THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 45, Issue of November 9, pp. 42077–42083, 2001
Printed in U.S.A.

Hyperglycemia-induced Production of Acute Phase Reactants in Adipose Tissue*

Received for publication, July 26, 2001, and in revised form, August 31, 2001
Published, JBC Papers in Press, September 6, 2001, DOI 10.1074/jbc.M107101200

Ying Lin‡§, Michael W. Rajala‡§, Joel P. Berger¶, David E. Moller¶, Nir Barzilai‖, and Philipp E. Scherer‡**‡‡**

*From the ‡Department of Cell Biology, ‖Department of Medicine, and **Diabetes Research and Training Center, Albert Einstein College of Medicine, Bronx, New York 10461 and ¶Department of Molecular Endocrinology, Merck Research Laboratories, Rahway, New Jersey 07065*

Chronic elevation of systemic levels of acute phase reactants and inflammatory cytokines found in patients with diabetes and the often-associated metabolic syndrome X (hypertriglyceridemia, low serum high density lipoprotein cholesterol, hypertension, and accelerated atherosclerosis) may be responsible for the increased incidence of cardiovascular problems in this population. Here we examine the contribution of adipose tissue to the systemic elevation of acute phase reactants associated with chronic hyperglycemia. We demonstrate that adipose tissue expresses a number of acute phase reactants at high levels, including serum amyloid A3 (SAA3), α1-acid glycoprotein, the lipocalin 24p3 as well as plasminogen activator inhibitor-1 (PAI-1). Additionally, we show SAA3 is expressed at low levels under normal conditions but in the diabetic state is dramatically up-regulated in adipose tissue while down-regulated in liver. Furthermore, pro-inflammatory stimuli and high glucose can lead to the induction of SAA3 in adipose tissue *in vivo* as well as in the 3T3-L1 adipocyte cell line. Adipose tissue may therefore play a major role in the pathogenic sequelae of Type II diabetes, in particular the cardiovascular problems associated with prolonged hyperglycemia.

The central regulatory role of the adipocyte in whole body energy homeostasis is well established. However, pre-adipocytes and adipocytes may also play an important physiological role in the regulation of both the innate and adaptive immune response. We have recently described the response of the adipocyte to various inflammatory stimuli including TNFα and IL-6, focusing primarily on the response to bacterial lipopolysaccharides (LPS), which are mediated through the newly identified Toll-like receptor family (TLRs) (1). The expression of molecules involved in the innate immune response in adipose tissue, such as complement factors D (adipsin), B, and C3 (2–4) as well as acute phase reactant proteins (5), has been demonstrated by our group as well as others. In addition, adipocytes actively secrete and respond to inflammatory cytokines such as TNFα, IL-1, and IL-6 (6–8). The latter two are primary cytokine mediators of the acute phase response. The transcription factors responsible for the downstream events of IL-1 and IL-6 include C/EBPβ as well as C/EBPδ, which are directly involved in the acute phase response of the liver (9, 10), typically considered the main contributor of circulating acute phase reactants. Because of the fact that adipocytes express significant levels of IL-1 and IL-6 receptors as well as high levels of C/EBPα, C/EBPβ, and C/EBPδ, it follows that adipocytes would be capable of producing high levels of acute phase reactant proteins in response to the proper stimuli (5). However, the relative contribution of adipose tissue to systemic acute phase reactant levels, particularly in the diabetic state, has not been studied to date.

Here we investigate the link between the inflammatory/acute phase reactant response and hyperglycemia/hyperinsulinemia at the level of the adipocyte. We show that hyperglycemia, but not hyperinsulinemia, leads to the induction and secretion of SAA3,[1] an acute phase reactant in the adipocyte. Adipose tissue, and not the liver, may therefore be responsible for the increased SAA levels in the diabetic state reported in a number of clinical studies.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium was purchased from Cellgro Inc. Murine TNFα and IL-6 was purchased from Pharmingen. LPS (*Escherichia coli*) was purchased from Sigma. All other chemicals were purchased from Fisher.

