Upregulation of Macrophage Lipoprotein Lipase in Patients With Type 2 Diabetes

Role of Peripheral Factors

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Atherosclerosis is the major complication of diabetes. Accumulating evidence indicates that lipoprotein lipase (LPL) produced by macrophages in the vascular wall may favor the development of atherosclerosis by promoting lipid accumulation within the lesion. We previously demonstrated that high glucose stimulates in vitro murine and human macrophage LPL production. In this study, we measured macrophage LPL mRNA expression, immunoreactive mass, and activity in normotriglyceridemic subjects with type 2 diabetes. Monocytes isolated from healthy control subjects and patients with type 2 diabetes were differentiated into macrophages in RPMI medium containing 20% autologous serum. After 5 days in culture, macrophage LPL mRNA expression, mass, and activity were determined. Macrophages of diabetic patients cultured in their own sera showed a significant increase in LPL mRNA levels, mass, and activity compared with macrophages of control subjects. Differentiation of macrophages of diabetic patients in sera obtained from control subjects significantly reduced these anomalies. Conversely, culturing macrophages of control subjects in sera of diabetic patients significantly increased LPL mass and activity in these cells. Besides LPL overproduction, macrophages of diabetic patients exhibited an increase in basal and LPL-induced tumor necrosis factor (TNF)-α release. TNF-α alterations were reduced by exposing these cells to sera of control subjects. Overall, these data demonstrate that macrophages of diabetic patients overexpress LPL and TNF-α and that peripheral factors dysregulated in diabetes are, at least in part, responsible for these alterations. *Diabetes* 49:597–602, 2000

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AGE, advanced glycation end product; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; LPL, lipoprotein lipase; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TNF, tumor necrosis factor.

Diabetes is associated with accelerated atherosclerosis (1–3). Evidence has been provided that differentiation of monocytes into lipid-laden macrophages may favor the development and progression of atherosclerotic lesions (4,5). Macrophages constitutively synthesize lipoprotein lipase (LPL) (6,7), a key enzyme in the catabolism of triglyceride-rich lipoproteins (8). Several studies have supported a major role of LPL in the atherogenic process. It has been shown that LPL activity is low in normal arteries and increases during the progression of atherosclerotic lesions (9). Macrophages have been documented to express LPL mRNA and protein in atherosclerotic lesions in vivo (10,11), and high macrophage LPL secretion has been documented in atherosclerosis-prone mice (12). The proatherogenic effects of LPL include its ability to facilitate retention of apolipoprotein E–containing lipoproteins after binding to cells or extracellular matrix, to modify lipoprotein particles to more atherogenic forms, and to mediate uptake of lipoprotein particles through receptors, independent of its catalytic activity (13–17). LPL also acts as a monocyte adhesion molecule (18,19) and as a signaling molecule for gene regulation of the proatherogenic factor tumor necrosis factor (TNF)-α (20,21).

Despite the high incidence of atherosclerosis in diabetic patients and the potential key role of macrophage LPL in atherogenesis, the regulation of macrophage LPL in diabetes has been poorly investigated. To the best of our knowledge, macrophage LPL mRNA expression has thus far been measured in only a small number of hypertriglyceridemic patients with type 2 diabetes (22), and the levels of macrophage LPL activity and secretion in these patients have not been determined. Our previous observation that high glucose stimulates in vitro human macrophage LPL production (23) supports the possibility that induction of macrophage LPL may occur in type 2 diabetes and that hormonal and metabolic factors dysregulated in diabetes may play a key role in this alteration.

Along with LPL, macrophage TNF-α may represent a major factor contributing to the development of atherosclerosis in type 2 diabetes. Accumulating evidence suggests that macrophage TNF-α production is increased in human type 2 diabetes. First, TNF-α production is increased in monocytes of patients with type 2 diabetes and in macrophages of diabetic mice (24,25). Second, human macrophages incubated with high glucose concentrations or in the presence of advanced glycation end products (AGEs) overproduce TNF-α

(23,26,27). In the present study, we sought to investigate the regulation of macrophage LPL and TNF-α secretion in patients with type 2 diabetes and to evaluate the effect of peripheral factors on these parameters.

