# Homocysteine Induces Protein Kinase C Activation and Stimulates c-Fos and Lipoprotein Lipase Expression in Macrophages

**Marie-Claude Beauchamp and Geneviève Renier**

**Hyperhomocysteinemia is an independent risk factor for cardiovascular disease in human diabetes. Among the multiple factors that may account for the atherogenicity of homocysteine (Hcys) in patients with diabetes, macrophage (Mo) lipoprotein lipase (LPL) has unique features in that it is increased in human diabetes and acts as a proatherogenic factor in the arterial wall. In the present study, we determined the direct regulatory effect of Hcys on Mo LPL gene expression and secretion. Incubation of J774 Mo with Hcys increased, in a time- and dose-dependent manner, LPL mRNA expression and secretion. Induction of LPL gene expression was biphasic, peaking at 1 and 6 h. Whereas Hcys treatment increased protein kinase C (PKC) activity in Mo, pretreatment of Mo with PKC inhibitors totally suppressed Hcys-induced LPL mRNA expression. Hcys also increases the levels of c-fos mRNA in Mo and enhanced nuclear protein binding to the AP-1 sequence of the LPL gene promoter. Overall, these results demonstrate that Hcys stimulates Mo LPL at both the gene and protein levels and that Hcys-induced LPL mRNA expression requires PKC activation. They also suggest a possible role of c-fos in the stimulatory effect of Hcys on Mo LPL mRNA expression. These observations suggest a new mechanism by which Hcys may exert its proatherogenic effects in human diabetes. *Diabetes* 51:1180–1187, 2002**

Cardiovascular diseases are the leading causes of morbidity and mortality in patients with diabetes. The high risk of cardiovascular disease in these individuals cannot be totally explained by classic risk factors. Hyperhomocysteinemia is an independent risk factor for cardiovascular disease in the general population (1–5). In the diabetic population, the association between hyperhomocysteinemia and cardiovascular disease seems even stronger (6–10). Furthermore, this risk factor may predict mortality in patients with type 2 diabetes (9,10).

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DTT, dithiothreitol; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Hcys, homocysteine; LPL, lipoprotein lipase; MAP, mitogen-activated protein; Mo, macrophage; PKC, protein kinase C; PPAR, peroxisome-proliferator activated receptor.

Several mechanisms have been proposed to explain the atherogenic properties of homocysteine (Hcys). In particular, it has been shown that Hcys promotes smooth muscle cell proliferation (11); activates the protein kinase C (PKC)/c-fos signaling pathway (12); and induces platelet aggregation (13), lipoprotein oxidation, and activation of the coagulation pathway (13).

Lipoprotein lipase (LPL), a key enzyme in lipid metabolism, is secreted by macrophages (Mo) in atherosclerotic lesions (14). Mo LPL produced in the vascular wall acts as a proatherogenic protein. Indeed, this enzyme mediates the uptake of lipoproteins by Mo (15–17), promotes lipoprotein retention to the extracellular matrix (18,19), induces the expression of the proatherogenic cytokine tumor necrosis factor-α (20,21), increases monocyte adhesion to endothelial cells (22–24), and increases proliferation of vascular smooth muscle cells (25). Recent evidence shows that Mo LPL promotes foam cell formation and atherosclerosis in vivo (26–29). The regulation of Mo LPL in the arterial wall is poorly understood but may involve metabolic factors. This hypothesis is supported by our previous findings that Mo LPL is increased in patients with type 2 diabetes (30) and that metabolic factors dysregulated in diabetes, such as glucose (31) and fatty acids (32), enhance Mo LPL expression. On the basis of these findings and of the high levels of serum Hcys in patients with diabetes, we sought to evaluate the direct regulatory effect of Hcys on Mo LPL expression and to determine the molecular mechanism(s) involved in this effect.

## RESEARCH DESIGN AND METHODS

**Reagents.** FCS was purchased from Hyclone Laboratories (Logan, UT); DL-Hcys, L-cysteine, and heparin were obtained from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium and Trizol reagent were purchased from Gibco BRL (Burlington, Ontario, Canada). Calphostin C was obtained from Calbiochem (La Jolla, CA).

**Murine macrophages.** The J774 murine Mo cell line was obtained from the American *Type Culture* Collection (Rockville, MD). J774 cells were cultured in Dulbecco's modified Eagle's medium containing 10% FCS and 100 μg/ml penicillin-streptomycin (Gibco BRL).

**Analysis of mRNA expression.** Six million J774 cells were plated in plastic Petri dishes (100 × 20 mm; Falcon, Lincoln Park, NJ). After treatment with Hcys, cells were lysed with Trizol reagent. Total RNA was isolated and separated in a 1.2% agarose gel containing 2.2 mol/l formaldehyde, as previously described (33). The blots were prehybridized for 8 h. The mRNA expression was analyzed by hybridization with [$^{32}$P]dCTP-labeled LPL, c-fos, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. Hybridization was detected by autoradiography with Kodak X-Omat-AR films (Rochester, NY). mRNA expression was quantified by high-resolution optical densitometry (Alpha Imager 2000; Packard Instruments, Meriden, CT).

**Electrophoretic mobility shift assay.** The isolation of the nuclei was performed as follows. Briefly, $5 \times 10^7$ J774 cells were collected, washed with