Cell Culture—3T3-L1 murine fibroblasts (a generous gift of Dr. Charles Rubin, Albert Einstein College of Medicine) were propagated and differentiated as previously described (11). In brief, the cells were propagated in medium (Dulbecco's modified Eagle's medium, 10% fetal calf serum (JRH Biosciences) and 100 units/ml each penicillin and streptomycin) and allowed to reach confluence (Day −2). After 2 days (Day 0), the medium was changed to fresh medium with the addition of 160 nM insulin, 250 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine. Two days later (Day 2), the medium was replaced with fresh medium containing only 160 nM insulin. After another 2 days, the cells were then propagated in medium only. NIH-3T3 cells were grown and propagated in Dulbecco's modified Eagle's medium containing 10% donor calf serum and antibiotics.

Animals—Male FVB or C56Bl/6 mice were bred in-house and used as indicated. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were housed in individual cages and subjected to a standard light (6:00 a.m. to 6:00 p.m.)-dark (6:00 p.m. to 6:00 a.m.) cycle. All rats were 3 months of age (~300 g, $n = 12$) and were fed *ad*

* This work was supported in part by Juvenile Diabetes Foundation Fellowship 3-2000-176 (to Y. L.), Juvenile Diabetes Foundation Research Grant 1-2001-780 (to P. E. S.), National Institutes of Health (NIH) Medical Scientist Training Grant T32-GM07288 (to M. R.), NIH Grant R01 AG18381 (to N. B.), and NIH Grant 1R01-DK55758 (to P. E. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Contributed equally to the work.

‡‡ To whom correspondence should be addressed: Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Tel.: 718-430-2928; Fax: 718-430-8574; E-mail: scherer@aecom.yu.edu.

[1] The abbreviations used are: SAA3, serum amyloid A3; LPS, lipopolysaccharide; TNFα, tumor necrosis factor α; IL-6, interleukin-6; PAI-1, plasminogen activator inhibitor-1; C/EBP, CCAAT/enhancer-binding protein; RT, reverse transcription; PCR, polymerase chain reaction; AGP, α1-acid glycoprotein; AGU, adipose glucose uptake; HDL, high density lipoprotein.

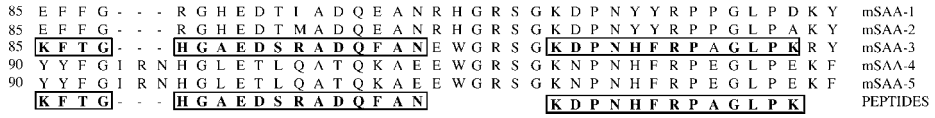

FIG. 1. **Peptide sequencing of a 10-kDa protein highly induced in 3T3-L1 adipocytes by TNFα.** Peptide sequences obtained are aligned with all known members of the SAA family. The fragments obtained unambiguously identify the protein as SAA3.

libitum using regular rat chow that consisted of 64% carbohydrate, 30% protein, and 6% fat with a physiological fuel value of 3.3 kcal/g chow. One week before the *in vivo* studies, rats were anesthetized with methoxyflurane, which allows fast recovery and normal food consumption after 1 day. A venous indwelling catheter was placed into the right jugular vein and extended to the level of the right atrium, and an indwelling arterial catheter was inserted into the left carotid artery and advanced to the level of the aortic arch. The rats were allowed to recover until body weight was within 3% of the pre-operative weight (~4–6 days). These stable catheterized rats were studied after ~24 h of fasting, while awake and unstressed.

Hyperglycemic Clamp Studies—Both somatostatin (1.5 μg/kg/min) and a 25% glucose solution (in phosphate-buffered saline) were infused into the arterial catheter of lean and obese rats to prevent endogenous insulin secretion and raise the plasma glucose concentration acutely to ~18 mM. Plasma glucose concentration was maintained at that level throughout the study (3 h) using a variable infusion of glucose periodically adjusted to maintain plasma glucose levels. All rats also received a primed-continuous (15–40 μCi/min) infusion of high performance liquid chromatograph-purified [3-^{3}H]glucose (PerkinElmer Life Sciences) throughout the study to determine glucose uptake. Similarly, a bolus of [U-^{14}C] 2-deoxyglucose (20 μCi) was administered 30 min before the end of the studies. Plasma samples for determination of plasma [U-^{14}C] 2-deoxyglucose-specific activity were obtained at 5-min intervals during the remainder of the clamp studies.

At the end of the study, rats were sacrificed by intravenous injection of 60 mg of pentobarbital-sodium/kg. The abdomen was quickly opened, and subcutaneous adipose tissue samples were freeze-clamped *in situ* with aluminum tongs pre-cooled in liquid nitrogen.

