Altered Adipose and Plasma Sphingolipid Metabolism in Obesity

A Potential Mechanism for Cardiovascular and Metabolic Risk

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The adipose tissue has become a central focus in the pathogenesis of obesity-mediated cardiovascular and metabolic disease. Here we demonstrate that adipose sphingolipid metabolism is altered in genetically obese (*ob/ob*) mice. Expression of enzymes involved in ceramide generation (neutral sphingomyelinase [NSMase], acid sphingomyelinase [ASMase], and serine-palmitoyl-transferase [SPT]) and ceramide hydrolysis (ceramidase) are elevated in obese adipose tissues. Our data also suggest that hyperinsulinemia and elevated tumor necrosis factor (TNF)-α associated with obesity may contribute to the observed increase in adipose NSMase, ASMase, and SPT mRNA in this murine model of obesity. Liquid chromatography/mass spectroscopy revealed a decrease in total adipose sphingomyelin and ceramide levels but an increase in sphingosine in *ob/ob* mice compared with lean mice. In contrast to the adipose tissue, plasma levels of total sphingomyelin, ceramide, sphingosine, and sphingosine 1-phosphate (S1P) were elevated in *ob/ob* mice. In cultured adipocytes, ceramide, sphingosine, and S1P induced gene expression of plasminogen activator inhibitor-1, TNF-α, monocyte chemoattractant protein-1, interleukin-6, and keratinocyte-derived chemokine. Collectively, our results identify a novel role for sphingolipids in contributing to the prothrombotic and proinflammatory phenotype of the obese adipose tissue currently believed to play a major role in the pathogenesis of obesity-mediated cardiovascular and metabolic disease. *Diabetes* 55:2579–2587, 2006

Obesity is reaching epidemic proportions in Western societies. Indeed, >65% of the adult U.S. population is either overweight or obese (1). Although obesity is a strong risk factor for the development of insulin resistance, type 2 diabetes, and cardiovascular disease (1), the molecular changes in obesity that promote these conditions are not completely understood. During the last several years, the role of the adipose tissue in contributing to obesity-associated cardiovascular and metabolic risk has gained much attention. Adipose tissues secrete a variety of prothrombotic and proinflammatory molecules that affect both insulin sensitivity and cardiovascular risk (2–4). For example, plasminogen activator inhibitor-1 (PAI-1), the primary inhibitor of plasminogen activation in vivo and an established risk factor for cardiovascular disease is dramatically elevated in adipose tissues of obese mice and humans (3,5). Adipose tissues also synthesize and secrete a variety of proinflammatory mediators including tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-6, and keratinocyte-derived chemokine (KC) (a functional homolog of human IL-8), and expressions of these cytokines and chemokines are elevated in the adipose tissues in obesity (2,6–8). Despite the increasing evidence implicating the adipose tissue in contributing to obesity-related cardiovascular and metabolic risk, the molecular mechanisms that contribute to increased adipose expression of prothrombotic (e.g., PAI-1) and proinflammatory molecules shown to be associated with these health complications have not been completely defined.

Numerous studies have demonstrated that sphingolipids contribute to the pathogenesis of a variety of diseases (9–12). Sphingolipids, in addition to their role as structural molecules of the plasma membrane, are also important bioactive mediators for a variety of cellular processes (11–13). In particular, biological functions of ceramide, sphingosine, and sphingosine 1-phosphate (S1P) have been extensively studied (12). The production of ceramide is mediated by the hydrolysis of membrane sphingomyelin by acid sphingomyelinase (ASMase) or neutral sphingomyelinase (NSMase) or by de novo synthesis via serine palmitoyl transferase (SPT) and ceramide synthase (12,13). Ceramide is subsequently metabolized into sphingosine through the action of ceramidases (alkaline or acid ceramidase), and sphingosine can be further converted to S1P via the action of sphingosine kinase (12,13). Accumulating evidence suggests that these sphingomyelin metabolites (e.g., ceramide, sphingosine, and S1P) in turn serve as signaling molecules involved in multiple signaling pathways regulating a variety of physiological and pathological biological events including cell growth, survival, and death (12,13). Abnormalities in sphingolipid metabolism have been implicated in the pathogenesis of obesity/diabetes (14–16) and atherosclerosis (10,17–20). Although some studies have addressed the expression and the role of

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ASMase, acid sphingomyelinase; GM3, *N*-acetylneuraminylgalactosylceramide; HPLC, high-performance liquid chromatography; IL, interleukin; KC, keratinocyte-derived chemokine; MCP-1, monocyte chemoattractant protein-1; NSMase, neutral sphingomyelinase; PAI-1, plasminogen activator inhibitor-1; S1P, sphingosine 1-phosphate; SPT, serine palmitoyl transferase; TMS, tandem mass spectrometry; TNF-α, tumor necrosis factor-α.