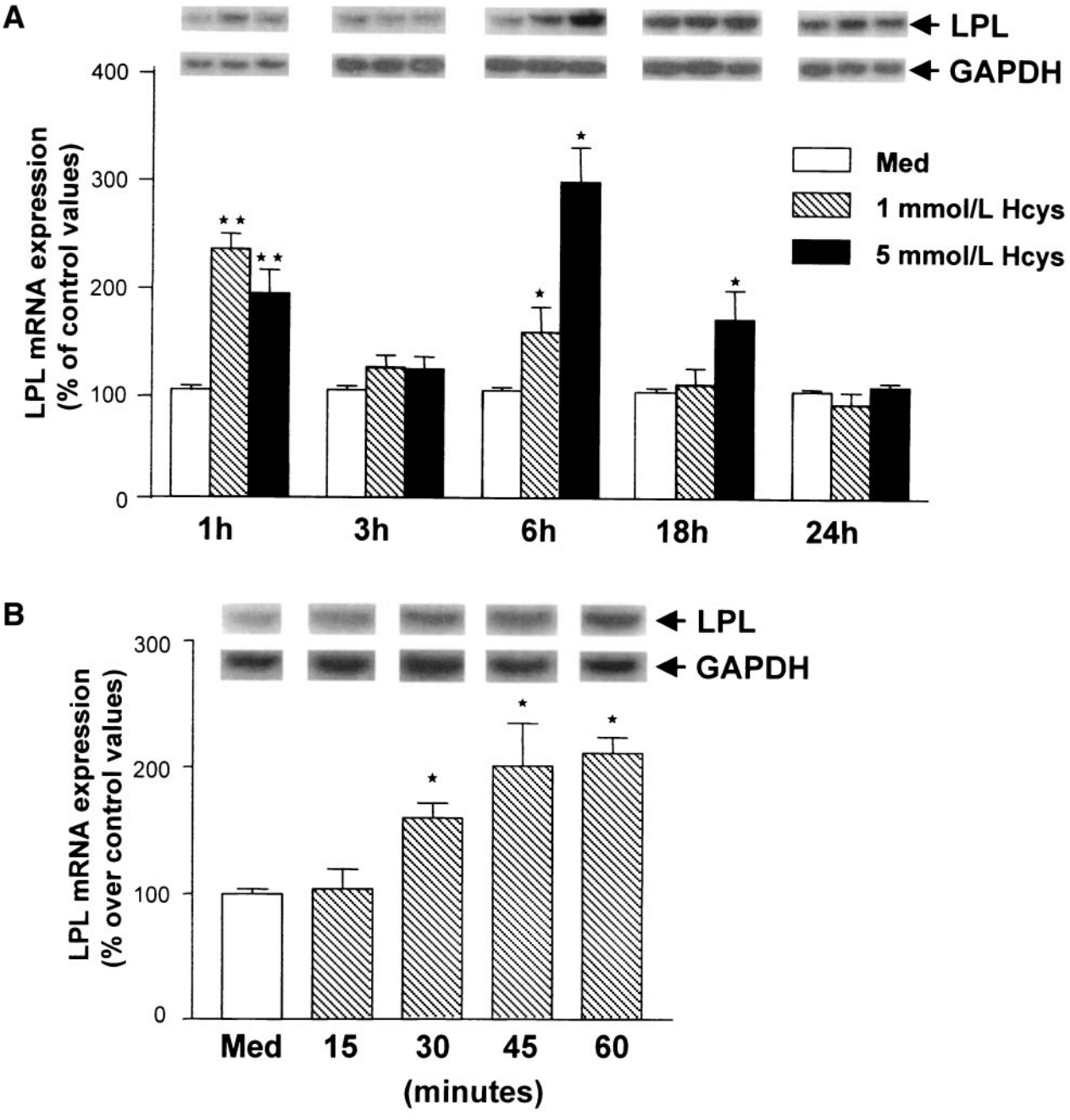


**FIG. 1. Effect of Hcys on Mo LPL mRNA levels. J774 cells were cultured in the presence of 1 or 5 mmol/l Hcys for 1, 3, 6, 18, and 24 h (*A*) or in the presence of 1 mmol/l Hcys for 15, 30, 45, and 60 min (*B*). At the end of these incubation periods, cells were lysed and total RNA was extracted and analyzed by Northern blot analysis for LPL and GAPDH mRNA expression. Levels of LPL mRNA were normalized to the levels of GAPDH mRNA. Results represent the mean ± SE of four different experiments. *$P < 0.05$; **$P < 0.01$ versus medium (Med).**

cold phosphate-buffered salt solution, and lysed in 1 ml of ice-cold buffer A (15 mmol/l KCL, 2 mmol/l $MgCl_2$, 10 mmol/l HEPES, 0.1% phenylmethylsulfonyl fluoride, and 0.5% Nonidet-P-40). After a 10-min incubation on ice, lysed cells were centrifuged, and the nuclei were washed with buffer A without Nonidet P-40. The nuclei were then lysed in a buffer containing 2 mol/l KCL, 25 mmol/l HEPES, 0.1 mmol/l EDTA, and 1 mmol/l dithiothreitol (DTT). After a 15-min incubation period, a dialysis buffer (25 mmol/l HEPES, 1 mmol/l DTT, 0.1% phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 0.1 mmol/l EDTA, and 11% glycerol) was added to the nuclei preparation. Nuclei were collected by centrifugation for 20 min at 13,000 rpm. Aliquots (50 μl) of the supernatants were frozen at −70°C, and protein concentration was determined. DNA retardation (mobility shift) electrophoresis assays were performed as previously described by Fried and Crothers (34). Briefly, 5 μg of nuclear extracts was incubated for 15 min in the presence of 5× binding buffer (125 mmol/l HEPES [pH 7.5], 50% glycerol, 250 mmol/l NaCl, 0.25% Nonidet P-40, and 5 mmol/l DTT). End-labeled double-stranded consensus sequences of the LPL promoter AP-1–enhancing element (20,000 cpm per sample) were then added to the samples for 30 min. Samples were analyzed on a 4% nondenaturing polyacrylamide gel containing 0.01% Nonidet P-40. The specificity of the nuclear protein binding was assessed by incubating the nuclear proteins isolated from murine Mo with labeled DNA probe in the presence of a 1,000-fold molar excess of unlabeled DNA probe.

**DNA probes.** The cDNA probe for detection of murine LPL was prepared by the polymerase chain reaction technique. cDNA was obtained from total RNA using a reverse transcription reaction. Two synthetic primers spanning bases 255–287 and 1,117–1,149 of the LPL cDNA were used to enzymatically amplify a 894-bp region of the LPL probe. The cDNA probes for murine c-fos and GAPDH were purchased from American *Type Culture* Collection. A 20-mer double-stranded oligonucleotide (5′-GGGCACCT GACTAAGGCCAG-3′; 5′-TGTGCTGGCCTTAGTCAGGT-3′) containing the

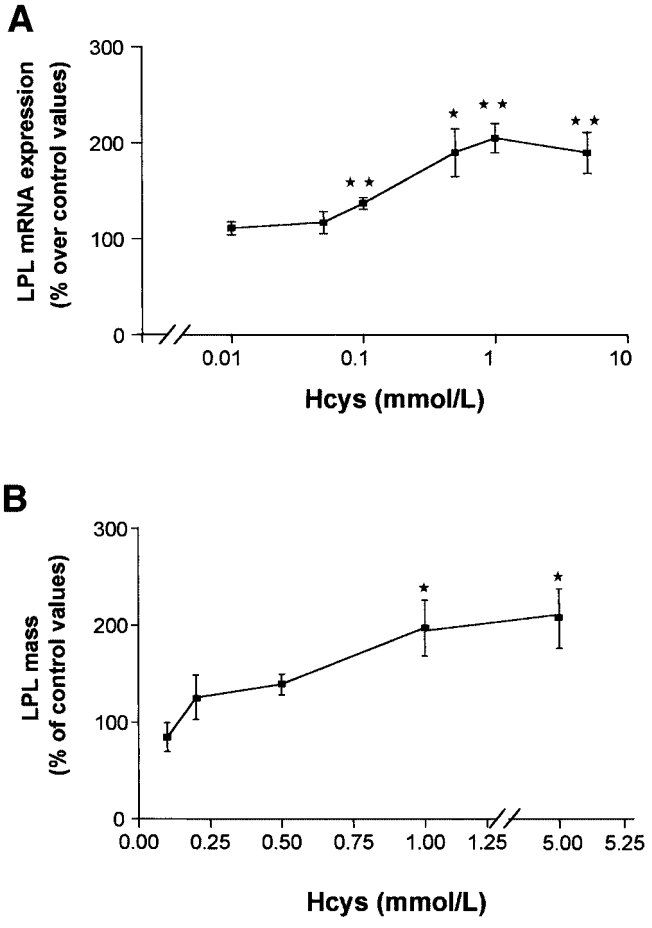


**FIG. 2. Dose-dependent effect of Hcys on Mo LPL mRNA levels and immunoreactive mass. *A*: J774 cells were cultured in the presence of 0.01, 0.05, 0.1, 0.5, 1, and 5 mmol/l Hcys for 60 min. At the end of this incubation period, cells were lysed and total RNA was extracted and analyzed by Northern blot analysis for LPL and GAPDH mRNA expression. Levels of LPL mRNA were normalized to the levels of GAPDH mRNA. *B*: J774 cells were cultured in the presence of 0.1, 0.2, 0.5, 1, and 5 mmol/l Hcys for 24 h. At the end of this incubation period, LPL immunoreactive mass was measured in the culture medium and normalized to total cell protein levels. Results represent the mean ± SE of four different experiments. *$P < 0.05$; **$P < 0.01$ versus medium.**