Hyperinsulinemic Euglycemic Clamp Studies—Lean rats received a primed continuous insulin infusion (3 milliunits/kg/min) and a variable infusion of 25% glucose periodically adjusted to maintain the plasma glucose concentration at the basal level of 7 mM for the duration of the study (3 h). This method enabled us to match the rate of glucose uptake observed in the above hyperglycemic clamp studies while maintaining euglycemia. Lean control rats received an infusion of an equivalent volume of saline for 3 h.

Streptozotocin Treatment of Mice—Four-week-old male C57Bl/6 mice were injected intraperitoneally with streptozotocin (100 mg/kg). Daily blood glucose measurements were monitored with a Precision Q-I-D glucose meter (Medisence, Abbott Laboratories, Inc.) from a drop of blood drawn by tail bleeding. Tissues were harvested 7 days after streptozotocin injection when blood glucose levels were between 400 and 500 mg/dl. All study protocols were reviewed and approved by the Animal Care and Use Committee of the Albert Einstein College of Medicine.

Flotation Assay—Fat pads were excised, minced, and collagenase-treated. Adipocytes were isolated as described in Hotamisligil *et al.* (6) and Rodbell (12).

Total RNA Isolation, Northern Blot Analysis, and RT-Polymerase Chain Reaction (PCR)—Total RNA from tissue and cultured cells was isolated with Trizol (Life Technologies, Inc.). Twenty micrograms of total RNA was used for Northern blot analysis as previously described (13). Blots were prehybridized in Ultrahyb (Ambion) for 2 h at 42 °C. Denatured ^{32}P-labeled DNA probes were added (2×10^{6} cpm/ml), and blots were hybridized overnight at 42 °C. The filters were washed in 2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), 0.1% SDS (×2, 10 min) and 0.1× SSC, 0.1% SDS (×2, 5 min) at 42 °C before autoradiography. RNA concentrations were quantitated with Ribogreen® according to the manufacturer's protocol (Molecular Probes, Eugene, OR). First-strand cDNA was synthesized from 5 μg of total RNA using Superscript™ II RT (Life Technologies) according to the provided protocol. Two microliters of first-strand cDNA either straight or diluted 1:10 was used for semiquantitative RT-PCR with primers for SAA3 with the following program: 94 °C 2 min and (94 °C 30 s, 60 °C 30 s, 72 °C 30 s) for 25 cycles. Total RNA samples for Taqman® analysis were prepared from cells and tissue using the Ultraspec RNA isolation kit (Biotecx, Houston, TX) and then DNase-treated with DNA-free® (Ambion, Austin, TX). Expression levels of SAA3 mRNAs were quantified using quantitative fluorescent real time PCR. RNA was first reverse-transcribed using random hexamers in a protocol provided by the manufacturer (PE Applied Biosystems, Foster City, CA). Amplification of each target cDNA was then performed with TaqMan® PCR reagent kits in the ABI Prism 7700 sequence detection system according to the protocols provided by the manufacturer (PE Applied Biosystems). The levels of mRNA were normalized to the amount of 18 S ribosomal RNA (primers and probes commercially available from PE Biosystems) detected in each sample.

Antibodies—A GST fusion protein construct comprising the mature portion of the murine SAA3 protein was generated in pGEX4T-1 (Amersham Pharmacia Biotech). The protein was expressed in *E. coli* BL21 pLys and purified on a glutathione-Sepharose column, and the mature SAA3 protein was cleaved from the GST moiety by Factor X. Polyclonal antibodies were raised in rabbits (Covance Inc., Denver, PA).

Immunoblotting—Separation of proteins by SDS-polyacrylamide gel electrophoresis, fluorography, and immunoblotting were performed as described previously (14). Primary and secondary antibodies were diluted in Tris-buffered saline with 0.1% Tween 20 and 1% bovine serum albumin. Horseradish peroxidase-conjugated secondary antibodies were detected with enhanced chemiluminescence according to the manufacturer's instructions (PerkinElmer Life Sciences).