RESEARCH DESIGN AND METHODS

Reagents. Fetal calf serum (FCS) was purchased from Hyclone Laboratories (Logan, UT). RPMI 1640 medium, Hanks' balanced salt solution, and TRIzol reagent were obtained from Gibco BRL (Grand Island, NY). Lymphoprep and penicillin-streptomycin were purchased from Nycomed Pharma (Oslo, Norway) and Flow (McLean, VA), respectively. Bovine LPL and heparin were obtained from Sigma Chemical (St. Louis, MO). LPL was dialyzed against saline using 10,000 molecular weight cut-off Slide-A-Lyzer dialysis cassettes purchased from Pierce (Rockford, IL). Purity of the LPL preparation, as assessed by silver-stained SDS-PAGE, was found to be >90%. LPL preparation was detoxified using an endotoxin removal resin from Associates of Cape Cod (Falmouth, MA). Endotoxin content of the LPL preparation (1 μg/ml) was determined by the limulus amebocyte lysate assay (E-toxate; Sigma Chemical) and was consistently found to be <6 pg/ml. Treatment of macrophages with 6 pg/ml lipopolysaccharide (LPS) did not induce any TNF-α production. LPL concentration was determined by the Bradford method (29) using a colorimetric assay (Bio-Rad, Mississauga, ON, Canada).

Patients. The study group comprised 10 patients with type 2 diabetes and 10 healthy control subjects. The diabetic patients, five women and five men, gave written consent to participate in this study, which was approved by the Centre Hospitalier de l'Université de Montréal Research and Ethics Committees. All patients were recruited from our diabetic outpatient clinic. Their mean age (± SE) was 48.5 ± 2.7 years (range 37–64), BMI 26.8 ± 0.86 kg/m^2 (range 24.4–32), and duration of diabetes 5.6 ± 2.3 years (range 1–22). All the patients except one had optimal to acceptable diabetes control (mean level of serum glycated hemoglobin, 0.059 ± 0.006; normal values, 0.038–0.052). All the patients except two were normotriglyceridemic (serum triglyceride levels <2.3 mmol/l) and were treated with glyburide and metformin. None of the patients was primarily insulin dependent. One patient was hypertensive and treated with enalapril, two had macroangiopathy, and one had microangiopathy (microalbuminuria). Characteristics of the study population are presented in Table 1. Healthy control subjects, matched with patients for sex, age, and BMI, were recruited from the hospital staff and relatives. Subjects who had infectious or inflammatory conditions or cardiac, renal, or pulmonary decompensated diseases or who were treated with anti-inflammatory or antioxidant drugs were excluded from the study.

Human macrophages. Peripheral blood mononuclear cells were isolated by density centrifugation using Ficoll, allowed to aggregate in the presence of FCS, and further purified using the rosetting technique (30). After density centrifugation, highly purified monocytes (85–90%) were recovered, as assessed by flow cytometry (FACScan; Becton Dickinson, Rutherford, NJ). In every experiment, monocytes were isolated from one diabetic patient and one control subject and serum from each was collected. Monocytes were differentiated into macrophages by culturing the cells (2 million per milliliter) in 24-well plates in RPMI 1640 medium supplemented with 1% (vol/vol) penicillin-streptomycin and 20% (vol/vol) of their own sera. The cells were incubated for 5 days at 37°C in a humidified 5% CO_2, 95% air atmosphere. The culture medium was changed at day 4. In experiments aimed at assessing the role of peripheral factors on macrophage LPL production, monocytes isolated from the diabetic patient were differentiated in the serum of the control subject, and monocytes isolated from the control subject were differentiated in the serum obtained from the diabetic patient. The biochemical composition of the control and diabetic sera used in the experiments was determined by measuring the levels of serum endotoxin, glucose, glycated hemoglobin, LPL mass, and LPL activity. Whereas levels of serum endotoxin, glycated hemoglobin, and LPL mass and activity did not differ between the control and diabetic populations, increased fasting serum glucose levels were observed in the diabetic patients compared with the control subjects (Table 1). The low- and high-glucose RPMI media were prepared by adding appropriate amounts of glucose to glucose-free RPMI medium (Gibco BRL) to achieve the desired final glucose concentrations.