consensus sequence for the AP-1–responsive element of the murine LPL gene promoter (35,36) was synthesized with the aid of an automated DNA synthesizer. After annealing, the double-stranded oligonucleotide was labeled with [γ-$^{32}$P]ATP by using the Boehringer Mannheim 5′ end-labeling kit (Indianapolis, IN).

**Determination of human LPL immunoreactive mass and activity.** One hour before the end of the incubation period, 50 units/ml heparin was added to the medium. The amount of LPL immunoreactive mass was measured by enzyme-linked immunosorbent assay using the Markit-F LPL kit (Dainippon, Pharmaceutical, Osaka, Japan) (37). Extracellular LPL activity was determined using the Confluolip kit (Progen, Heidelberg, Germany) (38). LPL mass and activity values were normalized to the levels of total cell proteins.

**Measurement of PKC activity.** Adherent murine J774 Mo were recovered and homogenized (Dounce: 15 strokes) in 500 μl of ice-cold buffer A (20 mmol/l Tris [pH 7.5], 0.5 mmol/l EDTA, 0.5 mmol/l EGTA, 25 μg/ml aprotinin, and 25 μg/ml leupeptin). The membrane and cytosolic fractions were separated by ultracentrifugation (100,000*g* for 30 min at 4°C). After recovery of high-speed supernatants containing cytosolic PKC, the corresponding membrane pellets were homogenized in 500 μl of buffer A containing 0.5% Triton X-100. The enzyme from both fractions was partially purified through DE52 chromatography columns. After removal of unbound proteins by washing of the columns with buffer B (20 mmol/l Tris [pH 7.5], 0.5 mmol/l EDTA, 0.5 mmol/l EGTA, and 10 mmol/l β-mercaptoethanol), fractions containing PKC were eluted with buffer C (Tris [pH 7.5], 0.5 mmol/l EDTA, 0.5 mmol/l EGTA, 10 mmol/l β-mercaptoethanol, and 0.2 mol/l NaCl). Eluates were analyzed for PKC activity, following the optimum conditions of the assay, by measuring the incorporation of $^{32}$P into the synthetic peptide Ac-myelin basic protein(4-14). The specificity of the assay was determined by subtracting the radioactivity obtained in the presence of the pseudosubstrate inhibitor PKC(19-36) from total radioactivity. PKC activity was normalized to the levels of total cell proteins. Data were expressed as percentages, considering the control as 100% activity.

**Determination of total protein concentrations.** Total protein content was measured according to the Bradford method (39) using a colorimetric assay (Bio-Rad, Mississauga, Ontario, Canada).

**Statistical analysis.** All values were expressed as the mean ± SE. Data were analyzed by Student's *t* test for comparison of two groups or by one-way ANOVA followed by the Student-Newman-Keuls test or the Dunn's method for multiple comparisons. A value of $P < 0.05$ was considered significant.

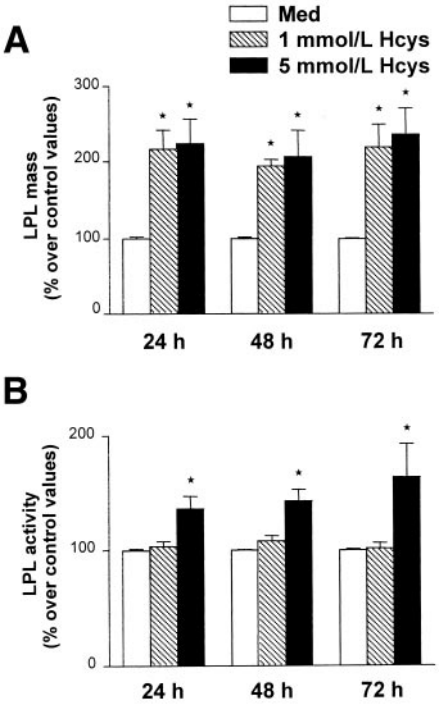


**FIG. 3. Effect of Hcys on Mo LPL immunoreactive mass (*A*) and activity (*B*). J774 cells were cultured in the presence of 1 or 5 mmol/l Hcys for 24, 48, and 72 h. LPL immunoreactive mass (*A*) and activity (*B*) were measured in the culture medium and normalized to total cell protein levels. Data represent the mean ± SE of four different experiments. *$P < 0.05$ versus medium (Med).**

## RESULTS

**Effect of Hcys on Mo LPL mRNA expression.** Incubation of J774 cells for 1–24 h with Hcys increased Mo LPL gene expression in a time-dependent manner. Induction of Mo LPL mRNA levels in response to Hcys (1–5 mmol/l) was biphasic, peaking at 1 and 6 h (Fig. 1*A*). Maximal effect was observed after a 6-h incubation period in the presence of 5 mmol/l Hcys. Under these experimental conditions, no modulation of the mRNA expression of GAPDH, used as internal control, was observed. LPL mRNA levels normalized to the levels of GAPDH mRNA are illustrated in Fig. 1*A*. No stimulatory effect of cysteine (1 mmol/l) was observed on Mo LPL mRNA levels, thereby indicating a specific effect of Hcys on Mo LPL gene expression (LPL mRNA levels [percent increase of control values]: cysteine 1 mmol/l: 1 h, 94.2 ± 8.2; 6 h, 100.0 ± 9.1).

To characterize further the kinetics of the early induction of LPL gene expression in response to Hcys, J774 cells were incubated in the presence of 1 mmol/l Hcys for 15, 30, 45, and 60 min. As shown in Fig. 1*B*, a significant increase

in Mo LPL mRNA levels was detected after a 30-min incubation period with Hcys. Maximal effect was observed at the 45- and 60-min incubation periods. LPL mRNA levels normalized to the levels of GAPDH are presented in Fig. 1*B*.