RESULTS

Induction of SAA3 by Pro-inflammatory Stimuli in 3T3-L1 Adipocytes—Adipocytes are highly responsive to inflammatory stimuli (1). We wanted to determine whether adipocytes are capable of secreting acute phase reactants in response to different pro-inflammatory stimuli. Treatment of fully differentiated 3T3-L1 adipocytes with TNFα markedly induced levels of a ~10-kDa protein in the tissue culture media as visualized by a Coomassie-stained SDS-polyacrylamide gel electrophoresis gel. Microsequencing identified this protein as murine SAA3, a member of a family of closely related acute phase reactant proteins (Fig. 1). To confirm this observation, 3T3-L1 adipocytes were subjected to treatment with TNFα as well as high levels of insulin, the peroxisome proliferator-activated receptor-γ agonist rosiglitazone, IL-6, and LPS, all of which have strong transcriptional effects on 3T3-L1 adipocytes (1, 15). After a 12-h treatment with each stimulus, total RNA was isolated and utilized for Northern blot analysis with probes specific for SAA3 (Fig. 2*A*). Consistent with the previous observation, the mRNA for SAA3 is dramatically up-regulated in 3T3-L1 cells in response to LPS as well as TNFα. All other treatments, including exposure to IL-6, had no affect on SAA3 levels.

Induction of Additional Acute Phase Reactants by Pro-inflammatory Stimuli—To gain further insight into whether the same kind of pro-inflammatory stimuli exert similar effects on additional acute phase reactants, the same Northern was analyzed with a few additional probes.

24p3, also known as Neu-related calin (NRL) or neutrophil gelatinase-associated lipocalin (NGAL), is a member of the lipocalin protein family (16) and a systemic acute phase reactant protein induced by endotoxemia (17). Interestingly, similar to SAA3, LPS strongly stimulates 24p3 transcription. However, TNFα fails to induce 24p3 in adipocytes. This indicates that the mechanisms by which various acute phase reactants are induced in the adipocyte are divergent and specific for the protein being examined. Furthermore, the adipocyte displays some cell-specific induction patterns. For example, either LPS or TNFα induced SAA3 in adipocytes, whereas only LPS and

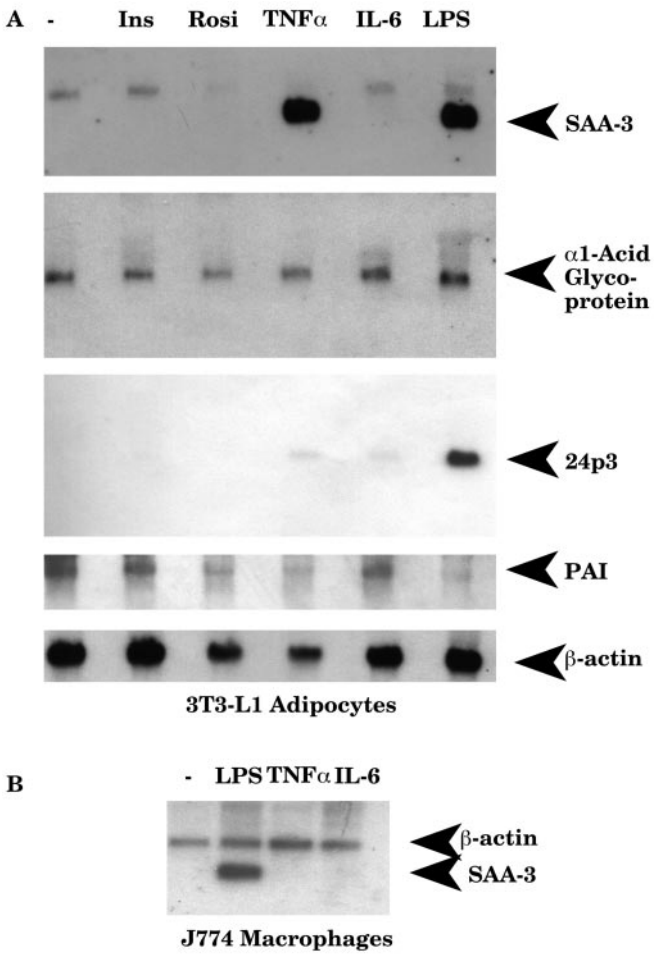

FIG. 2. **Northern blot analysis of acute phase reactant expression in 3T3-L1 adipocytes.** Fragments comprising the open reading frame of mouse SAA3, α1-acid glycoprotein, 24p3, PAI, and β-actin (as an equal loading control) were used to probe a Northern blot as described under "Experimental Procedures." RNA was isolated from 3T3-L1 adipocytes treated for 12 h in serum-free medium without or with either160 nM insulin (*INS*), 1 μM rosiglitazone (*Rosi*), 5 nM TNFα, 5 nM IL-6, or 100 ng/ml LPS.

