (200 μg/ml) in the presence of 20 mmol/l glucose (**Fig. 2A**). Although AGEs did not change LPL secretion by macrophages exposed for 24 or 48 h to a high-glucose environment (data not shown), a significant ($P = 0.03$) stimulatory effect of AGEs on LPL immunoreactive mass was observed in human macrophages exposed to high glucose for 72 h (Fig. 2B). Levels of LPL activity secreted by AGE-treated macrophages remained unchanged regardless of the experimental conditions applied (data not shown).

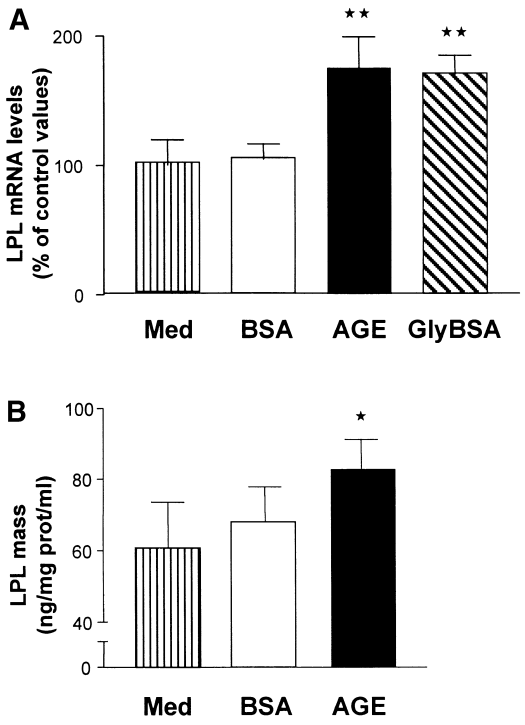

Fig. 2. Effect of AGEs on LPL mRNA levels and secretion in human monocyte-derived macrophages. Human monocyte-derived macrophages were cultured for 24 h (LPL mRNA) or 72 h (LPL mass) in 20 mmol/l glucose in the presence of BSA, AGEs, or GlyBSA (200 μg/ml). At the end of these incubation periods, cells were lysed and total RNA was extracted. LPL and GAPDH mRNA expression were analyzed by RT-PCR. A: LPL mRNA levels were normalized to the levels of GAPDH mRNA. B: LPL mass was measured in supernatants and normalized to the levels of total cell proteins. Results represent means ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$ versus medium.

Role of RAGEs in AGE-induced macrophage LPL gene expression

To determine the nature of the RAGEs mediating the stimulatory effect of AGEs on macrophage LPL expression, J774 cells cultured in a high-glucose medium were pretreated for 1 h with anti-RAGE, SR-A, or CD36 antibodies (1 μg/ml) before exposure to BSA or AGE (200 μg/ml). Immunoneutralization of RAGE totally abolished AGE-induced macrophage LPL gene expression (**Fig. 3**). In contrast, neither anti-SRA and anti-CD36, nor irrelevant IgG_1 and anti-FC-γ receptor II antibodies, significantly reduced LPL gene expression (data not shown). A similar inhibitory effect of RAGE antibody was observed in human macrophages [LPL mRNA levels (% of control values): AGEs, 174.9 ± 24.4; RAGE + AGEs, 77.0 ± 32.91 ($P < 0.05$ vs. AGEs)].

Effect of antioxidants on AGE-induced macrophage LPL mRNA levels

To assess the role of oxidative stress as a mediator of the stimulatory effect of AGEs on LPL mRNA levels, J774 cells were pretreated for 1 h with the antioxidant DMSO (0.5%) or NAC (10 mmol/l) and then exposed to AGEs