Analysis of LPL mRNA expression. Expression of the LPL gene in human diabetic and control macrophages was performed by polymerase chain reaction (PCR). Total RNA for use in the PCR reaction was extracted from human macrophages by an improvement of the acid-phenol technique of Chomczynski and Sacchi (31). Briefly, cells were lysed with TRIzol reagent, and chloroform was added to the solution. After centrifugation, the RNA present in the aqueous phase was precipitated and resuspended in diethyl pyrocarbonate water. cDNA was synthesized from RNA by incubating total cellular RNA with 0.1 μg oligo dT (Pharmacia, Piscataway, NJ) for 5 min at 98°C, then by incubating the mixture with reverse transcription buffer for 60 min at 37°C. The cDNA obtained was amplified by using 0.8 μmol/l of two synthetic primers specific for human LPL (5′-GA GATTTCTCTGTATGGCACC-3′ and 5′-CTGCAAATGAGACACTTTCTC-3′) and human GAPDH (5′-CCCTTCATTGACCTCAACTACATGG-3′ and 5′-AGTCTTCTG GGTGGCAGTGATGG-3′) as internal standard in the PCR reaction mixture. A 277-bp human LPL cDNA fragment and a 456-bp human GAPDH cDNA fragment were amplified enzymatically by 27 and 30 repeated cycles for GAPDH and LPL, respectively, at 95°C for 60 s, 60°C for 40 s, and 72°C for 90 s. An aliquot of each reaction mixture was then subjected to electrophoresis on a 1% agarose gel containing ethidium bromide. The intensity of the bands was measured by an image analysis scanning system (Alpha Imager 2000; Packard Instruments, Meriden, CT).

Determination of human macrophage LPL immunoreactive mass and activity. Monocytes isolated from the control subjects and the diabetic patients were incubated for 5 days in RPMI medium containing 20% serum of control subjects or diabetic patients. LPL activity and mass were measured in the supernatants 24 h after the final medium change. One hour before the end of the incubation period, 0.5 U/ml heparin was added to the medium. The amount of human LPL immunoreactive mass was measured by enzyme-linked immunosorbent assay (ELISA) using the Markit-F LPL kit (Dainippon Pharmaceutical, Osaka, Japan) (32). Medium LPL activity was determined using the Confluolip kit (Progen, Heidelberg, Germany) (33).

Determination of human macrophage TNF-α immunoreactive mass. Monocytes isolated from control subjects and diabetic patients were incubated for 5 days in RPMI medium containing 20% serum of control subjects or diabetic patients. Twenty-four hours after the final medium change, TNF-α immunoreactive mass released in the culture medium was determined by a double-sandwich ELISA (Quantikine; R&D Systems, Minneapolis, MN). In some experiments, the effect of LPL on TNF-α secretion was determined by adding 1 μg/ml LPL to the medium.

Statistical analysis. All values are expressed as the mean ± SE. For single comparisons, data were analyzed using Student's t test or the Mann-Whitney rank-sum test. For multiple comparisons, data were analyzed by analysis of variance followed by Tukey's test or Dunnett's method.

RESULTS

Levels of LPL mRNA in macrophages of control subjects and diabetic patients. Monocyte-derived macrophages of diabetic patients cultured in their own sera demonstrated a significant increase in LPL mRNA levels compared with those isolated from the control subjects (Fig. 1*A*). Under these experimental conditions, no modulation of the mRNA expression of GAPDH was observed (Fig. 1*B*). LPL mRNA levels normalized to the levels of GAPDH mRNA are presented in Fig. 1*C*.