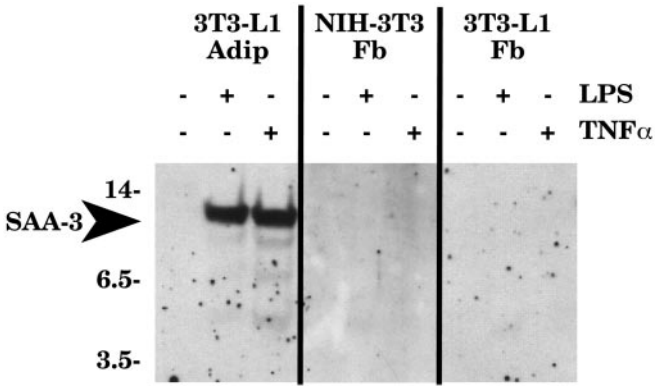

FIG. 3. **Western blot analysis of 3T3-L1 adipocytes (*Adip*), 3T3-L1 fibroblasts (*Fb*), and NIH-3T3 cells treated with LPS or TNFα.** Cells were treated with the indicated conditions (untreated, 5 nM TNFα or 100 ng/ml LPS) for 2 h in serum-free medium containing 25 mM glucose. Cells were subsequently lysed in hot SDS sample buffer. 50 μg of protein was applied to each lane. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and Western blot analysis with antibodies to SAA3. *Numbers* on the *left* indicate molecular mass (in kDa).

but not TNFα induced SAA3 in J774 macrophages (Fig. 2*B*). Expression of another acute phase reactant, α1-acid glycoprotein (AGP), was also examined. Our results show that AGP is constitutively expressed in the adipocyte. A number of treatments, including treatment with rosiglitazone, lead to a down-regulation of AGP. No further increase in AGP transcription after exposure to any of the other pro-inflammatory stimuli can be observed. This is in contrast with data from the liver that show a large increase in AGP after LPS exposure (18). Similarly, PAI-1, which is constitutively expressed in adipocytes and elevated in the diabetic state, shows no stimulation under these conditions.

We wanted to confirm our initial observations of transcriptional induction of SAA3 by LPS and TNFα at the protein level. An antibody raised against mature mouse SAA3 was used for Western blot analysis. Lysates from 3T3-L1 adipocytes, 3T3-L1 fibroblasts, and NIH-3T3 cells treated with either LPS or TNFα showed that 3T3-L1 adipocytes display a similar up-regulation of SAA3 at the protein level (Fig. 3). In contrast, neither 3T3-L1 fibroblasts nor NIH-3T3 fibroblasts were able to induce SAA3 expression under these conditions despite the fact that both are highly responsive to LPS and TNFα (1). The TNFα-induced up-regulation may be particularly relevant, since it is well documented that diabetes is associated with a local elevation of TNFα in adipose tissue (for review, see Ref. 19).

Finally, to determine whether SAA3 has identical regulation *in vivo*, adipose tissue was isolated from mice 24 h after intraperitoneal administration of LPS (Fig. 4). Northern blot analysis reveals *in vivo* data that is consistent with the *in vitro* results for SAA3 and 24p3, demonstrating the expected induction upon exposure to endotoxin. Interestingly, AGP is actually slightly reduced (~50%) under these conditions. In addition, Western blot analysis of total serum (Fig. 4, *bottom panel*, compare *lanes 1* and 2 *versus lanes 3* and *4*) confirms a dramatic increase in circulating SAA3 post-injection of LPS *in vivo* as well.

Induction of SAA3 in Adipose Tissue of Diabetic Mouse Models—The previous experiments demonstrate that SAA3 as well as other acute phase reactants can be induced in adipocytes in response to very strong pro-inflammatory stimuli such as endotoxin and TNFα. However, it was not known whether the mild chronic subclinical inflammatory state observed in the diabetic state is sufficient to result in chronically elevated levels of acute phase reactants in adipose tissue. To address this issue, adipose tissue was isolated from a number of mouse models for both Type I and Type II diabetes and their respective wild type controls.

Northern blot analysis on adipose tissue from *ob*/*ob* mice that suffer from severe hyperglycemia and hyperinsulinemia and wild type littermates was analyzed. SAA3 is hardly detectable in the control mice. In *ob*/*ob* mice, SAA3 is drastically increased at the transcriptional level (Fig. 5*A*).