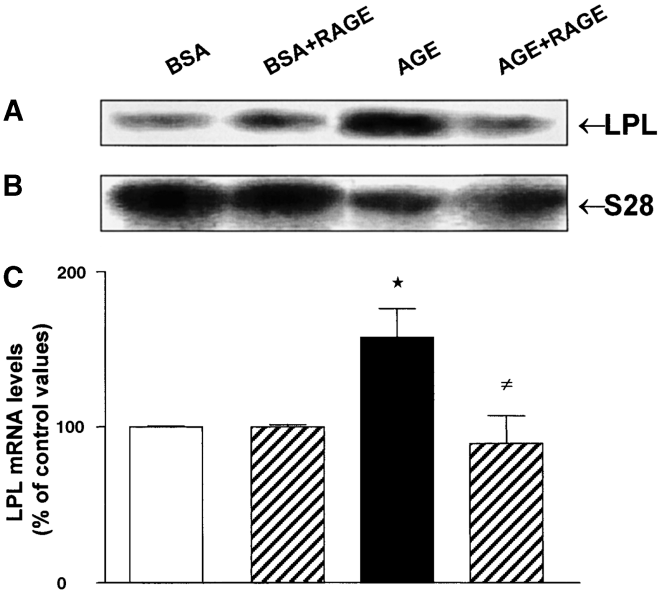

Fig. 3. Role of AGE receptor (RAGE) on AGE-induced macrophage LPL mRNA expression. J774 cells were preincubated for 1 h with antibody against RAGE (1 μg/ml) and then incubated for 24 h in the presence of 20 mmol/l glucose with BSA or AGEs (200 μg/ml). At the end of these incubation periods, total cellular RNA was extracted and analyzed by Northern blot for LPL (A) and S28 (B) mRNA expression. C: LPL mRNA levels were normalized to the levels of S28 mRNA. Results represent means ± SEM of four independent experiments. * $P < 0.05$ versus BSA; $^{\neq}$ $P < 0.05$ versus AGEs.

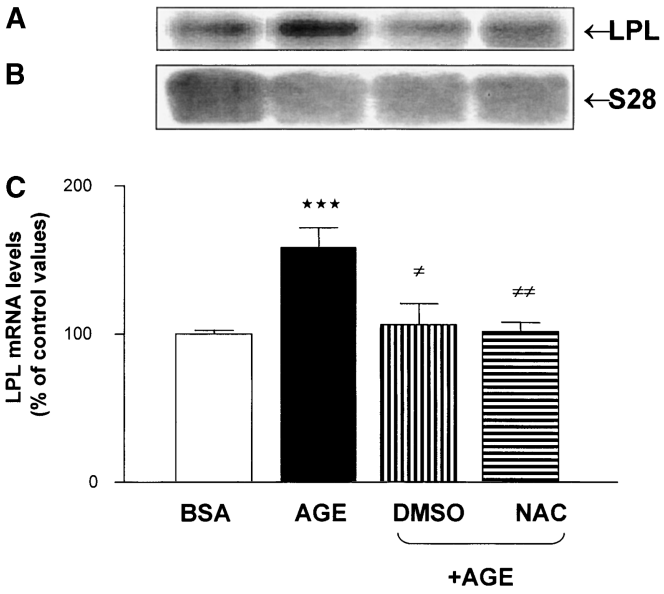

Fig. 4. Effect of antioxidants on AGE-induced macrophage LPL mRNA expression. J774 cells were preincubated in the presence of 0.5% DMSO or 10 mmol/l *N*-acetyl-L-cysteine (NAC) for 1 h and then cultured for 24 h in 20 mmol/l glucose in the presence of BSA or AGEs (200 μg/ml). At the end of these incubation periods, total cellular RNA was extracted and analyzed by Northern blot for LPL (A) and S28 (B) mRNA expression. C: LPL mRNA levels were normalized to the levels of S28 mRNA. Results represent means ± SEM of four independent experiments. *** $P < 0.001$ versus BSA; $^{\neq}$ $P < 0.05$, $^{\neq\neq}$ $P < 0.01$ versus AGEs.