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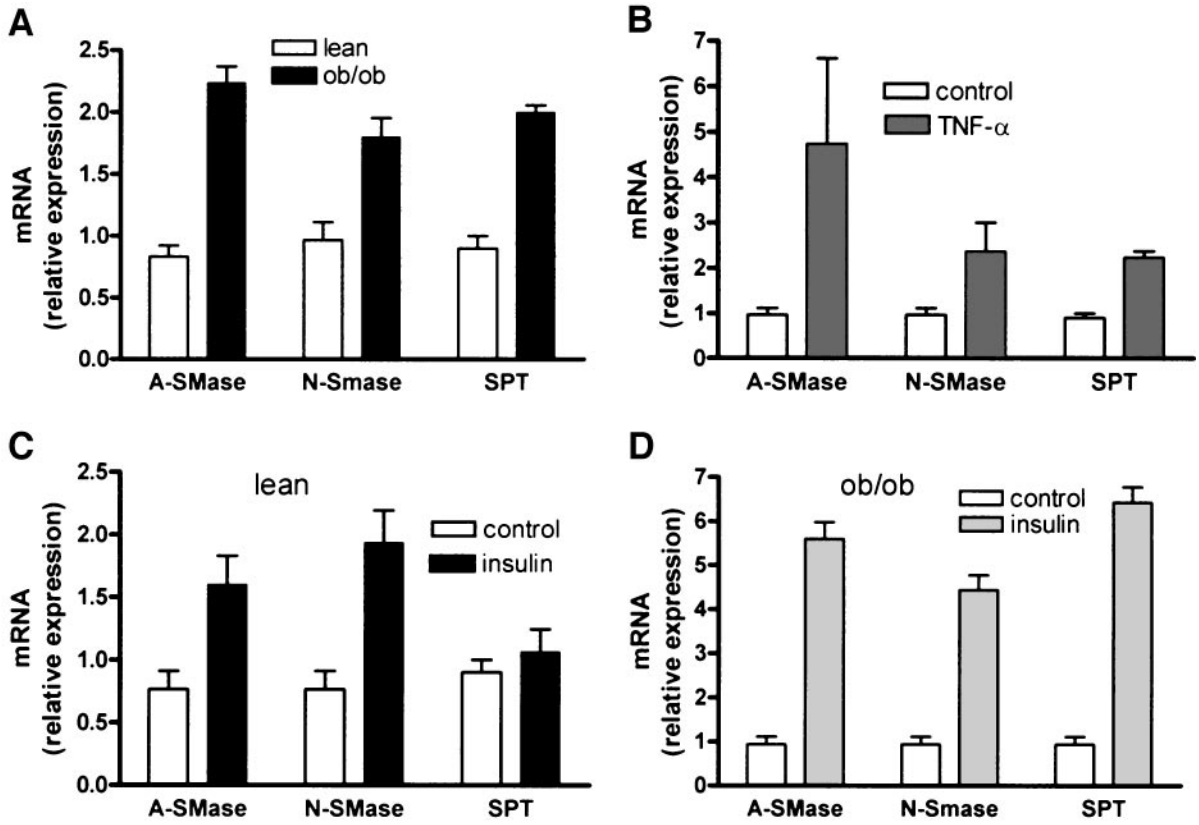

FIG. 1. Expression and regulation of ASMase, NSMase, and SPT mRNA. *A*: ASMase, NSMase, and SPT mRNA levels were determined by real-time RT-PCR from epididymal fat pads of lean and *ob/ob* mice. Data are means ± SD (n = 4). Statistical comparison between lean and *ob/ob* mice indicate that $P < 0.001$ for ASMase mRNA and SPT mRNA; $P < 0.05$ for NSMase mRNA. *B*: ASMase, NSMase, and SPT mRNA levels were determined from epididymal fat pads of lean C57BL/6J mice 3 h after intraperitoneal injection with either 4 μg recombinant mouse TNF-α or saline. Data are means ± SD (n = 4). For control versus TNF-α–treated mice: $P < 0.05$ for ASMase mRNA and NSMase mRNA; $P < 0.01$ for SPT mRNA. *C* and *D*: ASMase, NSMase, and SPT mRNA levels were determined from epididymal fat pads of lean C57BL/6J (*C*) or *ob/ob* (*D*) mice 3 h after intraperitoneal injection with either 5 units insulin or saline. Data are means ± SD (n = 4). For control versus insulin-treated lean mice: $P < 0.05$ for ASMase and NSMase mRNA. For control versus insulin-treated *ob/ob* mice: $P < 0.001$ for ASMase, NSMase, and SPT mRNA.

sphingolipids in tissues such as the muscle, liver, and pancreas in the pathogenesis of obesity/diabetes (14–16), to our knowledge, information regarding the expression, regulation, and functional significance of sphingolipids in adipose tissues in obesity is lacking. Additionally, very little is known regarding plasma levels of sphingolipids in obesity. The studies described in this article demonstrate that sphingolipid metabolism is differentially altered in adipose tissue and plasma in genetically obese mice and identify a novel role for sphingolipids in contributing to the induction of prothrombotic and proinflammatory proteins from the adipose tissues in obesity and thereby to the pathogenesis of obesity-mediated cardiovascular and metabolic risk.

RESEARCH DESIGN AND METHODS

All animal studies were reviewed and approved by our institutional animal care and use committee and the animal research committee, in accordance with public health policy regarding the use and care of laboratory animals. Adult male obese mice (C57BL-*ob/ob*) 14–16 weeks of age and their lean counterparts (C57BL/6J) were obtained from The Jackson Laboratory (Bar Harbor, ME). In some experiments, C57BL/6J mice were injected intraperitoneally with recombinant murine TNF-α (4 μg/mouse in 100 μl of sterile saline; a kind gift of Richard Ulevitch, The Scripps Research Institute), insulin (5 units/mouse, Humulin R; Eli Lilly, Indianapolis, IN), or equivalent volumes of saline. The concentrations of TNF-α and insulin used resulted in increases in the levels of these mediators in lean mice that very closely approximate the levels of TNF-α and insulin observed in obese mice (data not shown). Three hours later, mice were anesthetized with methoxyflurane (Metofane; Pitman-Moore, Mundelein, IL), and their blood was collected into 20 mmol/l (final concentration) EDTA, pH 8.0, and epididymal adipose tissues were removed and processed for the preparation of total RNA or sphingolipid analysis.

Cell culture. 3T3-L1 mouse embryo fibroblasts were obtained from the American Type Culture Collection (Rockville, MD) and grown and differentiated into adipocytes as described previously (21,22). Adipocytes were treated either with recombinant murine TNF-α (8 ng/ml; Genzyme Diagnostics, Cambridge, MA) or insulin (100 nmol/l bovine insulin; Sigma Chemical, St. Louis, MO) after a 24-h pretreatment in serum-free medium containing 0.2% BSA. Cells were harvested 3 h later, and total RNA was isolated using the Ultraspec RNA isolation system according to manufacturer's directions (Biotecx Laboratories, Houston, TX). Relative gene expression of each of the specific genes analyzed in the study was determined using real-time RT-PCR.

RNA analysis. Real-time RT-PCR was performed as previously described (22,23) in an iCycler (Bio-Rad, Hercules, CA). Real-time RT-PCR amplifications were performed from 2.5 μl of cDNA diluted 1:2 using each of the gene-specific primer sets (Invitrogen Life Technologies, Grand Island, NY), designed from published cDNA sequences. Each primer set was used at a concentration of 150 nmol/l in a final volume of 25 μl using the SYBR Green Master Mix (PE Applied Biosystems). Gene expression is expressed as relative mRNA levels compared with a control, calculated after normalization to β-actin.