Levels of LPL immunoreactive mass in macrophages of control subjects and diabetic patients. Monocyte-derived macrophages of diabetic patients cultured in their own sera secreted significantly higher LPL mass than macrophages of control subjects ($P < 0.001$) (Fig. 2). Culture of macrophages of diabetic patients in sera of control subjects significantly reduced this alteration ($P < 0.005$) (Fig. 2). In contrast, differentiation of macrophages of control subjects in sera of

TABLE 1
Characteristics of the study population

	Patients with type 2 diabetes	Control subjects
Age (years)	48.5 ± 2.7	39.2 ± 3.8
BMI (kg/m^2)	26.8 ± 0.9	26.1 ± 0.8
Fasting glucose (mmol/l)	7.9 ± 0.7*	5.0 ± 0.1
Triglycerides (mmol/l)	1.96 ± 0.21†	1.07 ± 0.14
Cholesterol (mmol/l)		
Total	5.18 ± 0.27	4.86 ± 0.26
HDL	1.10 ± 0.06	1.44 ± 0.12
LDL	3.18 ± 0.26	2.81 ± 0.24
Endotoxin (pg/ml)	<6	<6
LPL mass (ng/ml)	76.58 ± 15.20	76.58 ± 6.14
LPL activity (pmol/ml)	11.57 ± 0.48	10.66 ± 0.15

Data are means ± SE. *$P < 0.005$, diabetic vs. control subjects; †$P < 0.05$, diabetic vs. control subjects.

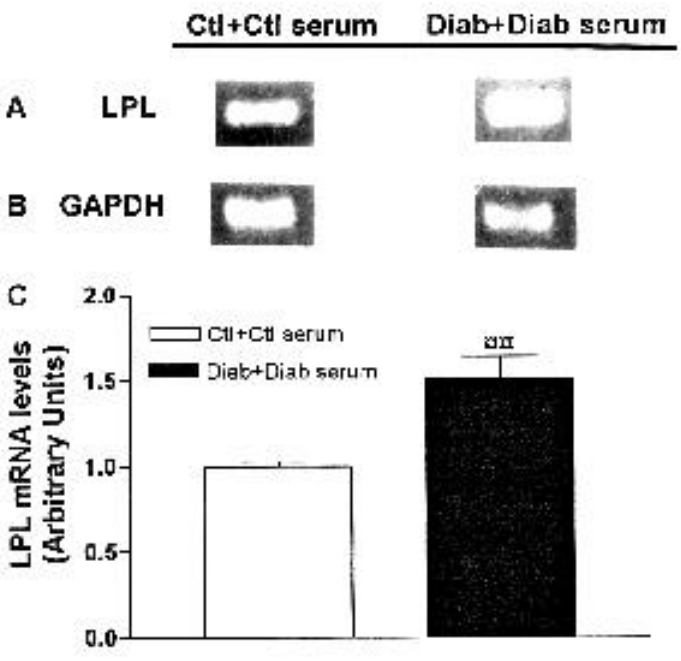

FIG. 1. LPL mRNA levels in macrophages of control subjects and diabetic patients. Monocytes of control subjects (Ctl) or diabetic patients (Diab) were cultured for 5 days in RPMI medium containing 20% of their own sera. Twenty-four hours after the final medium change, cells were lysed and LPL (*A*) and GAPDH (*B*) mRNA expression was analyzed by reverse transcription PCR. *C*: LPL mRNA levels (arbitrary units) normalized to the levels of GAPDH mRNA. Data represent means ± SE of results obtained in 10 control subjects and 10 diabetic patients. ¤¤$P < 0.005$ vs. control subjects.

diabetic patients resulted in a significant increase in the amounts of LPL secreted by these cells ($P < 0.001$) (Fig. 2).

LPL activity in macrophages of control subjects and diabetic patients. Monocyte-derived macrophages of diabetic patients cultured in their own sera secreted significantly higher LPL activity than macrophages of control subjects ($P < 0.005$) (Fig. 3). Incubation of macrophages of diabetic patients in sera of control subjects partially reversed this anomaly ($P < 0.001$) (Fig. 3). Conversely, differentiation of monocytes isolated from the control subjects into macrophages in sera of diabetic patients significantly enhanced LPL activity in these cells (Fig. 3).

Basal TNF-α secretion by macrophages of control subjects and diabetic patients. Macrophages of diabetic patients cultured in their own sera secreted significantly higher amounts of TNF-α than macrophages of control subjects ($P < 0.001$) (Fig. 4). Incubation of macrophages of diabetic patients in sera of control subjects dramatically decreased basal TNF-α production by these cells ($P < 0.001$) (Fig. 4). In macrophages of control subjects differentiated in sera of diabetic patients, a twofold increase in basal TNF-α secretion was also observed ($P < 0.005$) (Fig. 4).