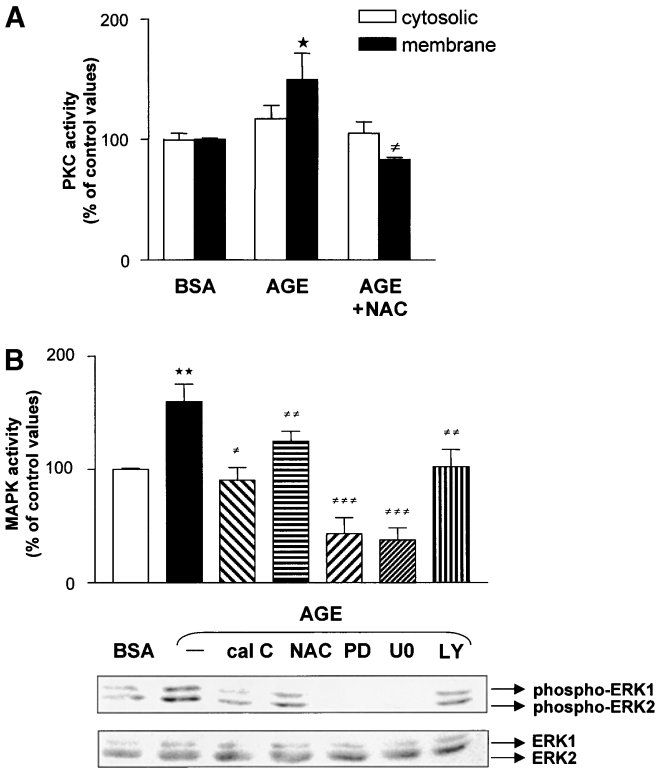

Fig. 5. Effect of AGEs on protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) activation in J774 macrophages. J774 cells were pretreated or not with NAC (10 mmol/l), calphostin C (cal C; 0.1μg/ml), PD98059 (PD; 100 μmol/l), U0126 (U0; 20 μmol/l), or LY379196 (LY; 30 nmol/l) for 1 h and then incubated with 20 mmol/l glucose in the presence of BSA or AGEs (200 μg/ml) for 30 min. A: PKC activity in cytosolic and particulate fractions was determined as described in Methods. Results represent means ± SEM of three independent experiments. * $P < 0.05$ versus BSA; $^{\neq}$ $P < 0.05$ versus AGEs. B: Extracellular signal-regulated protein kinase (ERK) phosphorylation was assessed by Western blot using phospho-specific or specific ERK1/2 antibodies. Results represent means ± SEM of four independent experiments. One representative blot is shown. ** $P < 0.01$ versus BSA; $^{\neq}$ $P < 0.05$, $^{\neq\neq}$ $P < 0.01$, $^{\neq\neq\neq}$ $P < 0.001$ versus AGEs.

for 24 h. As shown in **Fig. 4**, pretreatment of the cells with these agents negated the stimulatory effect of AGEs on LPL mRNA expression. A similar inhibitory effect of these antioxidants was noted in human macrophages [LPL mRNA levels (% of control values): AGEs, 174.9 ± 24.4; NAC + AGEs, 88.3 ± 31.5 ($P < 0.05$ vs. AGEs); DMSO + AGEs, 63.5 ± 14.6 ($P < 0.01$ vs AGEs)].

Role of PKC and MAPK as intracellular signaling pathways mediating the stimulatory effect of AGEs on macrophage LPL mRNA expression

To evaluate the role of PKC and MAPK in AGE-induced LPL gene expression, we first measured PKC and ERK1/2 phosphorylation in J774 macrophages treated for up to 30 min with AGEs. As shown in **Fig. 5A**, treatment of J774 macrophages with AGEs increased membrane PKC activity in these cells by 1.5-fold. This effect was totally reversed by preincubation of the cells with NAC (Fig. 5A). AGEs also induced ERK1/2 activation as assessed by immunoblot analysis of phosphospecific ERK1/2 (Fig. 5B). The maximal effect of AGEs on ERK1/2 activation was found at 30 min (data not shown). AGE-induced ERK1/2 activation was inhibited by calphostin C, LY379196, NAC, U0126, and PD98059 (Fig. 5B). In contrast, inhibitors alone or solvent controls did not affect basal or AGE-induced ERK1/2 activation, respectively (data not shown). To further investigate the involvement of PKC and MAPK in AGE-induced LPL gene expression, J774 and human macrophages were pretreated with the specific PKC and MAPK inhibitors calphostin C (0.1 μg/ml) and PD98059 (100 μmol/l), respectively. These compounds inhibited the induction of the LPL gene by AGEs in both murine (**Fig. 6**) and human cells [human macrophage LPL mRNA levels (% of control values): AGEs, 174.9 ± 24.4; calphostin C + AGEs, 83.67 ± 13.04; PD98059 + AGEs, 89.33 ± 14.68 ($P < 0.05$ vs. AGEs)]. A similar effect was noted when J774 macrophages were incubated with the classic PKC inhibitor GF109203X and the MAPK inhibitor U0126 (data not shown). To determine the role of PKC-β in the regulation of macrophage LPL gene expression by AGEs, J774 cells were pretreated with the specific PKC-β inhibitor LY379196 (30 nmol/l) before exposure to AGEs. As shown in Fig. 6, this compound totally inhibited AGE-induced LPL mRNA expression. None of these inhibitors affected basal LPL mRNA expression (data not shown).

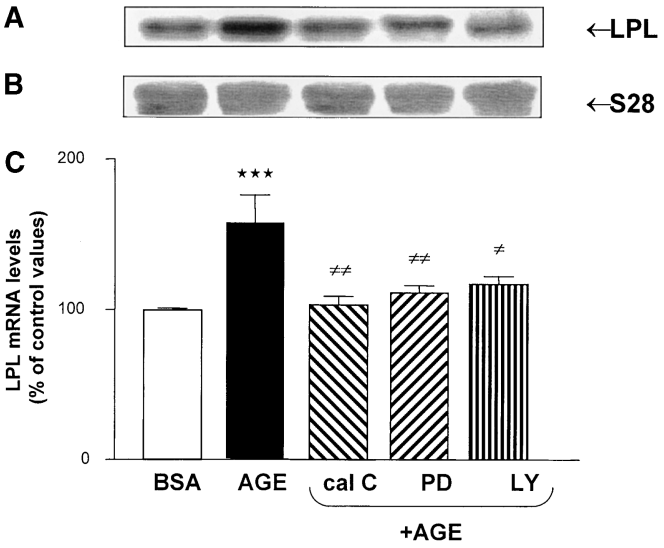

Fig. 6. Effect of PKC and ERK1/2 inhibitors on AGE-induced macrophage LPL mRNA expression. J774 cells were preincubated for 1 h with the PKC inhibitors calphostin C (cal C; 0.1 μg/ml) and LY379196 (LY; 30 nmol/l) or with the ERK1/2 inhibitor PD98059 (PD; 100 μmol/l) and then cultured for 24 h in 20 mmol/l glucose in the presence of BSA or AGEs (200 μg/ml). At the end of these incubation periods, total cellular RNA was extracted and LPL (A) and S28 (B) mRNA expression was analyzed by Northern blot. C: LPL mRNA levels were normalized to the levels of S28 mRNA. Results represent means ± SEM of three independent experiments. *** $P < 0.01$ versus BSA; $^{\neq}P < 0.05$, $^{\neq\neq}P < 0.01$ versus AGEs.