Sphingolipid analysis. Sphingolipids (sphingomyelin, ceramide, sphingosine, and S1P) were analyzed by high-performance liquid chromatography (HPLC)/tandem mass spectroscopy (TMS) as described previously (24). Adipose tissue homogenates (in buffer containing 0.25 mol/l sucrose, 25 mmol/l KCl, 50 mmol/l Tris, 0.5 mmol/l EDTA; pH 7.4) or plasma samples were fortified with internal standards *N*-hexanoyl-1-(2-phosphorylcholine)-sphingosine for sphingomyelin determination and C_{17} base D-*erythro*-sphingosine, C_{17} sphingosine-1-phosphate, *N*-palmitoyl-D-*erythro*-C_{13} sphingosine, and heptadecanoyl-D-*erythro*-sphingosine. Samples were extracted into a one-phase neutral organic solvent system (ethyl acetate-isopropanol-water; 60:30:10 vol/vol/vol), evaporated, and reconstituted in methanol and analyzed by a Surveyor/TSQ 7000 liquid chromatography/mass spectrometry system. Quali-

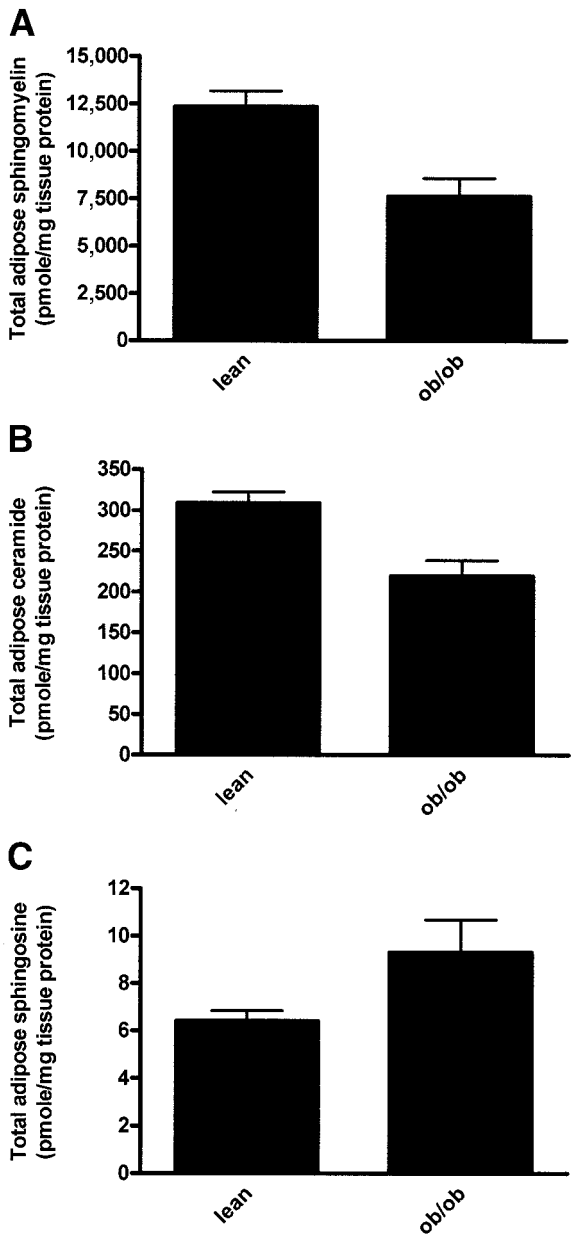

FIG. 2. Analysis of adipose sphingolipids in epididymal fat pads. Levels of sphingomyelin (*A*), ceramide (*B*), and sphingosine (*C*) from epididymal fat pads were measured by HPLC/TMS. Total sphingomyelin and ceramides were the sum of individual sphingomyelin and ceramide species, respectively. Data are means ± SD (n = 4). For lean versus *ob/ob* mice: $P < 0.05$ for total adipose sphingomyelin, total adipose ceramide, and adipose sphingosine.

tative analysis of sphingolipids was performed by a parent ion scan of a common fragment ion characteristic for a particular class of sphingolipids. Quantitative analysis was performed in a positive multiple reaction monitoring mode, on the basis of calibration curves generated by spiking an artificial matrix with known amounts of target analytes, synthetic standards, and an equal amount of internal standard. The calibration curves were constructed by plotting the peak area ratios of analyte to the respective internal standard against concentration using a linear regression model.

Statistical analysis. Statistical comparison of results was performed using the unpaired Student's t test.

RESULTS

Expression and regulation of enzymes involved in ceramide synthesis in adipose tissues in obesity. In adipose tissues of *ob/ob* mice, significant increases in the mRNA levels of ASMase, NSMase, and SPT were observed compared with those in the lean counterparts (Fig. 1*A*). Similar increases in the mRNA expression of these genes also were observed for the related leptin receptor–deficient obese *db/db* mice (data not shown). Obesity is associated with increased expression of TNF-α in adipose tissues (5). Because TNF-α has been shown to activate ASMase, NSMase, and the de novo pathway of ceramide generation (13), we were interested to see whether TNF-α regulated sphingomyelinase and SPT gene expression in adipose tissue. The adipose tissue mRNA levels of all of these enzymes were increased significantly in response to an intraperitoneal injection of TNF-α into C57/BL6 mice (Fig. 1*B*). These studies demonstrate that the mRNAs of enzymes involved in ceramide generation are elevated in the adipose tissues of obese mice and suggest that TNF-α may contribute to their increased expression in adipose tissues in obesity.