**Dose-dependent effect of Hcys on Mo LPL mRNA expression and immunoreactive mass.** To assess the optimal concentration of Hcys required to stimulate Mo LPL gene expression, we incubated J774 cells for 60 min in the presence of increasing concentrations (0.01, 0.05, 0.1, 0.5, 1, or 5 mmol/l) of Hcys. Whereas Mo LPL mRNA expression remained unchanged in cells exposed to 0.01 and 0.05 mmol/l Hcys, a significant increase in this parameter was found in the presence of 0.1 mmol/l Hcys (Fig. 2*A*). Maximal levels of LPL mRNA were observed after culture of the cells with 0.5–1 mmol/l Hcys. A similar dose-dependent effect of Hcys on LPL mRNA expression was observed in Mo exposed for 6 h to Hcys (data not shown). Recovery of enhanced amounts of LPL immunoreactive mass from the culture media reflected the increase in LPL mRNA expression of Hcys-treated J774 cells. To determine the dose-dependent effect of Hcys on Mo LPL secretion, we incubated J774 cells for 24 h with increasing concentrations (0.1, 0.2, 0.5, 1, or 5 mmol/l) of Hcys. Whereas a small induction of LPL secretion was already observed in Mo exposed to 0.2–0.5 mmol/l, the effect of Hcys on Mo LPL secretion reached statistical significance only at concentrations of 1–5 mmol/l (Fig. 2*B*).

**Time-dependent effect of Hcys on Mo LPL mass and activity.** Mo exposed for 24–72 h to Hcys (1–5 mmol/l) showed a sustained twofold increase in LPL mass secretion (Fig. 3*A*). Determination of Mo LPL activity in the supernatants of J774 cells exposed to 5 mmol/l Hcys showed a moderate increase in this parameter (Fig. 3*B*). In contrast, the levels of extracellular LPL activity in cells treated with 1 mmol/l Hcys remained unchanged (Fig. 3*B*).

**Role of PKC on Hcys-stimulated LPL gene expression.** To determine whether PKC may represent the signaling pathway involved in the upregulation of Mo LPL gene expression by Hcys, we pretreated J774 cells for 30 min in the presence or absence of the specific PKC inhibitor Calphostin C (0.5 μg/ml), and then we treated cells for 1 h with 1 mmol/l Hcys. As shown in Fig. 4*A*, calphostin C totally abolished the stimulatory effect of Hcys on Mo LPL gene expression. A similar effect was observed when J774 cells were pretreated with the PKC inhibitors GF109203X (20 nmol/l) and chelerythrine (0.5 μmol/l) (LPL mRNA levels [percent of control values]: controls: 100.0 ± 1.6; Hcys: 145.2 ± 5.5; GF109203X: 79.0 ± 11.8, GF109203X + Hcys: 73.0 ± 11.1, $P < 0.05$ versus Hcys; chelerythrine: 95.3 ± 7.2, chelerythrine + Hcys: 94.3 ± 7.7, $P < 0.05$ versus Hcys).

To establish further the involvement of PKC in Hcys-stimulated Mo LPL mRNA expression, we measured PKC activity in cytosolic and particulate fractions of Hcys-treated J774 cells (Fig. 4*B*). A maximal increase in PKC activity in the membrane fraction of Mo was observed after a 10-min exposure of the cells to 1 mmol/l Hcys.

**Effect of Hcys on Mo c-fos mRNA levels.** To assess the effect of Hcys on Mo c-fos gene expression, we incubated J774 cells for 15 to 60 min with 1 mmol/l Hcys (Fig. 5*A*). Exposure of Mo for 15 min to Hcys led to a significant induction of basal c-fos gene expression. Maximal effect was observed after a 30- to 45-min incubation period. As illustrated in Fig. 5*B*, induction of Mo c-fos gene expression was dose-dependent, with a maximal effect occurring at a Hcys concentration of 1 mmol/l.

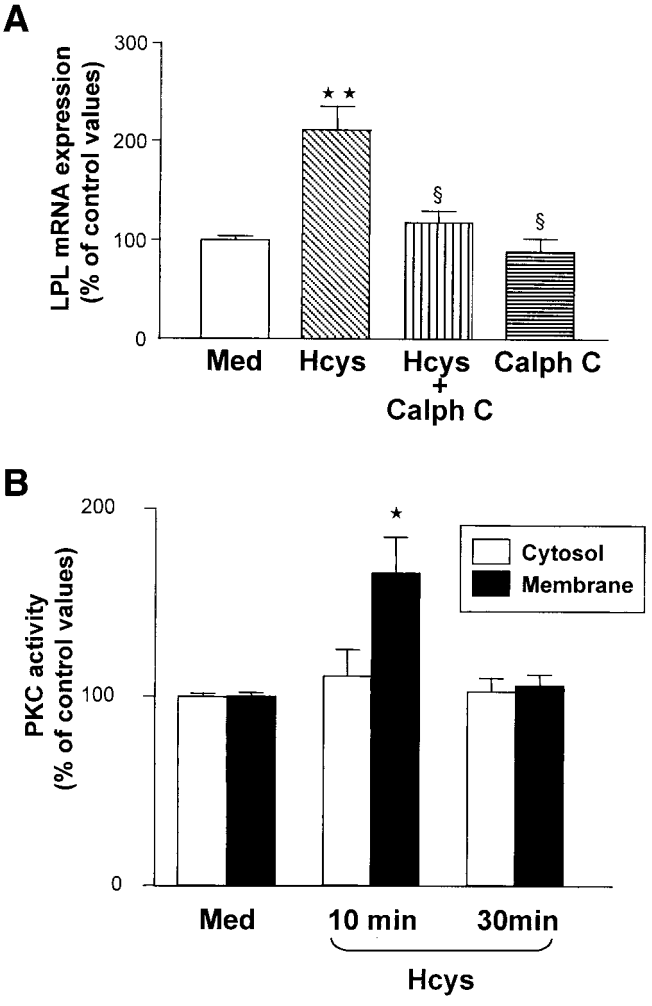


**FIG. 4. *A*: Effect of Calphostin C on Hcys-stimulated Mo LPL mRNA levels. J774 cells were pretreated for 30 min with calphostin C (0.5 μg/ml) before being exposed for 60 min to 1 mmol/l Hcys. At the end of this incubation period, cells were lysed and total RNA was extracted and analyzed by Northern blot analysis for LPL and GAPDH mRNA expression. LPL mRNA levels were normalized to the levels of GAPDH mRNA. Data represent the mean ± SE of four different experiments. **$P < 0.01$ versus medium (Med); §$P < 0.05$ versus Hcys-treated cells. *B*: Effect of Hcys on PKC activity. J774 cells were incubated with 1 mmol/l Hcys for 10 and 30 min. PKC activity in cytosolic and particulate fractions was determined as described in RESEARCH DESIGN AND METHODS. Data represent the mean ± SE of four different experiments. *$P < 0.05$ versus medium (Med).**

**Effect of Hcys 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 Hcys might induce changes at the level of LPL gene promoter binding proteins. We found that a 15- to 30-min exposure of J774 cells to Hcys led to a major increase in the binding of nuclear proteins to the AP-1 consensus sequence of the LPL promoter. Maximal effect was observed after a 15-min incubation period (Fig. 6). This binding complex was specifically competed in the presence of a 1,000-fold molar excess of the unlabeled AP-1 oligonucleotide.