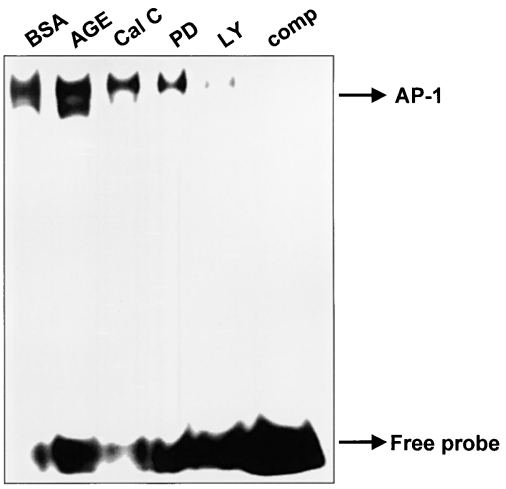

Fig. 7. Effect of AGEs on the binding of nuclear proteins extracted from J774 cells to the regulatory activated protein-1 (AP-1) sequence of the murine LPL gene promoter. J774 cells were preincubated for 1 h with the PKC inhibitors calphostin C (cal C; 0.1 μg/ml) and LY379196 (LY; 30 nmol/l) or with the ERK1/2 inhibitor PD98059 (PD; 100 μmol/l) and then cultured for 16 h in 20 mmol/l glucose in the presence of BSA or AGE (200 μg/ml). The nuclear proteins isolated from these cells were incubated with the double-stranded AP-1 regulatory element of the LPL gene promoter. Retardation was assessed by gel electrophoresis. Results from one representative experiment out of four are shown. comp, competitor.

Effect of AGEs on the binding of nuclear proteins to the regulatory AP-1 sequence of the murine LPL gene promoter

We next determined whether incubation of J774 cells in the presence of AGEs might induce changes at the level of the LPL gene promoter binding proteins. We found that a 16 h exposure of J774 cells to AGEs led to an increase in the binding of nuclear proteins to the AP-1 consensus sequence of the LPL promoter (**Fig. 7**). This binding complex was specifically competed for in the presence of a 1,000-fold molar excess of the unlabeled AP-1 oligonucleotide. To assess the role of PKC and ERK1/2 in this effect, the effects of calphostin C, PD98059, and LY379196 on AGE-induced AP-1 activation were further evaluated. As shown in Fig. 7, these compounds inhibited the enhanced nuclear proteins binding to AP-1. These results suggest that AP-1 binding to the LPL promoter occurs downstream of PKC and MAPK activation in response to AGEs.

DISCUSSION

Previous studies have demonstrated that the interaction of AGEs with RAGEs expressed on macrophages induces several biological events linked to the development of vascular lesions (34–36). Among these, increased monokine secretion has been documented in AGE-treated human monocytes and macrophages (34, 36). Interestingly, it has also been reported that these compounds act synergistically with high glucose to enhance human monocyte tumor necrosis factor-α and interleukin-6 production (36). In accordance with these data, our study demonstrates that AGEs, although ineffective per se, act synergistically with glucose to induce macrophage LPL secretion. These results further emphasize the critical role of AGEs as key regulators of macrophage function.

On the basis of our previous results showing that high glucose stimulates macrophage LPL at the transcriptional level, we evaluated whether the AGEs' effect on macrophage LPL secretion may involve transcriptional events. Our findings that changes in LPL mRNA levels in AGE-treated macrophages correlate with changes in LPL secretion support this possibility. Among the signaling pathways initiated by the AGEs' interaction with vascular cells, the induction of oxidative stress and the activation of kinases have captured considerable interest (37–39). Evidence that oxidant stress and PKC are involved in the regulation of macrophage LPL (40–42) suggests a role for these events in the induction of macrophage LPL in response to AGEs. Our results demonstrating that antioxidants inhibit AGE-induced macrophage LPL gene expression indicate that oxidative stress evoked by the AGEs-macrophage interaction is involved in this process.