Obesity is also associated with insulin resistance and hyperinsulinemia. Our previous studies demonstrating that the hyperinsulinemia associated with obesity may contribute to the adipose expression of prothrombotic and proinflammatory genes, such as PAI-1, TNF-α, and MCP-1 (3,6,21), prompted us to examine the role of insulin in the regulation of enzymes involved in ceramide synthesis in obesity. In adipose tissues of insulin-injected lean mice, significant increases in ASMase and NSMase mRNAs were observed with no significant change in SPT mRNA expression (Fig. 1*C*). However, insulin treatment induced dramatic and significant induction of expression of all three enzymes in the adipose tissues of *ob/ob* mice (Fig. 1*D*). Interestingly, the insulin-mediated increase in these genes in *ob/ob* mice was substantially larger than what was observed in lean mice. Thus, in addition to TNF-α, hyperinsulinemia associated with obesity may also contribute to the altered expression of genes involved in ceramide generation in adipose tissues in obesity.

Analysis of sphingolipids in adipose tissues from lean and obese (*ob/ob*) mice. Having shown changes in the expression of enzymes involved in ceramide generation, we next measured endogenous levels of sphingomyelin and ceramide in adipose tissues from lean and obese (*ob/ob*) mice using HPLC/TMS as described under RESEARCH DESIGN AND METHODS. Interestingly, there was a significant reduction in both the total sphingomyelin (Fig. 2*A*) and ceramide levels (Fig. 2*B*) and an increase in the level of the downstream ceramide metabolite sphingosine (Fig. 2*C*) in adipose tissue of *ob/ob* mice. Adipose levels of S1P were very low and below the detection limit of the assay. Similar changes in sphingolipid levels also were observed for the genetically obese *db/db* mice (data not shown). When individual sphingomyelin species were examined in adipose tissues from lean and *ob/ob* mice, the results demonstrated a general decrease for every species of sphingomyelin examined (Table 1), with the largest and significant decreases of ~61–67% being observed for C20, C20:1, C22:1, C24, and C24:1 sphingomyelin. Although decreases in sphingomyelin levels of 25–55% were also observed for C14, C16, C18, and C18:1 in the adipose tissues from *ob/ob* mice, these changes were not significant. When individual ceramide species were examined there was a decrease in most ceramide species in adipose tissues from *ob/ob* mice, with the decrease being significant for C18:1 (32%), C24 (46%), and C24:1 (27%) ceramide (Table 1). Interestingly, in contrast with other ceramide

TABLE 1
Adipose sphingomyelin and ceramide

	Lean	Composition	*ob/ob*	Composition
Sphingomyelin (SM)				
C14 SM	96 ± 61	0.78	50.5 ± 10.5	0.67
C16 SM	7,617 ± 164.5	61.87	5,720 ± 1,380	75.35
C18 SM	1,140 ± 555	9.26	510 ± 57	6.72
C18:1 SM	230 ± 116	1.87	139 ± 26	1.83
C20 SM	679 ± 224	5.52	223 ± 37*	2.94
C20:1 SM	203.5 ± 75.5	1.65	76.25 ± 16.2*	1.00
C22:1 SM	356.3 ± 120	2.90	125 ± 19*	1.65
C24 SM	571 ± 108	4.64	200 ± 33*	2.63
C24:1 SM	1,430 ± 52	11.61	547.5 ± 108†	7.21
Ceramide (Cer)				
C14Cer	2.2 ± 0.6	0.69	4.8 ± 0.5*	2.14
C16 Cer	178.6 ± 28.8	56.19	124 ± 28.7	55.18
C18 Cer	32.0 ± 6.5	10.07	23.3 ± 4.9	10.37
C18:1 Cer	11.25 ± 0.45	3.54	7.6 ± 1.2†	3.38
C20 Cer	9.5 ± 4.3	0.03	11.7 ± 0.1	5.21
C24 Cer	38 ± 6.5	11.96	20.4 ± 2.9*	9.08
C24:1 Cer	38.5 ± 2.2	12.11	27.8 ± 3.7*	12.37
DHC16	7.8 ± 2.3	2.45	5.1 ± 0.65	2.27

Data are means ± SD (pmol/mg tissue protein) or percent (n = 4). For lean vs. *ob/ob* mice: *$P < 0.05$; †$P < 0.01$.

species, there was a significant increase in C14 ceramide (54%).

The decrease in total sphingomyelin and ceramide levels, with the parallel increase in sphingosine, in the adipose tissues of obese mice suggests that sphingomyelin may be hydrolyzed to ceramide, which may be further converted to sphingosine through the action of the ceramidase enzymes. Indeed, in accordance with this hypothesis, we observed a significant increase in the mRNA levels of both acid ceramidase and alkaline ceramidase in the adipose tissues of *ob/ob* mice compared with those in lean mice (Fig. 3*A*). The ceramidases hydrolyze ceramide to sphingosine and the increase in the expression of these enzymes in the adipose tissues of *ob/ob* mice suggests that ceramide hydrolysis may be increased in obese adipose tissue compared with lean adipose tissue. Alternately, the observed decrease in adipose ceramide may also reflect ceramide conversion into complex sphingolipids such as glucosylceramide. To test this possibility, we measured the mRNA levels of glucosylceramide synthase and *N*-acetylneuraminylgalactosylceramide (GM3) synthase in adipose tissues from lean and *ob/ob* mouse (Fig. 3*B*). Glucosylceramide synthase and GM3 synthase mRNA expressions were significantly reduced in adipose tissues from *ob/ob* mice, suggesting that this pathway probably does not contribute to the decrease in adipose ceramide observed in *ob/ob* mice.