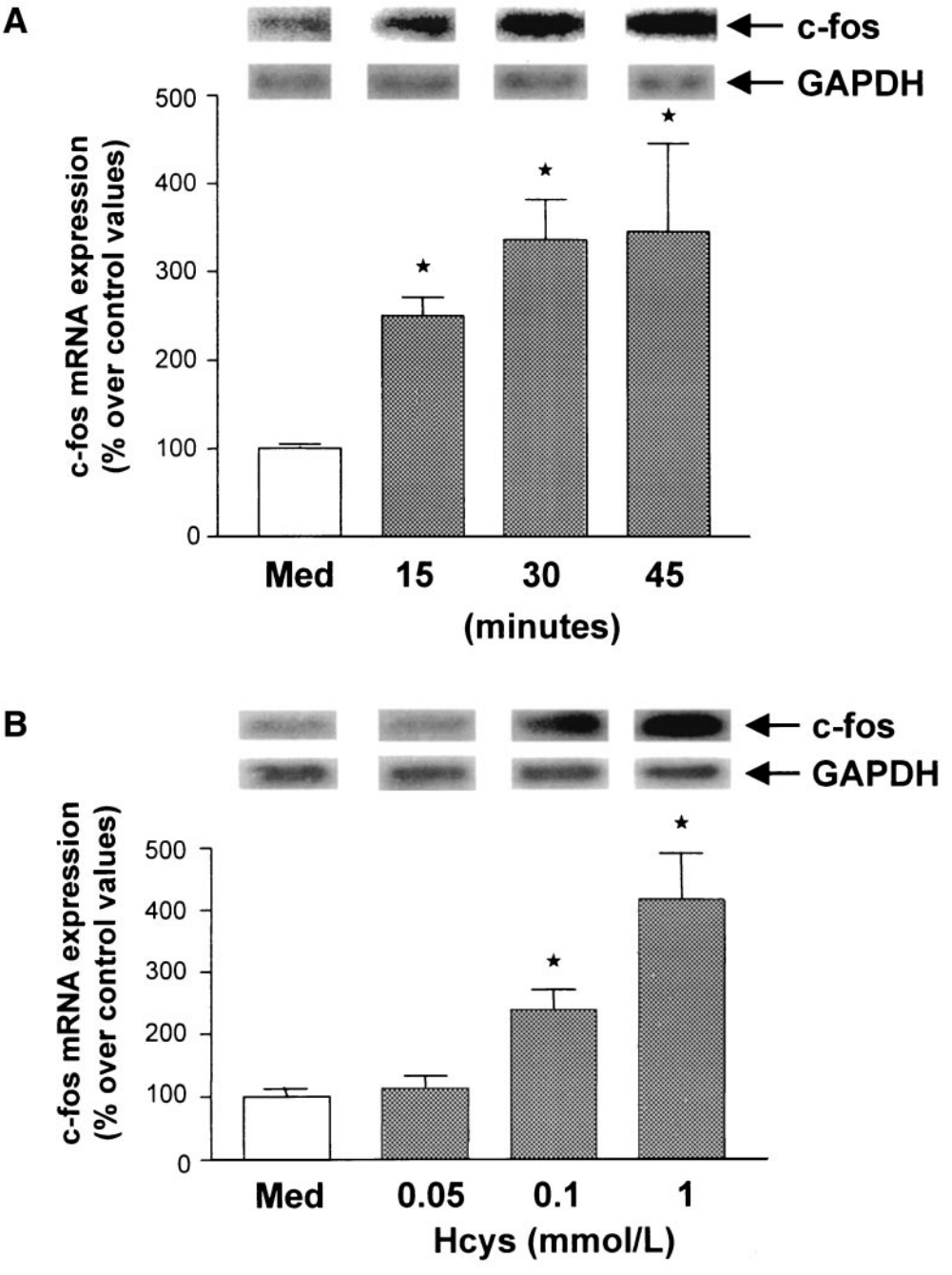


**FIG. 5. Effect of Hcys on Mo c-fos mRNA levels. J774 cells were cultured in the presence of 1 mmol/l Hcys for 15, 30, and 45 min (*A*) or with increasing concentrations of Hcys for 45 min (*B*). At the end of these incubation periods, cells were lysed and total RNA was extracted and analyzed by Northern blot analysis for c-fos and GAPDH mRNA expression. Levels of c-fos mRNA were normalized to the levels of GAPDH mRNA. Results represent the mean ± SE of four different experiments. *$P < 0.05$ versus medium (Med).**

## DISCUSSION

Hyperhomocysteinemia is an independent risk factor for cardiovascular disease (1–5). Although Hcys has been implicated in many biological events associated with atherogenesis, the mechanism(s) by which this amino acid exerts its proatherogenic effects is still not well known.

Our results demonstrate, for the first time, that Hcys stimulates Mo LPL secretion in vitro. This observation is relevant in light of recent studies showing that Mo LPL production in the arterial wall promotes foam cell formation and atherosclerosis in vivo (26–29). Our finding that Hcys induces to a similar extent LPL mRNA levels and LPL mass suggests that the stimulatory effect of Hcys on Mo LPL secretion may, at least partly, involve transcriptional events. It is widely known that the regulation of LPL gene expression is controlled by several *cis*- and *trans*-acting factors surrounding the LPL transcriptional start site (40,41). Induction of LPL gene expression by Hcys may theoretically involve different signaling pathways. On the basis of the presence of an AP-1 site in the regulatory sequence of the LPL gene and of the previously documented effect of Hcys on PKC and c-fos activation (12), one may suggest that increased AP-1 activity resulting from PKC-induced

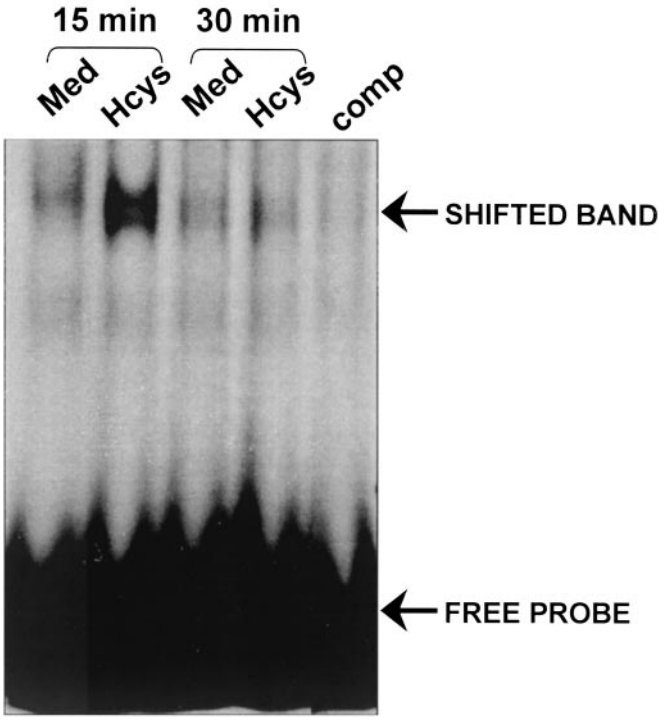


**FIG. 6. Effect of Hcys on the binding of nuclear proteins extracted from J774 cells to the regulatory AP-1 sequence of the murine LPL gene promoter. J774 cells were cultured in the presence of 1 mmol/l Hcys for 15 and 30 min. The nuclear proteins isolated from these cells were incubated with double-stranded AP-1 regulatory element of the LPL gene promoter. Retardation was assessed by gel electrophoresis in 4% polyacrylamide gel. Figure shows one representative experiment out of four.**