Recent data have demonstrated that AGEs activate PKC and MAPK in mesangial cells and human monocytes, respectively (37, 39). In accordance with these results, we found that AGEs induce PKC activity in macrophages and that this event is required for the transcriptional effect of AGEs on macrophage LPL gene expression. Evidence that

the PKCβ isoform-specific inhibitor LY379196 inhibits AGE-induced LPL mRNA levels further supports a role for PKCβ in this effect. This finding, together with the previously documented effect of AGEs on PKCβ activation in vascular renal cells (37), emphasizes the critical role of this PKC isoform in diabetic vascular complications (43, 44). The requirement of high glucose levels to see increased LPL by AGEs may result from the differential kinetic pattern of PKC activation in response to glucose and AGEs. Indeed, in contrast to the rapid and transient activation of PKC that we reported in AGE-treated macrophages, we found that PKC activation in high-glucose-treated macrophages was evident only after several hours (unpublished observations). From these data, it is tempting to speculate that AGEs, although ineffective per se, may induce macrophage LPL by speeding up PKC activation in high-glucose-treated macrophages. Because oxidative stress induces PKC (45), activation of PKC in AGE-treated macrophages may result from the generation of reactive oxygen species. This possibility is supported by our results showing that NAC totally suppresses PKC activation in AGE-treated macrophages. Among the major downstream targets of PKC in human diabetes, MAPK is likely to constitute an important point of regulation of vascular cells. Indeed, it has been demonstrated that coactivation of PKC and MAPK occurs in cells maintained in high glucose (44, 46) and that PKC can activate MAPK (47, 48). Furthermore, evidence has been provided that AGEs activate ERK in human monocytes (39) and that MAPK isoforms constitute downstream effectors of AGE-induced PKC activation in smooth muscle cells (49). Our study clearly demonstrates that AGEs activate ERK1/2 in macrophages and suggests a role of this signaling pathway in AGE-induced macrophage LPL expression. Despite these observations, the use of pharmacologic inhibitors, particularly at high concentrations, can be misleading, and further studies are required to discern the precise role of PKC and ERK1/2 in this effect.

Recent studies have demonstrated that AGEs increase the activities of two major transcription factors involved in the regulation of the macrophage LPL gene, namely AP-1 (49, 50) and peroxisome proliferator-activated receptors (PPARs) (52). Evidence that AGEs activate PKC and MAPK in macrophages and that these kinases regulate the activities of AP-1 (52, 53) and PPARs (54) suggests a potential role of these transcription factors as mediators of the transcriptional effect of AGEs on macrophage LPL gene expression. Our results demonstrating that AGEs enhance the binding of nuclear proteins to the AP-1 sequence of the LPL gene and that this effect is abrogated by PKC and MAPK inhibitors support this hypothesis.

Binding and uptake of AGE proteins by macrophages is mediated by various receptors, including SR-A, CD36, and RAGE (55–57). Evidence that blockade of RAGE, but not of other AGE receptors, inhibits the stimulatory effect of AGEs on LPL indicates that AGEs operate via this receptor to induce macrophage LPL.

Although the potentiation of high-glucose effects by AGEs may be relevant for diabetic vascular complications, the significance of our observations to atherogenesis remains unclear. Given the very significant stimulation of LPL expression by high glucose alone that we previously documented at 96 and 120 h (18), the relatively modest effect of AGEs on glucose-induced LPL mass at 72 h may not have much physiological consequence. It remains to be determined whether AGEs isolated from diabetic sources at concentrations found in the arterial wall produce a similar effect. Finally, lack of an AGE effect on macrophage LPL activity may lessen the relevance of our data to atherogenesis. Although evidence that noncatalytically active LPL promotes atherogenesis partly addresses this issue (58), further studies are clearly needed to evaluate the physiological consequences of the AGE effect that we report here. In conclusion, this study demonstrates that AGEs potentiate the stimulatory effect of high glucose on macrophage LPL expression. This effect is mediated through oxidative stress and appears to require PKC and MAPK activation.