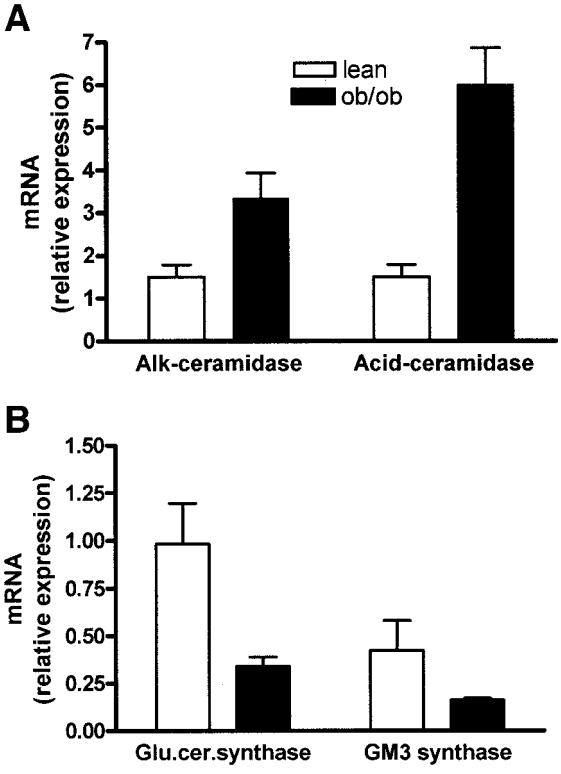

FIG. 3. Adipose expression of ceramidase, glucosylceramide synthase (Glu.cer.synthase), and GM3 synthase mRNA. *A*: Alkaline (Alk) and acid ceramidase mRNA levels were determined from epididymal fat pads of lean and *ob/ob* mice. Data are means ± SD (n = 4). For lean versus *ob/ob* mice: $P < 0.05$ for alkaline ceramidase; $P < 0.01$ for acid ceramidase. *B*: Glucosylceramide synthase and GM3 synthase mRNA from epididymal fat pads of lean and *ob/ob* mice. For lean versus *ob/ob* mice: $P < 0.01$ for glucosylceramide synthase; $P < 0.05$ for GM3 synthase.

Analysis of sphingolipids in plasma from lean and obese (*ob/ob*) mice. Obesity is an established risk factor for atherosclerosis, and recent data suggest that sphingomyelin and ceramide may be involved in the pathogenesis of atherosclerosis (10,18,20). In contrast to adipose tissue, a significant increase in total sphingomyelin and total ceramide was observed in the plasma of *ob/ob* mice over that in lean mice (Fig. 4*A* and *B*). Moreover, the levels of the downstream ceramide metabolites, sphingosine and S1P, also were significantly increased in *ob/ob* mice (Fig. 4*C*). When plasma levels of sphingomyelin isoforms were examined (Table 2), the data show an increase in most of the sphingomyelin isoforms, with the largest increase (69%) being observed for C14 sphingomyelin. C22, C22:1, and C24 sphingomyelins were decreased by 52, 33, and 16%, respectively, in the plasma of *ob/ob* mice compared with lean mice. All ceramide species that were detectable in the plasma were increased in the plasma of *ob/ob* mice compared with that of lean mice (Table 2). A dramatic and

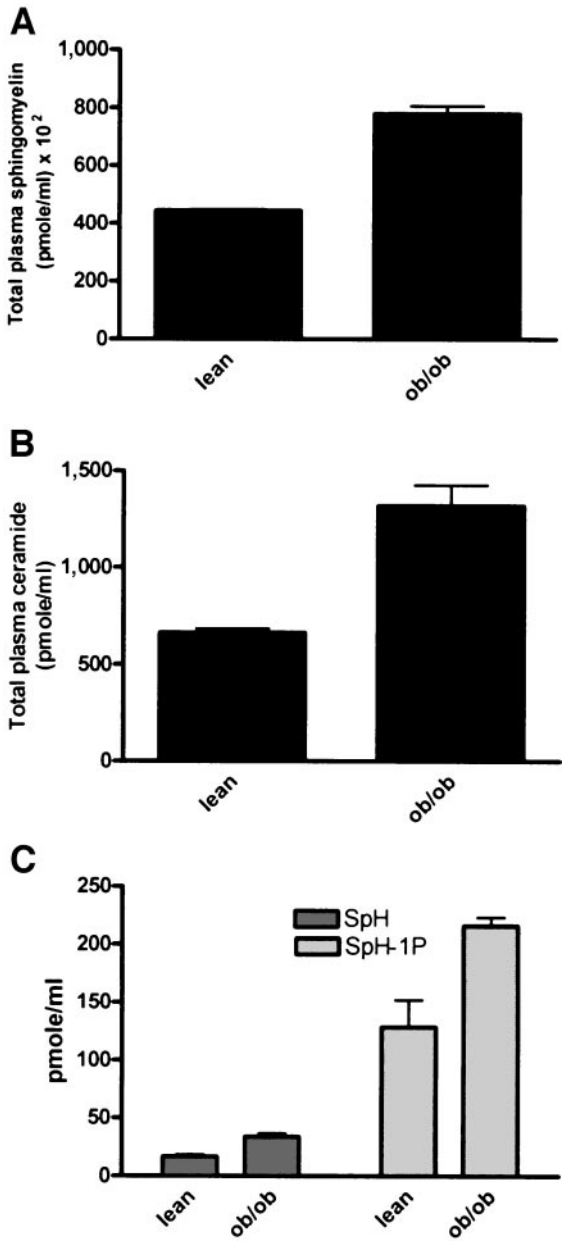

FIG. 4. Analysis of plasma sphingolipids. Levels of sphingomyelin (*A*), ceramide (*B*), sphingosine, and S1P (*C*) were measured by HPLC/TMS. Total sphingomyelin and ceramides were the sum of individual sphingomyelin and ceramide species, respectively. Data are means ± SD (n = 4). For lean versus *ob/ob* mice: $P < 0.01$ for total plasma sphingomyelin and total plasma ceramide; $P < 0.05$ for plasma sphingosine and plasma S1P.

highly significant increase of 86% was observed for C18 ceramide in the plasma of *ob/ob* mice. Significant increases also were observed for C16 (55%), C24 (50%), and C24:1 (56%) ceramides in *ob/ob* mice compared with lean mice. In light of recent observations implicating a role for plasma sphingolipids in the pathogenesis of atherosclerosis (10,18,20), our observations demonstrating increased levels of sphingolipids (sphingomyelin, ceramide, sphingosine, and S1P) in the plasma of obese mice provide an additional molecular link for the increased cardiovascular risk associated with the obese phenotype.