c-fos expression could mediate the stimulatory effect of Hcys on Mo LPL gene expression. Our findings that Hcys led to PKC activation in Mo and that specific PKC inhibitors totally abolished the induction of Mo LPL gene expression in response to Hcys clearly demonstrated that PKC activation is required for the stimulatory effect of Hcys on Mo LPL gene expression. Furthermore, our observations that Hcys induces parallel changes in Mo LPL and c-fos mRNA levels and that Hcys induces the binding of nuclear proteins to the AP-1 sequence suggest a role for c-fos in the transcriptional regulation of Mo LPL gene expression by Hcys. From these observations, one may postulate that one signaling event mediating the increase of the transcriptional rate of the LPL gene in response to Hcys involves the activation of PKC and the interaction of its well-known target c-fos with the AP-1 sequence. Several lines of evidence indicate that PKC activates the mitogen-activated protein (MAP) kinase signal transduction pathway and that induction of MAP kinase activity results subsequently in c-fos expression. Members of the MAP kinase family include the extracellular signal-regulated kinases (ERKs) 1 and 2 and the stress-induced kinases Jun $NH_2$-terminal kinase and p38. It has been demonstrated that Hcys activates the Jun $NH_2$-terminal kinase signaling pathway in human endothelial cells (42) and stimulates ERK1 and -2 phosphorylation in smooth muscle cells and NIH/3T3 cells (43–45). On the basis of these findings and on the previously documented role of the MAP kinase pathway in the regulation of adipocyte LPL expression (46), it is tempting to speculate that PKC-dependent MAP kinase activation could mediate the upregulation of Mo LPL gene expression by Hcys. This question is especially relevant considering the close correlation between the reported biphasic activation of ERK, which rises 10 min and 4 h after stimulation (47), and the presently documented Hcys-induced early and delayed stimulation of Mo LPL gene expression that we observed at 1 and 6 h after Hcys exposure, respectively.

Besides c-fos, other transcription factors, such as peroxisome-proliferator activated receptors (PPARs), could mediate the upregulation of Mo LPL gene expression in response to Hcys. Indeed, evidence has been provided that PPARs regulate Mo LPL gene expression (32,48) by binding to a peroxisome-proliferator response element present in the LPL promoter (49). Because Hcys (50), PKC (51,52), and MAP kinases (53–57) regulate the expression and/or activation of PPARs, these transcription factors may play a key role in the upregulation of Mo LPL gene expression in response to Hcys. Our preliminary observations showing that Hcys induces the binding of nuclear proteins to the peroxisome-proliferator response element of the LPL gene support this possibility.

Our results finally demonstrated that Hcys increases Mo LPL secretion. Whereas the increment of LPL mass paralleled the induction in LPL mRNA, increase of LPL activity was not observed in cells exposed to 1 mmol/l Hcys and was well below the reported elevation in the enzyme mRNA levels in Mo treated with 5 mmol/l Hcys. Because the antibody used to measure LPL mass recognized both active and inactive LPL, these findings suggest that Hcys induces the release of mostly inactive LPL. The complex posttranslational processing of LPL allows for the expectation of different sites for this modulation of LPL activity by Hcys. Additional research will be necessary to determine exactly which steps of the process of LPL synthesis are target sites for the modulation of LPL activity by Hcys.

Recent data suggest that Hcys may be a risk factor for cardiovascular disease in the diabetic population. Plasma Hcys concentrations are elevated in patients with diabetes (58,59), and a relationship between hyperhomocysteinemia and increased risk of cardiovascular morbidity and mortality has been established in patients with type 2 diabetes (6–10). We previously demonstrated that Mo LPL, a proatherogenic protein, is increased in patients with type 2 diabetes (30) and that peripheral factors are, at least partly, responsible for this alteration (30–32). Our finding that Hcys increases Mo LPL secretion in vitro suggests that Hcys may contribute to the induction of Mo LPL in human diabetes.

One limitation of the present study is the supraphysiological Hcys concentrations needed to enhance Mo LPL expression in vitro. Indeed, we found that the DL-Hcys concentrations required to stimulate Mo LPL in the present study were >10 times those found in the plasma of patients with diabetes. Because the inactive D-isomer constitutes ~50% of the DL-Hcys, use of the L-isomer of Hcys exclusively could have lowered the Hcys concentration needed to elicit a biological response. It is also possible that concentrations of Hcys in the limited environment of the atherosclerotic plaque may be sufficient to

favor the induction of Mo LPL in vivo. Because LPL exerts its proatherogenic effects through both its structural and catalytic properties (60–65), generation of increased amounts of LPL, even in its inactive form, may contribute to the accelerated atherosclerosis associated with diabetes.

In conclusion, this study demonstrates for the first time that Hcys stimulates Mo LPL gene expression and secretion in vitro. Our data show that induction of Mo LPL mRNA expression by Hcys involves PKC activation and suggest a role of c-fos in the transcriptional regulation of Mo LPL expression. These results provide a new mechanism by which Hcys may promote atherosclerosis in human diabetes.