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REFERENCES

1. Savage, P. J. 1998. Treatment of diabetes mellitus to reduce its chronic cardiovascular complications. *Curr. Opin. Cardiol.* **13:** 131–138.
2. O'Brien, K. D., D. Gordon, S. Deeb, M. Ferguson, and A. Chait. 1992. Lipoprotein lipase is synthesized by macrophage-derived foam cells in human coronary atherosclerotic plaques. *J. Clin. Invest.* **89:** 1544–1550.
3. Yla-Herttuala, S., B. A. Lipton, M. E. Rosenfeld, I. J. Goldberg, D. Steinberg, and J. L. Witztum. 1991. Macrophages and smooth muscle cells express lipoprotein lipase in human and rabbit atherosclerotic lesions. *Proc. Natl. Acad. Sci. USA.* **88:** 10143–10147.
4. Eisenberg, S., E. Sehayek, T. Olivecrona, and I. Vlodavsky. 1992. Lipoprotein lipase enhances binding of lipoproteins to heparan sulfate on cell surfaces and extracellular matrix. *J. Clin. Invest.* **90:** 2013–2021.
5. Saxena, U., M. G. Klein, T. M. Vanni, and I. J. Goldberg. 1992. Lipoprotein lipase increases low density lipoprotein retention by subendothelial cell matrix. *J. Clin. Invest.* **89:** 373–380.
6. Olivecrona, G., and T. Olivecrona. 1995. Triglyceride lipases and atherosclerosis. *Curr. Opin. Lipidol.* **6:** 291–305.
7. Renier, G., E. Skamene, J. B. DeSanctis, and D. Radzioch. 1994. Induction of tumor necrosis factor alpha gene expression by lipoprotein lipase. *J. Lipid Res.* **35:** 271–278.
8. Mamputu, J. C., and G. Renier. 1999. Differentiation of human monocytes to monocyte-derived macrophages is associated with increased lipoprotein lipase-induced tumor necrosis factor-alpha expression and production: a process involving cell surface proteoglycans and protein kinase C. *Arterioscler. Thromb. Vasc. Biol.* **19:** 1405–1411.
9. Mamputu, J. C., A. C. Desfaits, and G. Renier. 1997. Lipoprotein lipase enhances human monocyte adhesion to aortic endothelial cells. *J. Lipid Res.* **38:** 1722–1729.
10. Saxena, U., N. M. Kulkarni, E. Ferguson, and R. S. Newton. 1992. Lipoprotein lipase-mediated lipolysis of very low density lipoproteins increases monocyte adhesion to aortic endothelial cells. *Biochem. Biophys. Res. Commun.* **189:** 1653–1658.
11. Obunike, J. C., S. Paka, S. Pillarisetti, and I. J. Goldberg. 1997. Lipoprotein lipase can function as a monocyte adhesion protein. *Arterioscler. Thromb. Vasc. Biol.* **17:** 1414–1420.