Role of sphingolipids in the regulation of prothrombotic and proinflammatory gene expression in adipocytes. Obesity is associated with changes in the adipose tissue expression of prothrombotic and proinflammatory genes, which may underlie the cardiovascular and metabolic risk associated with the obese phenotype (2,3,5,7,8,25,26). Increased expressions of genes encoding prothrombotic proteins such as PAI-1 and proinflammatory proteins (e.g., TNF-α, IL-6, MCP-1, and KC) have been consistently demonstrated in adipose tissues from obese animals and humans (2,5–8,26). To assess the role of sphingolipids (ceramide, sphingosine, and S1P) in mediating the transcriptional prothrombotic and inflammatory response to obesity, we determined the mRNA levels of these genes in response to ceramide, sphingosine, or S1P in mature 3T3-L1 adipocytes.

3T3-L1 adipocytes were treated with soluble short-chain ceramides (C2 or C6) or the ceramide metabolites sphingosine or S1P, and the expressions of PAI-1 and proinflammatory proteins were measured (Fig. 5). The data indicate a significant increase in PAI-1 in response to both C2 ($P < 0.001$) and C6 ($P < 0.01$) ceramide (Fig. 5*A*). Moreover, a significant increase in PAI-1 mRNA also was observed when adipocytes were treated with either sphingosine ($P < 0.01$) or S1P ($P < 0.05$).

We next investigated whether ceramide or its metabolites might also play a role in the expression of proinflammatory cytokines (TNF-α, MCP-1, IL-6, and KC) in adipocytes. C2 ceramide significantly induced MCP-1 ($P < 0.01$) and KC ($P < 0.05$) (Fig. 5*C* and *E*), but not TNF-α or IL-6 (Fig. 5*B* and *D*). C6 ceramide significantly ($P < 0.05$) induced the expression of TNF-α, MCP-1, and IL-6 in these cells (Fig. 5*B–D*), whereas induction of KC did not reach significance (Fig. 5*E*). The data therefore suggest that the adipocyte proinflammatory response in obesity can be mediated to various degrees by small-chain ceramides. Because ceramide is easily metabolized to sphingosine, which, in turn, is readily converted to S1P, we also determined whether sphingosine and S1P can regulate the expression of proinflammatory cytokines in adipocytes (Fig. 5*B–E*). TNF-α and KC expression were increased significantly ($P < 0.05$) in response to sphingosine and S1P (Fig. 5*B* and *E*, respectively). For MCP-1, a significant increase was observed when cells were treated with sphingosine ($P < 0.05$), whereas there was no effect in response to S1P (Fig. 5*C*), and IL-6 mRNA was significantly increased ($P < 0.05$) when adipocytes were treated with sphingosine and with the higher dose of S1P (Fig. 5*D*). Interestingly, treatment of 3T3-L1 adipocytes with the ganglioside GM3 also induced PAI-1, TNF-α, and IL-6 mRNA by fourfold ($P < 0.05$) (Fig. 5*F*). Taken together, these findings suggest that ceramide and/or its downstream metabolites to various degrees can regulate the gene expression of prothrombotic and inflammatory proteins from adipocytes, and, thus, ceramide metabolism may be a key component in the increased expression of prothrombotic and proinflammatory genes in obesity.

DISCUSSION

Although obesity is a complex metabolic disorder often associated with insulin resistance/hyperinsulinemia and type 2 diabetes, as well as with accelerated atherosclerosis, the molecular changes in obesity that promote these problems are still not completely understood. Sphingolipids have recently been indicated in the pathogenesis of a variety of diseases including obesity, diabetes, and atherosclerosis (9,10,14,15,17,18). Sphingolipid metabolism can

TABLE 2
Plasma sphingomyelin and ceramide

	Lean	Composition	*ob/ob*	Composition
Sphingomyelin (SM)				
C14 SM	4.6 ± 1.1	1.01	14.85 ± 1.25*	1.91
C16 SM	239 ± 16	52.29	534 ± 16.5*	69.00
C18 SM	12.35 ± 2.4	2.70	23 ± 3.4†	2.96
C18:1 SM	3.84 ± 1.8	0.84	11.23 ± 2.8	1.45
C20 SM	6.33 ± 0.7	1.38	7.4 ± 0.2	0.95
C20:1 SM	2.75 ± 0.46	0.60	4.9 ± 0.05†	0.63
C22 SM	52.7 ± 9.5	11.53	25.25 ± 1.5†	3.24
C22:1 SM	27 ± 3.2	5.91	17.98 ± 0.08†	2.31
C24 SM	29.22 ± 5.5	6.39	24.3 ± 2.07	3.12
C24:1 SM	79.3 ± 12.2	17.35	115.4 ± 13.57†	14.83
Ceramide (Cer)				
C16 Cer	49.2 ± 18.3	7.49	109.5 ± 1.5†	7.63
C18 Cer	15 ± 4.5	2.28	106 ± 2.0‡	7.38
C20 Cer	31 ± 4.0	4.72	55 ± 18	3.83
C24 Cer	370 ± 70	56.32	740 ± 40†	51.55
C24:1 Cer	182.6 ± 26	27.79	416 ± 20*	28.98
DHC16	9.2 ± 6.1	1.40	9.1 ± 1.8	0.63

Data are means ± SD (pmol/ml × 10^2) or percent ($n = 4$). For lean vs. *ob/ob* mice: *$P < 0.01$; †$P < 0.05$; ‡$P < 0.001$.

be activated by a variety of conditions such as proinflammatory cytokines, growth factors, and oxidative stress (11–13), conditions that characterize the local milieu of the adipose tissues in obesity (2,4,27,28). These observations suggest that sphingolipid metabolism may be altered in the adipose tissues in obesity. The present study was therefore undertaken to address specifically the expression, regulation, and functional significance of adipose sphingolipids as they relate to obesity and associated cardiovascular and metabolic risk.