The levels of circulating AGP have been shown by others to increase markedly in the diabetic state. The primary source of this circulating AGP is conventionally considered to be the liver. Adipose tissue, which constitutively expresses high levels of AGP, decreases AGP production in the diabetic state. Transcript levels for 24p3 are also decreased.

To address whether the liver shows similar regulatory patterns under these conditions, transcriptional levels in adipose tissue and liver for these acute phase reactants were directly compared. AGP is slightly up-regulated in the liver of *ob*/*ob* mice and slightly down-regulated in adipose tissue (Fig. 5*B*). Although adipose tissue represents a highly significant source of systemic AGP under normal conditions, its relative contributions in the diabetic state are therefore diminished. Similarly, 24p3 shows a marked induction in the liver of diabetic

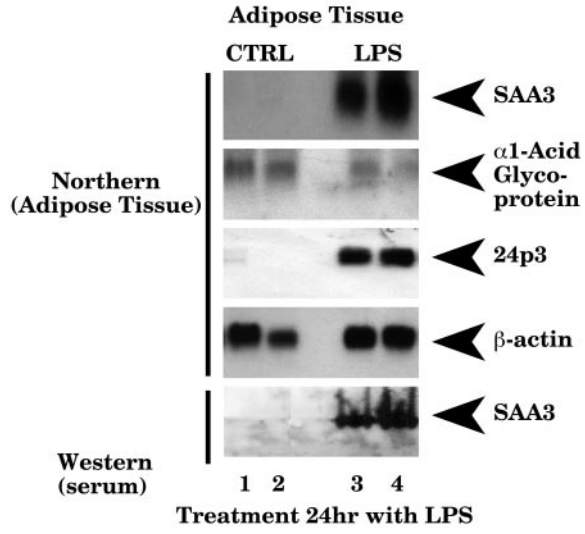

FIG. 4. ***In Vivo* effect of LPS administration on acute phase reactant expression in adipose tissue.** 6-Month-old FVB mice were injected with 100 ng/g body weight LPS ($n = 2$ for each condition). 24 h post-injection, adipose tissue was isolated and analyzed by Northern blot analysis for the expression levels of SAA3, α1-acid glycoprotein, 24p3, and β-actin. *CTRL*, control.

mice. In contrast, SAA3 is expressed at very low levels in both the wild type as well as the diabetic liver, whereas it is markedly induced in adipose tissue. Combined, this suggests that adipocytes may contribute to a significant extent toward the increased levels of SAA3 seen in the diabetic state.

SAA3 Is Induced in Adipocytes and Not in Interstitial Cells within the Fat Pad—To test whether the increased SAA3 mRNA levels observed in adipose tissue are indeed because of an induction in adipocytes and not in surrounding interstitial cells such as preadipocytes and macrophages, we performed a flotation assay that effectively separated adipocytes from all other cell types (Fig. 5*C*). SAA3 mRNA is detected only in the adipocyte fraction and not in the interstitial fraction.

To further confirm the induction of SAA3 in another genetic model of Type II diabetes, we analyzed RNA samples from liver and white adipose tissue of *db*/*db* mice by quantitative RT-PCR analysis (Taqman™). Fig. 5*D* shows that in lean heterozygote (*db*/+) littermates, there are approximately equal amounts of SAA3 expressed in liver and adipose tissue. In contrast, *db*/*db* mice show a 50% reduction in liver SAA3 expression, whereas in adipose tissue, there is a 3-fold increase when compared with white adipose tissue of *db*/+ controls. Overall, this induction of SAA3 expression in white adipose tissue and reduction in liver of *db*/*db* mice may indicate that white adipose tissue could be the major source of systemic SAA3 in the diabetic state.

Furthermore, we wanted to test if these observations would extend to Type I diabetes, a condition characterized by reduced levels of insulin and marked hyperglycemia. To mimic this disorder, mice were treated with streptozotocin, a chemical compound that selectively destroys pancreatic β-cells, thereby greatly reducing the endogenous insulin levels and causing severe hyperglycemia. These mice therefore serve as a model system for Type I diabetes, with loss of glycemic control due to very low insulin levels. As predicted, diabetes induced by streptozotocin treatment resulted in the up-regulation of SAA3 in adipose tissue (Fig. 5, *E* and *F*). Again, this induction was specific for adipose tissue with levels not significantly altered in liver when compared with untreated controls.