12. Mamputu, J. C., L. Levesque, and G. Renier. 2000. Proliferative effect of lipoprotein lipase on human vascular smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* **20:** 2212–2219.
13. Babaev, V. R., S. Fazio, L. A. Gleaves, K. J. Carter, C. F. Semenkovich, and M. F. Linton. 1999. Macrophage lipoprotein lipase promotes foam cell formation and atherosclerosis in vivo. *J. Clin. Invest.* **103:** 1697–1705.
14. Babaev, V. R., M. B. Patel, C. F. Semenkovich, S. Fazio, and M. F. Linton. 2000. Macrophage lipoprotein lipase promotes foam cell formation and atherosclerosis in low density lipoprotein receptor-deficient mice. *J. Biol. Chem.* **275:** 26293–26299.
15. Semenkovich, C. F., T. Coleman, and A. Daugherty. 1998. Effects of heterozygous lipoprotein lipase deficiency on diet-induced atherosclerosis in mice. *J. Lipid Res.* **39:** 1141–1151.
16. Van Eck, M., R. Zimmermann, P. H. Groot, R. Zechner, and T. J. van Berkel. 2000. Role of macrophage-derived lipoprotein lipase in lipoprotein metabolism and atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **20:** E53–E62.
17. Sartippour, M. R., and G. Renier. 2000. Upregulation of macrophage lipoprotein lipase in patients with type 2 diabetes: role of peripheral factors. *Diabetes.* **49:** 597–602.
18. Sartippour, M. R., A. Lambert, M. Laframboise, P. St. Jacques, and G. Renier. 1998. Stimulatory effect of glucose on macrophage lipoprotein lipase expression and production. *Diabetes.* **47:** 431–438.
19. Michaud, S. E., and G. Renier. 2001. Direct regulatory effect of fatty acids on macrophage lipoprotein lipase: potential role of PPARs. *Diabetes.* **50:** 660–666.
20. Beauchamp, M-C., and G. Renier. 2002. Homocysteine induces protein kinase C activation and stimulates c-Fos and lipoprotein lipase expression in macrophages. *Diabetes.* **51:** 1180–1187.
21. Wautier, J. L., and P. J. Guillausseau. 1998. Diabetes, advanced glycation endproducts and vascular disease. *Vasc. Med.* **3:** 131–137.
22. Kume, S., M. Takeya, T. Mori, N. Araki, H. Suzuki, S. Horiuchi, T. Kodama, Y. Miyauchi, and K. Takahashi. 1995. Immunohistochemical and ultrastructural detection of advanced glycation end products in atherosclerotic lesions of human aorta with a novel specific monoclonal antibody. *Am. J. Pathol.* **147:** 654–667.
23. Park, L., K. G. Raman, K. J. Lee, Y. Lu, L. J. Ferran, Jr., W. S. Chow, D. Stern, and A. M. Schmidt. 1998. Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. *Nat. Med.* **4:** 1025–1031.
24. Kuniyasu, A., S. Hayashi, and H. Nakayama. 2002. Adipocytes recognize and degrade oxidized low density lipoprotein through CD36. *Biochem. Biophys. Res. Commun.* **295:** 319–323.
25. Chao, S. K., R. F. Hamilton, J. C. Pfau, and A. Holian. 2001. Cell surface regulation of silica-induced apoptosis by the SR-A scavenger receptor in a murine lung macrophage cell line (MH-S). *Toxicol. Appl. Pharmacol.* **174:** 10–16.
26. Mentzer, S. J., P. M. Guyre, S. J. Burakoff, and D. V. Faller. 1986. Spontaneous aggregation as a mechanism for human monocyte purification. *Cell. Immunol.* **101:** 312–319.
27. Pongor, S., P. C. Ulrich, F. A. Bencsath, and A. Cerami. 1984. Aging of proteins: isolation and identification of a fluorescent chromophore from the reaction of polypeptides with glucose. *Proc. Natl. Acad. Sci. USA.* **81:** 2684–2688.
28. Yamagishi, S. I., H. Yonekura, Y. Yamamoto, K. Katsuno, F. Sato, I. Mita, H. Ooka, N. Satozawa, T. Kawakami, M. Nomura, and H. Yamamoto. 1997. Advanced glycation end products-driven angiogenesis in vitro. *J. Biol. Chem.* **272:** 8723–8730.
29. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162:** 156–159.
30. Ikeda, Y., A. Takagi, Y. Ohkaru, K. Nogi, T. Iwanaga, S. Kurooka, and A. Yamamoto. 1990. A sandwich-enzyme immunoassay for the quantification of lipoprotein lipase and hepatic triglyceride lipase in human postheparin plasma using monoclonal antibodies to the corresponding enzymes. *J. Lipid Res.* **31:** 1911–1924.
31. Duque, M., M. Graupner, H. Stutz, I. Wicher, R. Zechner, F. Paltauf, and A. Hermetter. 1996. New fluorogenic triacylglycerol analogs as substrates for the determination and chiral discrimination of lipase activities. *J. Lipid Res.* **37:** 868–876.
32. Hua, X. X., S. Enerback, J. Hudson, K. Youkhana, and J. M. Gimble. 1991. Cloning and characterization of the promoter of the murine lipoprotein lipase-encoding gene: structural and functional analysis. *Gene.* **107:** 247–258.
33. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
34. Vlassara, H., M. Brownlee, K. R. Manogue, C. A. Dinarello, and A. Pasagian. 1988. Cachectin/TNF and IL-1 induced by glucose-modified proteins: role in normal tissue remodeling. *Science.* **240:** 1546–1548.
35. Vlassara, H. 1994. Recent progress on the biologic and clinical significance of advanced glycosylation end products. *J. Lab. Clin. Med.* **124:** 19–30.
36. Morohoshi, M., K. Fujisawa, I. Uchimura, and F. Numano. 1995. The effect of glucose and advanced glycosylation end products on IL-6 production by human monocytes. *Ann. N. Y. Acad. Sci.* **748:** 562–570.
37. Scivittaro, V., M. B. Ganz, and M. F. Weiss. 2000. AGEs induce oxidative stress and activate protein kinase C-beta(II) in neonatal mesangial cells. *Am. J. Physiol. Renal Physiol.* **278:** F676–F683.
38. Yan, S. D., A. M. Schmidt, G. M. Anderson, J. Zhang, J. Brett, Y. S. Zou, D. Pinsky, and D. Stern. 1994. Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. *J. Biol. Chem.* **269:** 9889–9897.
39. Yeh, C. H., L. Sturgis, J. Haidacher, X. N. Zhang, S. J. Sherwood, R. J. Bjercke, O. Juhasz, M. T. Crow, R. G. Tilton, and L. Denner. 2001. Requirement for p38 and p44/p42 mitogen-activated protein kinases in RAGE-mediated nuclear factor-kappaB transcriptional activation and cytokine secretion. *Diabetes.* **50:** 1495–1504.
40. Auwerx, J. H., S. Deeb, J. D. Brunzell, G. Wolfbauer, and A. Chait. 1989. Lipoprotein lipase gene expression in THP-1 cells. *Biochemistry.* **28:** 4563–4567.
41. Renier, G., A. C. Desfaits, A. Lambert, and R. Mikhail. 1996. Role of oxidant injury on macrophage lipoprotein lipase (LPL) production and sensitivity to LPL. *J. Lipid Res.* **37:** 799–809.
42. Tengku-Muhammad, T. S., T. R. Hughes, A. Cryer, and D. P. Ramji. 1999. Involvement of both the tyrosine kinase and the phosphatidylinositol-3′ kinase signal transduction pathways in the regulation of lipoprotein lipase expression in J774.2 macrophages by cytokines and lipopolysaccharide. *Cytokine.* **11:** 463–468.
43. King, G. L., H. Ishii, and D. Koya. 1997. Diabetic vascular dysfunctions: a model of excessive activation of protein kinase C. *Kidney Int. Suppl.* **60:** 77–85.
44. Kunisaki, M., S. E. Bursell, A. C. Clermont, H. Ishii, L. M. Ballas, M. R. Jirousek, F. Umeda, H. Nawata, and G. L. King. 1995. Vitamin E prevents diabetes-induced abnormal retinal blood flow via the diacylglycerol-protein kinase C pathway. *Am. J. Physiol.* **269:** E239–E246.
45. Taher, M. M., J. G. Garcia, and V. Natarajan. 1993. Hydroperoxide-induced diacylglycerol formation and protein kinase C activation in vascular endothelial cells. *Arch. Biochem. Biophys.* **303:** 260–266.
46. Haneda, M., R. Kikkawa, T. Sugimoto, D. Koya, S. Araki, M. Togawa, and Y. Shigeta. 1995. Abnormalities in protein kinase C and MAP kinase cascade in mesangial cells cultured under high glucose conditions. *J. Diabetes Complications.* **9:** 246–248.
47. Dent, P., W. D. Jarvis, M. J. Birrer, P. B. Fisher, R. K. Schmidt-Ullrich, and S. Grant. 1998. The roles of signaling by the p42/p44 mitogen-activated protein (MAP) kinase pathway: a potential route to radio- and chemo-sensitization of tumor cells resulting in the induction of apoptosis and loss of clonogenicity. *Leukemia.* **12:** 1843–1850.
48. Hattori, Y., H. Kakishita, K. Akimoto, M. Matsumura, and K. Kasai. 2001. Glycated serum albumin-induced vascular smooth muscle cell proliferation through activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by protein kinase C. *Biochem. Biophys. Res. Commun.* **281:** 891–896.
49. Nakamura, N., H. Obayashi, M. Fujii, M. Fukui, K. Yoshimori, M. Ogata, G. Hasegawa, H. Shigeta, Y. Kitagawa, T. Yoshikawa, M. Kondo, M. Ohta, M. Nishimura, T. Nishinaka, and C. Y. Nishimura. 2000. Induction of aldose reductase in cultured human microvascular endothelial cells by advanced glycation end products. *Free Radic. Biol. Med.* **29:** 17–25.
50. Simm, A., G. Munch, F. Seif, O. Schenk, A. Heidland, H. Richter, S. Vamvakas, and R. Schinzel. 1997. Advanced glycation endproducts stimulate the MAP-kinase pathway in tubulus cell line LLC-PK1. *FEBS Lett.* **410:** 481–484.
51. Iwashima, Y., M. Eto, A. Hata, K. Kaku, S. Horiuchi, F. Ushikubi, and H. Sano. 2000. Advanced glycation end products-induced gene expression of scavenger receptors in cultured human mono-