LPL-induced TNF-α secretion by control and diabetic macrophages. Responsiveness to LPL, as assessed by TNF-α production, was significantly higher in monocyte-derived macrophages of diabetic patients than in cells of control subjects when culture was performed with autologous serum ($P = 0.005$) (Fig. 5). A significant decrease in LPL-induced TNF-α secretion by macrophages of diabetic patients was observed following exposure of these cells to sera of normal control subjects ($P < 0.005$) (Fig. 5). In contrast, a marked increase in TNF-α levels was found in supernatants harvested from macrophages of control subjects exposed to sera of diabetic patients ($P = 0.05$) (Fig. 5).

DISCUSSION

The present study establishes that macrophage LPL induction occurs in human type 2 diabetes at both the gene and protein levels. Our finding that LPL mRNA is overexpressed in type 2 diabetes is in accordance with the results of Creedon et al. (22), who found higher copy numbers of LPL mRNA in hypertriglyceridemic diabetic patients than in nondiabetic subjects. Although hypertriglyceridemia may contribute to the

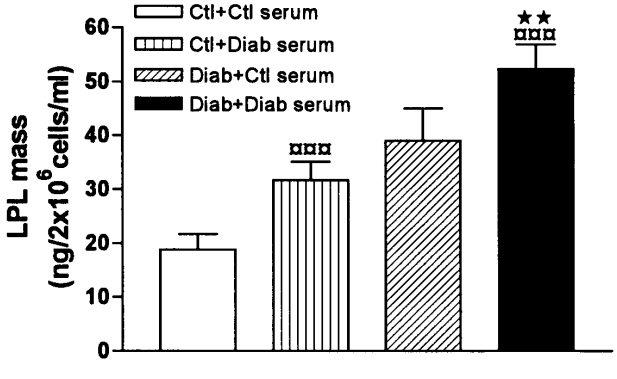

FIG. 2. LPL secretion by macrophages of control subjects and diabetic patients. Monocytes of control subjects (Ctl) or diabetic patients (Diab) were cultured in their own sera for 5 days. In some experiments, monocytes of control subjects were differentiated in sera of diabetic patients and monocytes of diabetic patients were differentiated in sera of control subjects. Twenty-four hours after the final medium change, LPL immunoreactive mass was determined in the medium. Values for LPL mass are expressed as nanograms per 2×10^6 cells per milliliter. Data represent means ± SE of results obtained in 10 control subjects and 10 diabetic patients. ¤¤¤$P < 0.001$ vs. Ctl + Ctl serum; ★★$P < 0.005$ vs. Diab + Ctl serum.

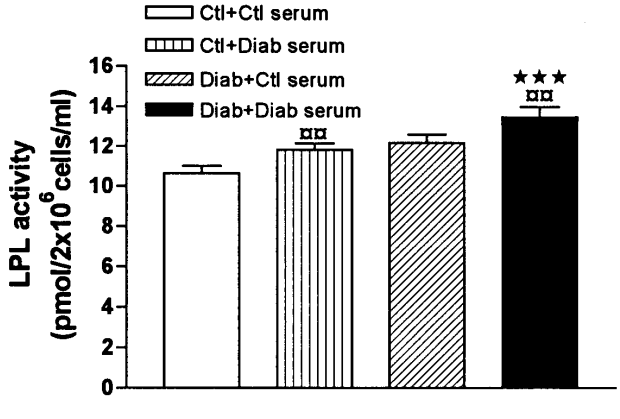

FIG. 3. LPL activity in macrophages of control subjects and diabetic patients. Monocytes of control subjects (Ctl) or diabetic patients (Diab) were cultured in their own sera for 5 days. In some experiments, monocytes of control subjects were differentiated in sera of diabetic patients and monocytes of diabetic patients were differentiated in sera of control subjects. Twenty-four hours after a medium change, LPL activity was determined in the supernatants. Values for LPL activity are expressed as picomoles per 2×10^6 cells per milliliter. Data represent means ± SE of results obtained in 10 control subjects and 10 diabetic patients. ¤¤$P < 0.005$ vs. Ctl + Ctl serum; ★★★$P < 0.001$ vs. Diab + Ctl serum.