## REFERENCES


1. Clarke R, Daly L, Robinson K, Naughten E, Cahalane S, Fowler B, Graham I: Hyperhomocysteinemia: an independent risk factor for vascular disease. *N Engl J Med* 324:1149–1155, 1991
2. Robinson K, Mayer EL, Miller DP, Green R, Lente FV, Gupta A, Kottke-Marchant K, Savon SR, Selhub J, Nissen SE, Kutner M, Topol EJ, Jacobsen DW: Hyperhomocysteinemia and low pyridoxal phosphate: common and independent reversible risk factors for coronary artery disease. *Circulation* 92:2825–2830, 1995
3. Malinow MR, Ducimetiere P, Luc G, Evans AE, Arveiler D, Cambien F, Upson BM: Plasma homocyst(e)ine levels and graded risk for myocardial infarction: findings in two populations at contrasting risk for coronary heart disease. *Atherosclerosis* 126:27–34, 1996
4. Loehrer FMT, Angst CP, Haefeli WE, Jordan PP, Ritz R, Fowler B: Low whole-blood S-adenosylmethionine and correlation between 5-methyltetrahydrofolate and homocysteine in coronary artery disease. *Arterioscler Thromb Vasc Biol* 16:727–733, 1996
5. Evans RW, Shaten J, Hempel JD, Cutler JA, Kuller LH: Homocyst(e)ine and risk of cardiovascular disease in the Multiple Risk Factor Intervention Trial. *Arterioscler Thromb Vasc Biol* 17:1947–1953, 1997
6. Okada E, Oida K, Tada H, Asazuma K, Eguchi K, Tohda G, Kosaka S, Takahashi S, Miyamori I: Hyperhomocysteinemia is a risk factor for coronary arteriosclerosis in Japanese patients with type 2 diabetes. *Diabetes Care* 22:484–490, 1999
7. Hoogeveen EK, Kostense PJ, Beks PJ, Mackaay AJ, Jakobs C, Bouter LM, Heine RJ, Stehouwer CDA: Hyperhomocysteinemia is associated with an increased risk of cardiovascular disease, especially in non-insulin-dependent diabetes mellitus. A population-based study. *Arterioscler Thromb Vasc Biol* 18:133–138, 1998
8. Smulders YM, Rakic M, Slaats EH, Treskes M, Sijbrands EJ, Odekerken DA, Stehouwer CD, Silberbusch J: Fasting and post-methionine homocysteine levels in NIDDM. Determinants and correlations with retinopathy, albuminuria, and cardiovascular disease. *Diabetes Care* 22:125–132, 1999
9. Stehouwer CD, Gall MA, Hougaard P, Jakobs C, Parving HH: Plasma homocysteine concentration predicts mortality in non-insulin-dependent diabetic patients with and without albuminuria. *Kidney Int* 55:308–314, 1999
10. Hoogeveen EK, Kostense PJ, Jakobs C, Dekker JM, Nijpels G, Heine RJ, Bouter LM, Stehouwer CD: Hyperhomocysteinemia increases risk of death, especially in type 2 diabetes: 5-year follow-up of the Hoorn Study. *Circulation* 101:1506–1511, 2000
11. Tsai JC, Perrella MA, Yoshizumi M, Hsieh CM, Haber E, Schlegel R, Lee ME: Promotion of vascular smooth muscle cell growth by homocysteine: a link to atherosclerosis. *Proc Natl Acad Sci U S A* 91:6369–6373, 1994
12. Dalton ML, Gadson PF Jr, Wrenn RW, Rosenquist TH: Homocysteine signal cascade: production of phospholipids, activation of protein kinase C, and the induction of c-fos and c-myb in smooth muscle cells. *FASEB J* 11:703–711, 1997
13. Durand P, Lussier-Cacan S, Blache D: Acute methionine load-induced hyperhomocysteinemia enhances platelet aggregation, thromboxane biosynthesis, and macrophage-derived tissue factor activity in rats. *FASEB J* 11:1157–1168, 1997
14. Chait A, Iverius PH, Brunzell JD: Lipoprotein lipase secretion by human monocyte-derived macrophages. *J Clin Invest* 69:490–493, 1982
15. Aviram M, Bierman EL, Chait A: Modification of low density lipoprotein by lipoprotein lipase or hepatic lipase enhances uptake and cholesterol accumulation in cells. *J Biol Chem* 263:15416–15422, 1988
16. Rumsey SC, Obunike JC, Arad Y, Deckelbaum RJ, Goldberg IJ: Lipoprotein lipase-mediated uptake and degradation of low density lipoproteins by fibroblasts and macrophages. *J Clin Invest* 90:1504–1512, 1992
17. Stein O, Ben-Naim M, Dabach Y, Hollander G, Halperin G, Stein Y: Can lipoprotein lipase be the culprit in cholesteryl ester accretion in smooth muscle cells in atheroma? *Atherosclerosis* 99:15–22, 1993
18. Rutledge JC, Goldberg IJ: Lipoprotein lipase (LPL) affects low density lipoprotein (LDL) flux through vascular tissue: evidence that LPL increases LDL accumulation in vascular tissue. *J Lipid Res* 35:1152–1160, 1994
19. Saxena U, Ferguson E, Bisgaier CL: Apolipoprotein E modulates low density lipoprotein retention by lipoprotein lipase anchored to the subendothelial matrix. *J Biol Chem* 268:14812–14819, 1993
20. Renier G, Skamene E, DeSanctis JB, Radzioch D: Induction of tumor necrosis factor alpha gene expression by lipoprotein lipase. *J Lipid Res* 35:271–278, 1994
21. Mamputu JC, Renier G: 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, 1999
22. Mamputu JC, Desfaits AC, Renier G: Lipoprotein lipase enhances human monocyte adhesion to aortic endothelial cells. *J Lipid Res* 38:1722–1729, 1997
23. Saxena U, Kulkarni NM, Ferguson E, Newton RS: Lipoprotein lipase-mediated lipolysis of very low density lipoproteins increases monocyte adhesion to aortic endothelial cells. *Biochem Biophys Res Commun* 189:1653–1658, 1992
24. Obunike JC, Paka S, Pillarisetti S, Goldberg IJ: Lipoprotein lipase can function as a monocyte adhesion protein. *Arterioscler Thromb Vasc Biol* 17:1414–1420, 1997
25. Mamputu JC, Levesque L, Renier G: Proliferative effect of lipoprotein lipase on human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 20:2212–2219, 2000
26. Babaev VR, Fazio S, Gleaves LA, Carter KJ, Semenkovich CF, Linton MF: Macrophage lipoprotein lipase promotes foam cell formation and atherosclerosis in vivo. *J Clin Invest* 103:1697–1705, 1999
27. Babaev VR, Patel MB, Semenkovich CF, Fazio S, Linton MF: Macrophage lipoprotein lipase promotes foam cell formation and atherosclerosis in low density lipoprotein receptor-deficient mice. *J Biol Chem* 275:26293–26299, 2000
28. Semenkovich CF, Coleman T, Daugherty A: Effects of heterozygous lipoprotein lipase deficiency on diet-induced atherosclerosis in mice. *J Lipid Res* 39:1141–1151, 1998
29. Van Eck M, Zimmerman R, Groot PH, Zechner R, Van Berckel TJ: Role of macrophage-derived lipoprotein lipase in lipoprotein metabolism and atherosclerosis. *Arterioscler Thromb Vasc Biol* 20:E53–E62, 2000
30. Sartippour MR, Renier G: Upregulation of macrophage lipoprotein lipase in patients with type 2 diabetes: role of peripheral factors. *Diabetes* 49:597–602, 2000
31. Sartippour MR, Lambert A, Laframboise M, St-Jacques P, Renier G: Stimulatory effect of glucose on macrophage lipoprotein lipase expression and production. *Diabetes* 47:431–438, 1998
32. Michaud SE, Renier G: Direct regulatory effect of fatty acids on macrophage lipoprotein lipase: potential role of PPARs. *Diabetes* 50:660–666, 2001
33. Thomas PS: Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc Natl Acad Sci USA* 77:5201–5205, 1980