cyte-derived macrophages. *Biochem. Biophys. Res. Commun.* **277:** 368–380.
52. Li, R. C., P. Ping, J. Zhang, W. B. Wead, X. Cao, J. Gao, Y. Zheng, S. Huang, J. Han, and R. Bolli. 2000. PKC epsilon modulates NF-kappaB and AP-1 via mitogen-activated protein kinases in adult rabbit cardiomyocytes. *Am. J. Physiol. Heart Circ. Physiol.* **279:** H1679–H1689.
53. Soh, J. W., E. H. Lee, R. Prywes, and I. B. Weinstein. 1999. Novel roles of specific isoforms of protein kinase C in activation of the c-fos serum response element. *Mol. Cell. Biol.* **19:** 1313–1324.
54. Hu, E., J. B. Kim, P. Sarraf, and B. M. Spiegelman. 1996. Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPAR gamma. *Science.* **274:** 2100–2103.
55. Araki, N., T. Higashi, T. Mori, R. Shibayama, Y. Kawabe, T. Kodama, K. Takahashi, M. Shichiri, and S. Horiuchi. 1995. Macrophage scavenger receptor mediates the endocytic uptake and degradation of advanced glycation end products of the Maillard reaction. *Eur. J. Biochem.* **230:** 408–415.
56. Ohgami, N., R. Nagai, M. Ikemoto, H. Arai, A. Miyazaki, H. Hakamata, S. Horiuchi, and H. Nakayama. 2002. CD36, serves as a receptor for advanced glycation endproducts (AGE). *J. Diabetes Complications.* **16:** 56–59.
57. Thornalley, P. J. 1998. Cell activation by glycated proteins. AGE receptors, receptor recognition factors and functional classification of AGEs. *Cell. Mol. Biol.* **44:** 1013–1023.
58. Mead, J. R., S. A. Irvine, and D. P. Ramji. 2002. Lipoprotein lipase: structure, function, regulation, and role in disease. *J. Mol. Med.* **80:** 753–769.