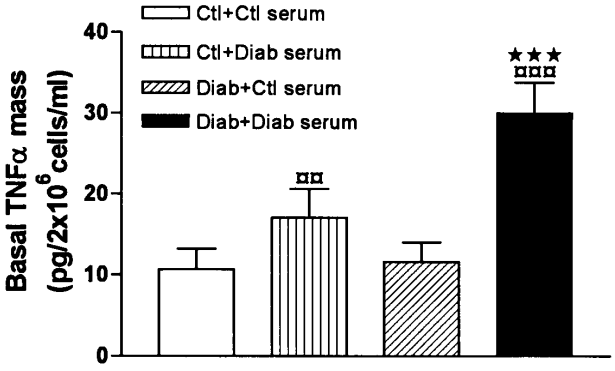

FIG. 4. Basal TNF-α secretion by macrophages of control subjects and diabetic patients. Monocytes of control subjects (Ctl) or diabetic patients (Diab) were cultured in their own sera for 5 days. In some experiments, monocytes of control subjects were differentiated in sera of diabetic patients and monocytes of diabetic patients were differentiated in sera of control subjects. Twenty-four hours after the final medium change, TNF-α immunoreactive mass was determined in the supernatants. Values for TNF-α mass are expressed as picograms per 2×10^6 cells per milliliter. Data represent means ± SE of results obtained in 8 control subjects and 8 diabetic patients. ¤¤$P < 0.005$ vs. Ctl + Ctl serum; ¤¤¤$P < 0.001$ vs. Ctl + Ctl serum; ★★★$P < 0.001$ vs. Diab + Ctl serum.

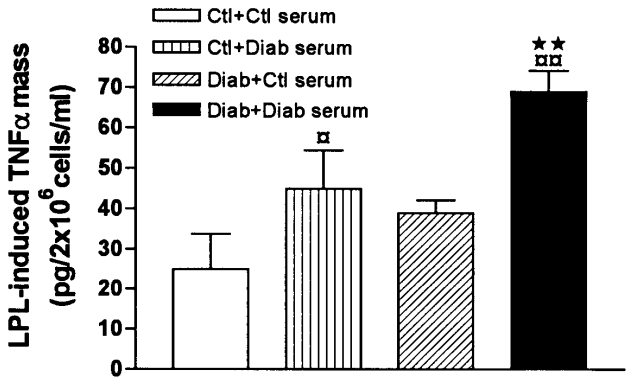

FIG. 5. LPL-induced TNF-α secretion by macrophages of control subjects and diabetic patients. Monocytes of control subjects (Ctl) or diabetic patients (Diab) were cultured in their own sera for 5 days. In some experiments, monocytes of control subjects were differentiated in sera of diabetic patients and monocytes of diabetic patients were differentiated in sera of control subjects. Twenty-four hours after the final medium change, exogenous bovine LPL (1 μg/ml) was added to the culture medium. The 24-h production of TNF-α was determined in the supernatants. Values for TNF-α mass are expressed as picograms per 2×10^6 cells per milliliter. Data represent means ± SE of results obtained in 5 control subjects and 5 diabetic patients. ¤P = 0.05 vs. Ctl + Ctl serum; ¤¤P = 0.005 vs. Ctl + Ctl serum; ★★$P < 0.005$ vs. Diab + Ctl serum.

increased transcript levels of LPL message in patients with type 2 diabetes (34), our finding that enhanced LPL mRNA expression also occurs in normotriglyceridemic subjects with type 2 diabetes clearly indicates that dyslipidemia is not the sole mechanism responsible for the induction of macrophage LPL mRNA expression in type 2 diabetes. Our observation that high glucose stimulates in vitro human macrophage LPL mRNA expression (23) suggests that other metabolic factors dysregulated in diabetes, including hyperglycemia, may play a key role in the macrophage LPL mRNA overexpression associated with type 2 diabetes. The molecular mechanisms responsible for the induction of macrophage LPL mRNA expression in macrophages isolated from diabetic patients are unknown. Although the limited amount of biological material extracted from human cells did not allow us to perform run-on experiments or evaluate LPL mRNA stability, our previous finding that transcriptional events are involved in the stimulatory effect of glucose on murine macrophage LPL expression (23) suggests that a similar mechanism may be responsible for the overexpression of LPL in macrophages isolated from patients with type 2 diabetes.