A novel and unexpected finding of this study was that the total sphingomyelin and ceramide contents were reduced, whereas the level of the ceramide metabolite sphingosine was increased in the adipose tissues of genetically obese *ob/ob* mice compared with their lean counterparts (Fig. 2*A*–*C*). These observations were consistent with an increase in gene expression of enzymes involved both in ceramide generation (ASMase, NSMase, and SPT) (Fig. 1*A*) and in ceramide hydrolysis (acid and alkaline ceramidase) (Fig. 3*A*) in adipose tissues of obese mice. Thus, in the obese adipose tissue, the equilibrium of sphingolipid metabolism appears to be shifted toward the generation of sphingosine (Fig. 6). The conversion of ceramide into complex sphingolipids such as glucosylceramide is another potential mechanism for the reduction of adipose ceramide. However, the mRNA levels of glucosylceramide synthase and GM3 synthase were decreased in the adipose tissues of *ob/ob* mice (Fig. 3*B*), suggesting that this pathway probably does not contribute to the observed reduction of adipose ceramide in *ob/ob* mice. Our results showing that GM3 synthase is decreased in epididymal fat pads of *ob/ob* mice are in contrast to previous reports in which GM3 synthase mRNA was shown to be increased in adipose tissues of Zucker *fa/fa* rats and *ob/ob* mice (29). We believe that differences in parameters such as the genetic background (B6 vs. Ks), age, sex, the use of appropriate littermate lean controls, feed composition, and housing conditions, which are known to influence not only diabetes and insulin resistance but also gene expression in these genetically obese murine models, may have led to the discrepancies observed between the two studies. Because the previously published study does not provide specific details regarding the above parameters, we are unable to draw parallel comparisons.

Ceramide has been shown to be antiproliferative and proapoptotic (30). In contrast, S1P has been implicated in cell proliferation and survival (31). The function of the intermediate sphingolipid, sphingosine, is less clear. Despite a number of studies supporting a role for sphingosine in inducing apoptosis (32,33), some recent literature suggests possible mechanisms for sphingosine-induced antiapoptotic pathways (34,35). In this instance, it is tempting to speculate that the reduction in ceramide and/or increase in sphingosine levels observed in the adipose tissues of obese mice may be associated with a survival advantage and protects adipocytes and perhaps other cells in the obese adipose tissue (e.g., infiltrating macrophages, endothelial cells, and preadipocytes) from apoptosis, thereby contributing to obesity-associated adipose hyperplasia.

In contrast with sphingolipid levels in adipose tissue of obese mice, total levels of sphingomyelin and ceramide were increased in the plasma of obese *ob/ob* mice compared with those in lean mice (Fig. 4*A* and *B*). Although there was variation with respect to individual sphingomyelin levels, all species of detectable ceramide were increased in the plasma with the largest increase of 86% being observed for C18 ceramide (Table 2). The ceramide metabolites sphingosine and S1P were also increased in the plasma of *ob/ob* mice compared with their lean counterparts (Fig. 4*C*). We believe that the changes in plasma sphingolipids observed in this study may be significant because only a fraction of the clinical complications of atherosclerosis are explained by known risk factors. Animal and human studies have shown that plasma sphingomyelin and ceramide levels are closely related to the development of atherosclerosis (18,36–38). Sphingomyelin carried into the arterial wall on atherogenic lipoproteins may be locally hydrolyzed by sphingomyelinase, promoting lipoprotein aggregation and macrophage foam cell formation (39). Ceramide signaling has also been shown to be responsible for certain inflammatory responses in the developing atherosclerotic lesions, such as smooth muscle cell proliferation (20). Moreover, ceramide also may con-