34. Fried M, Crothers DM: Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. *Nucleic Acids Res* 9:6505–6525, 1981
35. Zechner R, Newman TC, Steiner E, Breslow JL: The structure of the mouse lipoprotein lipase gene: a B1 repetitive element is inserted into the 3′-untranslated region of the mRNA. *Genomics* 11:62–76, 1991
36. Hua XX, Enerbäck S, Hudson J, Youkhana K, Gimble JM: Cloning and characterization of the promoter of the murine lipoprotein lipase-encoding gene: structural and functional analysis. *Gene* 107:247–258, 1991
37. Ikeda Y, Takagi A, Ohkaru Y, Nogi K, Iwanaga T, Kurooka S, Yamamoto A: 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, 1990
38. Duque M, Graupner M, Stutz H, Wicher I, Zechner R, Paltauf F, Hermetter A: New fluorogenic triacylglycerol analogs as substrates for the determination and chiral discrimination of lipase activities. *J Lipid Res* 37:868–876, 1996
39. Bradford MM: 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, 1976
40. Enerbäck S, Gimble JM: Lipoprotein lipase gene expression: physiological regulators at the transcriptional and post-transcriptional level. *Biochim Biophys Acta* 1169:107–125, 1993
41. Bey L, Etienne J, Tse C, Brault D, Noe L, Raisonnier A, Arnault F, Hamilton MT, Galibert F: Cloning, sequencing and structural analysis of 976 base pairs of the promoter sequence for the rat lipoprotein lipase gene. Comparison with the mouse and human sequences. *Gene* 209:31–38, 1998
42. Cai Y, Zhang C, Nawa T, Aso T, Tanaka M, Oshiro S, Ichijo H, Kitajima S: Homocysteine-responsive ATF3 gene expression in human vascular endothelial cells: activation of c-Jun $NH_2$-terminal kinase and promoter response element. *Blood* 96:2140–2148, 2000
43. Brown JC, Rosenquist TH, Monaghan DT: ERK2 activation by homocysteine in vascular smooth muscle cells. *Biochem Biophys Res Commun* 251:669–676, 1998
44. Woo DK, Dudrick SJ, Sumpio BE: Homocysteine stimulates MAP kinase in bovine aortic smooth muscle cells. *Surgery* 128:59–66, 2000
45. Shi SS, Day RM, Halpner AD, Blumberg JB, Suzuki YJ: Homocysteine and alpha-lipoic acid regulate p44/42 MAP kinase phosphorylation in NIH/3T3 cells. *Antioxid Redox Signal* 1:123–128, 1999
46. Boney CM, Gruppuso PA, Faris RA, Frackeltone AR Jr: The critical role of Shc in insulin-like growth factor-1-mediated mitogenesis and differentiation in 3T3–L1 preadipocytes. *Mol Endocrinol* 14:805–813, 2000
47. Meloche S, Seuwen K, Pages G, Pouyssegur J: Biphasic and synergistic activation of p44mapk (ERK1) by growth factors: correlation between late phase activation and mitogenicity. *Mol Endocrinol* 6:845–854, 1992
48. Sartippour MR, Renier G: Differential regulation of macrophage peroxisome proliferator-activated receptor expression by glucose. Role of peroxisome proliferator-activated receptors in lipoprotein lipase gene expression. *Arterioscler Thromb Vasc Biol* 20:104–110, 2000
49. Schoonjans K, Peinado-Onsurbe J, Lefebvre AM, Heyman RA, Briggs M, Deeb S, Staels B, Auwerx J: PPARα and PPARγ activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *EMBO J* 15:5336–5348, 1996
50. Brude IR, Finstad HS, Seljeflot I, Drevon CA, Solvoll K, Sandstad B, Hjermann I, Arnesen H, Nenseter MS: Plasma homocysteine concentration related to diet, endothelial function and mononuclear cell gene expression among male hyperlipidaemic smokers. *Eur J Clin Invest* 29:100–108, 1999
51. Iwashima Y, Eto M, Horiuchi S, Sano H: Advanced glycation end product-induced peroxisome proliferator-activated receptor gamma gene expression in the cultured mesangial cells. *Biochem Biophys Res Commun* 264:441–448, 1999
52. Feng J, Han J, Pearce SF, Silverstein RL, Gotto AM Jr, Hajjar DP, Nicholson AC: Induction of CD36 expression by oxidized LDL and IL-4 by a common signaling pathway dependent on protein kinase C and PPAR-gamma. *J Lipid Res* 41:688–696, 2000
53. Juge-Aubry CE, Hammar E, Siegrist-Kaiser C, Pernin A, Takeshita A, Chin WW, Burger AG, Meier CA: Regulation of the transcriptional activity of the peroxisome proliferator-activated receptor alpha by phosphorylation of a ligand-independent trans-activating domain. *J Biol Chem* 274:10505–10510, 1999
54. Camp HS, Tafuri SR: Regulation of peroxisome proliferator-activated receptor gamma activity by mitogen-activated protein kinase. *J Biol Chem* 272:10811–18816, 1997
55. Shao D, Rangwala SM, Bailey ST, Krakow SL, Reginato MJ, Lazar MA: Interdomain communication regulating ligand binding by PPAR-gamma. *Nature* 396:377–380, 1998
56. Hu E, Kim JB, Sarraf P, Spiegelman BM: Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARγ. *Science* 274:2100–2103, 1996
57. Passilly P, Schohn H, Jannin B, Malki MC, Boscoboinik D, Dauca M, Latruffe N: Phosphorylation of peroxisome proliferator-activated receptor alpha in rat Fao cells and stimulation by ciprofibrate. *Biochem Pharmacol* 58:1001–1008, 1999
58. Chico A, Perez A, Cordoba A, Arcelus R, Carreras G, de Leiva A, Gonzalez-Sastre F, Blanco-Vaca F: Plasma homocysteine is related to albumin excretion rate in patients with diabetes mellitus: a new link between diabetic nephropathy and cardiovascular disease? *Diabetologia* 41:684–693, 1998
59. Drzewoski J, Czupryniak L, Chwatko G, Bald E: Hyperhomocysteinemia in poorly controlled type 2 diabetes patients. *Diabetes Nutr Metab* 13:319–324, 2000
60. Lindqvist P, Ostlund-Lindqvist AM, Witztum JL, Steinberg D, Little JA: The role of lipoprotein lipase in the metabolism of triglyceride-rich lipoproteins by macrophages. *J Biol Chem* 258:9086–9092, 1983
61. Ishibashi S, Yamada N, Shimano H, Mori N, Mokuno H, Gotohda T, Kawakami M, Murase T, Takaku F: Apolipoprotein-E and lipoprotein lipase secreted from human monocyte-derived macrophages modulate very low density lipoprotein uptake. *J Biol Chem* 265:3040–3047, 1990
62. Eisenberg S, Sehayek E, Olivecrona T, Vlodavsky I: Lipoprotein lipase enhances binding of lipoproteins to heparan sulfate on cell surfaces and extracellular matrix. *J Clin Invest* 90:2013–2021, 1992
63. Tabas I, Li Y, Brocia RW, Xu SW, Swenson TL, Williams KJ: Lipoprotein lipase and sphingomyelinase synergistically enhance the association of atherogenic lipoproteins with smooth muscle cells and extracellular matrix. A possible mechanism for low density lipoprotein and lipoprotein(a) retention and macrophage foam cell formation. *J Biol Chem* 268:20419–20432, 1993
64. Saxena U, Klein MG, Vanni TM, Goldberg IJ: Lipoprotein lipase increases low density lipoprotein retention by subendothelial cell matrix. *J Clin Invest* 89:373–380, 1992
65. Williams KJ, Tabas I: The response-to-retention hypothesis of early atherogenesis. *Arterioscler Thromb Vasc Biol* 15:551–561, 1995