Previous studies have demonstrated that diabetes induces tissue-specific changes in the levels of LPL mRNA, immunoreactive protein, and activity. Whereas diabetes has been repeatedly shown to decrease adipose tissue LPL activity (35–38), LPL activity in skeletal muscle of diabetic patients has been found to be either unchanged or decreased (37,39–42). Our results, which demonstrate that induction of macrophage LPL immunoreactive protein and activity occurs in the diabetic state and parallels changes in LPL mRNA levels, indicate that in human macrophages, diabetes exerts a pretranslational control on LPL expression.

Although the mechanisms responsible for macrophage LPL induction in diabetes remain uncertain, our observation that culture of macrophages of diabetic patients in sera of control subjects markedly decreases this alteration indicates that metabolic or hormonal factors present in the sera of diabetic subjects are at least partly responsible for the overproduction of macrophage LPL in diabetes. Potential metabolic factors associated with macrophage LPL induction include glucose and AGE. Indeed, these factors accumulate in the plasma and vessel walls of diabetic patients (43,44) and stimulate in vitro human macrophage LPL production (23).

Along with peripheral factors, other mechanisms seem to be involved in the upregulation of macrophage LPL in type 2 diabetes. Indeed, we found that incubation of macrophages of diabetic patients with sera of normal control subjects did not totally normalize macrophage LPL induction. Although clear evidence demonstrating the insulin-sensitivity of macrophage LPL is still lacking (45), one possibility is that induction of macrophage LPL in diabetes could, at least in part, represent a compensatory mechanism to provide the cells with energy in the presence of diminished insulin-mediated glucose uptake. Studies of macrophage LPL regulation in insulin-resistant nondiabetic subjects are currently underway to investigate this possibility.

Just as TNF-α is overexpressed in adipose tissue and muscle of insulin-resistant subjects, increased TNF-α production has been documented in monocytes of patients with type 2 diabetes (24). Our results indicate a similar augmentation of basal TNF-α secretion by macrophages of diabetic patients. Our finding that culture of macrophages of diabetic subjects in sera of normal control subjects totally reverses the induction of macrophage TNF-α underlines the role of peripheral factors in this alteration. Data indicating a stimulatory effect of high glucose, AGE, and oxidized LDL on monocyte/macrophage TNF-α release (26,27,46) support a role of these factors in the dysregulation of macrophage TNF-α in diabetes.

Besides its role in lipid metabolism, LPL acts as an activator of macrophage function, inducing TNF-α secretion (20,21). The present report provides evidence that LPL-induced TNF-α production is higher in macrophages of diabetic patients than in those of control subjects. Because dia-

betes is associated with enhanced oxidative stress (44,47) and reactive oxygen species facilitate LPS-induced TNF-α production (48), one may speculate that oxidant stress may, at least in part, be responsible for the upregulation of macrophage responsiveness to LPL observed in diabetes. However, arguing against this possibility is our recent observation that reactive oxygen intermediates decrease LPL-induced TNF-α production (49). From these results, it clearly appears that future studies will be needed to address the mechanisms involved in this alteration.

Atherosclerosis is the leading complication of type 2 diabetes. The pathogenesis of atherosclerosis is poorly understood but clearly involves the production in the vascular wall of macrophage LPL and TNF-α. Data generated in the present study clearly demonstrate that human type 2 diabetes promotes the overproduction of these two proatherogenic factors and that metabolic or hormonal factors accumulating in the sera of diabetic subjects are, at least in part, responsible for these alterations. These results suggest that diabetes may tend to increase the in vivo production of LPL and TNF-α in the arterial wall and that these alterations may contribute to the development of atherosclerosis associated with diabetes. Better understanding of the pathobiology of atherosclerosis associated with diabetes gained from studies of the human diabetic macrophage should lead to the development of new methods for the prevention and treatment of diabetic atherosclerosis.

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