Elevated Glucose Levels Lead to Induction of SAA3 in 3T3-L1 Adipocytes—Prompted by the observation that both hyperinsulinemic and insulinopenic model systems display an elevated level of SAA3 in adipose tissue, we addressed the question as to whether hyperglycemia alone could trigger SAA3 expression *in vitro*. Because 3T3-L1 adipocytes are conventionally grown in the presence of 25 mM glucose, a concentration ~6 times greater than that seen in serum, it was necessary to decrease the glucose concentration of the medium to mimic *in vivo* levels (5 mM) for 6 h preceding the experiment. These adipocytes were then treated for an additional 6 h with varying concentrations of glucose intended to mimic a range of glucose levels from normoglycemia (~3 mM glucose) to hyperglycemia (~25 mM glucose). Western blot analysis shows a correlation between increasing levels of glucose and increasing synthesis of SAA3 (Fig. 6), suggesting that 3T3-L1 adipocytes can directly sense and respond to alterations in extracellular glucose. It is important to note though that the SAA3 levels induced under these conditions are much lower than the levels induced by an acute proinflammatory stimulus such as TNFα or endotoxin treatment, similar to the situation *in vivo*. As demonstrated in Fig. 3, Western blot analysis with signal intensities within the linear range for SAA3 after TNFα or endotoxin treatment in 3T3-L1 cells fail to visualize a signal for SAA3 under high glucose conditions.

Hyperglycemia per se, but Not Acute Hyperinsulinemia, Leads to Induction of SAA3 in Adipose Tissue—To determine whether elevated glucose and/or elevated insulin triggers increased SAA3 expression in adipose tissue *in vivo*, hyperinsulinemia or hyperglycemia were induced in rats by "clamping" glucose or insulin independent of compensating increases in the other. Specifically, either glucose levels were clamped within normal basal levels, and insulin was infused to sustain high circulating levels (euglycemic hyperinsulinemic clamp) (see "Experimental Procedures"), or insulin levels were kept near basal levels and glucose was infused to sustain elevated levels (hyperglycemic clamp) (Figs. 7, *A* and *B*). Physiological hyperinsulinemia (58 ± 4 units/ml) in lean animals resulted in an increase in total glucose uptake by approximately 2-fold. However, adipose tissue glucose uptake (AGU) increased by approximately 5-fold compared with the saline control (Table I). Adipose tissue from rats excised rapidly after the euglycemic hyperinsulinemic clamp did not reveal any induction in SAA3 mRNA as seen by Northern analysis (Fig. 7*A*). However, hyperglycemia *per se*, without the concomitant increase in insulin levels, leads to a significant increase in SAA3 within the relatively short time frame of the experiment (3 h) (Fig. 7*B*). This clamp study was performed on both young and old rats. Interestingly, the older control animal had a tendency for increased basal level expression of SAA3, suggesting an age-related increase of adipocyte-borne SAA3 even in non-diabetic animals. These *in vivo* results confirm the observations in tissue culture and suggest that elevated glucose levels independent of insulin are capable of mediating the induction of SAA3 in adipose tissue.

DISCUSSION

There is an increasing body of evidence that correlates the diabetic phenotype with chronically elevated systemic levels of acute phase reactants and inflammatory cytokines. It has been suggested that these elevated levels may contribute or be partially causative to some of the pathologies of the disease, in particular the increased incidence of cardiovascular problems. Pickup *et al.* (20) show a correlation between the increased levels of several acute phase reactants and IL-6 and the metabolic syndrome X. These authors report elevated levels of circulating α1-acid glycoprotein and serum amyloid A. In addition, other groups show that fibrinogen, another acute phase reactant protein associated with coronary heart disease, is