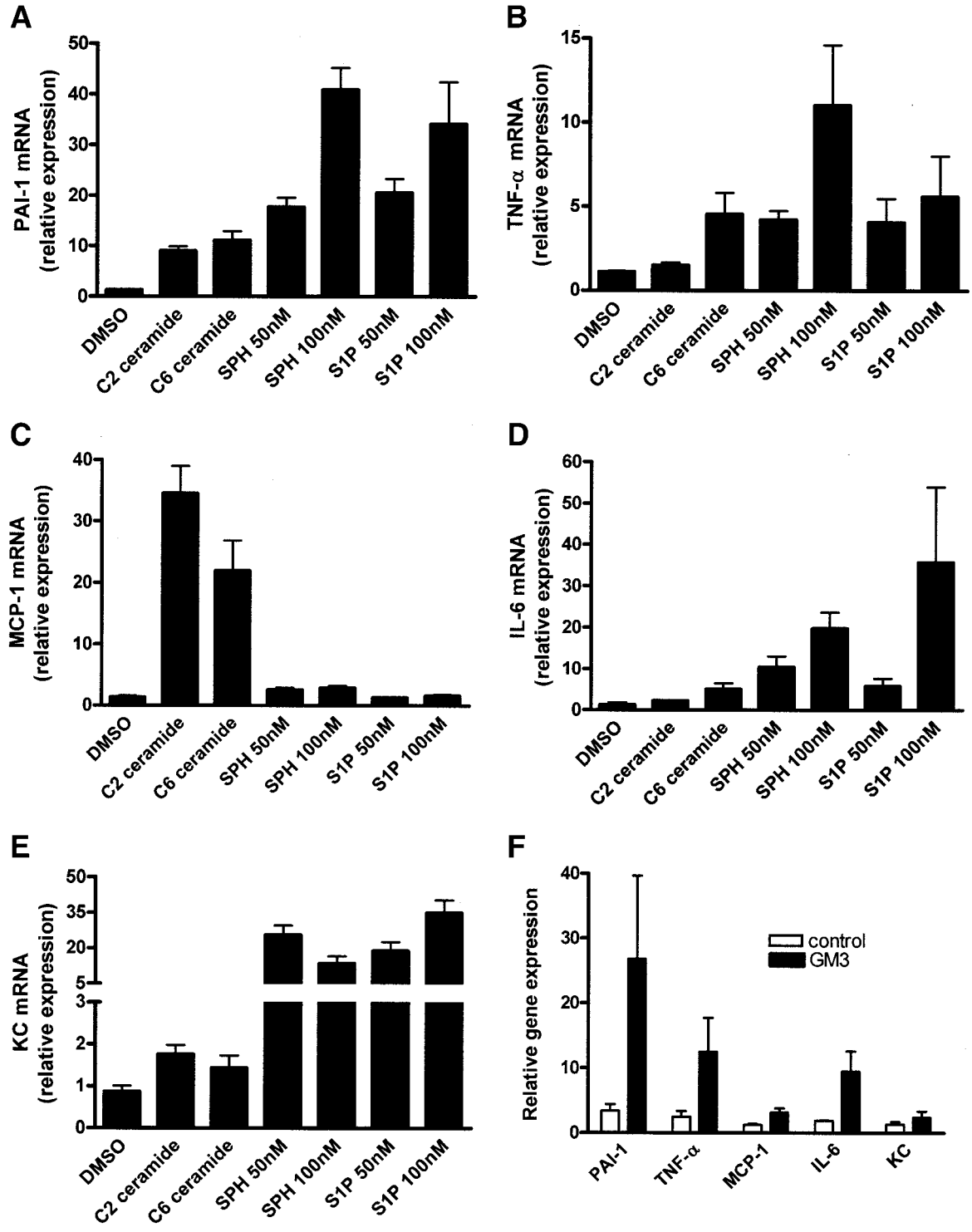

FIG. 5. Regulation of prothrombotic and proinflammatory gene expression in adipocytes by sphingolipids. 3T3-L1 adipocytes were grown and treated for 3 h with 25 μmol/l short-chain ceramides (C2 and C6), sphingosine, S1P, or GM3 (50 μmol/l). The mRNA levels of PAI-1, TNF-α, MCP-1, IL-6, and KC were determined. Data are means ± SD (n = 4). SPH, sphingosine.

tribute to the instability and rupture of atherosclerotic plaques because of its proapoptotic potential of macrophages and smooth muscle cells (40). These observations underscore the emerging significance of sphingolipids in the pathogenesis of atherosclerosis, and our data demonstrating increased levels of these sphingolipids in the plasma of obese mice provide an additional link between obesity and increased risk for cardiovascular disease.

Although TNF-α has been reported to induce the expression of enzymes involved in ceramide generation, little is known about the role of insulin in this response. In our study, insulin induced substantial expressions of ASMase, NSMase, and SPT mRNA in adipose tissues of lean and insulin-resistant *ob/ob* mice. The magnitude of induction of these genes was significantly higher in insulin-treated *ob/ob* mice compared with that in insulin-treated lean

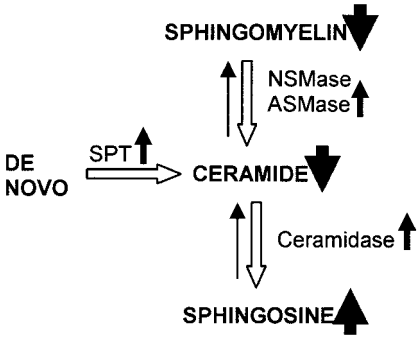

FIG. 6. Sphingolipid metabolism in adipose tissues of genetically obese mice. Increased expression of enzymes involved in ceramide generation and hydrolysis in obese adipose tissue leads to a decrease in the level of sphingomyelin and ceramide and an increase in sphingosine.

mice. These results suggest that the hyperinsulinemia that frequently accompanies obesity and insulin resistance may promote the abnormal expression of genes involved in the activation of the ceramide pathway in adipose tissues. We previously demonstrated that insulin-resistant adipocytes and insulin-resistant obese mice remained sensitive to insulin in terms of the expression of the prothrombotic gene PAI-1 (21). A similar increase in selective genes (e.g., IL-6) was recently described in insulin-resistant human subjects in response to exogenous insulin (41). Collectively, these observations raise the possibility that in the situation of metabolic insulin resistance accompanied by hyperinsulinemia, the expression of certain insulin-responding genes may dramatically increase in insulin target tissues. Expression profiling studies in adipocytes have identified a number of genes that continue to respond normally or are hyperresponsive to insulin even though the adipocytes themselves were metabolically insulin resistant; i.e., they displayed a significantly decreased rate of insulin-mediated glucose uptake (42). Identification of genes that continue to respond to insulin in an insulin-resistant state such as obesity, in concert with studies of the properties of these genes and the signaling pathways that regulate them, may provide novel insights into the molecular mechanisms that control abnormal gene expression in obesity and provide novel opportunities for rational drug development.

Because obesity is associated with increased expression of prothrombotic and proinflammatory proteins in the adipose tissue, we questioned whether sphingolipids (ceramide, sphingosine, and S1P) can regulate the expression of prothrombotic and inflammatory genes in adipocytes. We demonstrate that treatment of 3T3-L1 adipocytes with short-chain ceramide, sphingosine, or S1P induced the expression of the prothrombotic molecule PAI-1 and the expression of the proinflammatory molecules, TNF-α, IL-6, MCP-1, and KC (Fig. 5) to various extents. Genes such as PAI-1 and KC were induced by ceramide, sphingosine, and S1P. Alternately, MCP-1 was induced almost 20-fold by ceramide and only 2-fold by sphingosine with no induction observed with S1P. TNF-α and IL-6 were not induced by C2 ceramide but were induced by sphingosine and S1P. Additional studies demonstrate that leptin expression was induced in response to ceramide but not to sphingosine or S1P, and resistin was not induced by any of the sphingolipids tested (data not shown). These observations showing varying magnitude of induction and/or lack of induction in gene expression in response to the sphingolipids tested suggest that these effects are specific effects on specific genes. Importantly, our studies suggest a role for sphingolipid mediators in the regulation of prothrombotic and proinflammatory proteins in the adipose tissues in obesity. It should be noted that ceramide can be readily hydrolyzed to sphingosine, which in turn can be phosphorylated to S1P, and all of these reactions are reversible. Thus, these results demonstrating an increase in gene expression with these sphingolipids do not in themselves suggest that it is a ceramide-, sphingosine-, or S1P-specific event. Approaches using specific inhibitors or small interfering RNA of enzymes involved in these conversions are needed to specifically identify the roles of individual sphingolipids in the regulation of these prothrombotic and inflammatory genes observed in this study.

In conclusion, our study provides novel information on the expression, regulation, and functional significance of sphingolipids in adipose tissues and plasma of genetically obese mice. Moreover, our observations indicate a hitherto unrecognized and novel role for sphingolipid metabolism in the adipose tissues in obesity and suggest that altered sphingolipid metabolism may contribute not only to the pathogenesis of the adipose tissues in obesity but also to the prothrombotic and inflammatory milieu of the obese adipose tissue, which is now thought to contribute to many of obesity-associated pathological conditions, including cardiovascular and metabolic diseases.

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