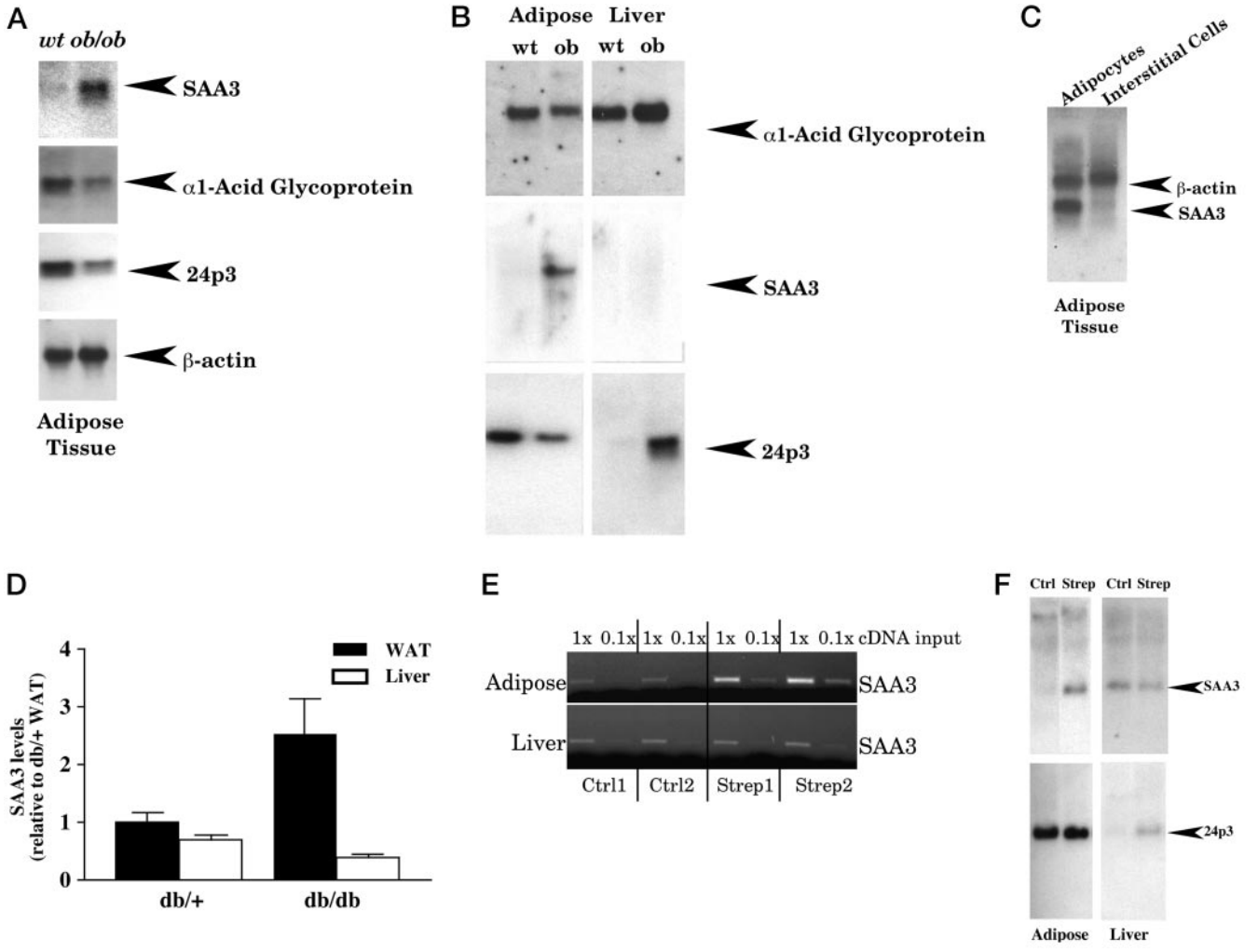

FIG. 5. **Induction of SAA3 in diabetic mouse models.** *A*, Northern blot analysis of adipose tissue isolated from wild type (*wt*) and *ob/ob* mice (male, age-matched with otherwise identical genetic background). The Northern blot was probed with SAA3, α1-acid glycoprotein, 24p3, and β-actin (*top* to *bottom*). *B*, direct comparison by Northern blot analysis of α1-acid glycoprotein, SAA3, and 24p3 levels in adipose and liver tissue from the mice used in *A*. *C*, flotation assay for the separation of adipocytes from stromal cells demonstrating the presence of SAA3 in the mRNA isolated from the adipose and the stromal cell fraction. *D*, liver and white adipose tissue (*WAT*) of *db/db* mice was analyzed by quantitative RT-PCR analysis (Taqman™) for the presence of SAA3; *E*, semi-quantitative PCR analysis of SAA3 expression in adipose tissue and liver from streptozotocin-treated mice. PCR was performed for 25 cycles with first-strand cDNA. Two different levels (1× and 0.1×) were used. 10-fold elevated levels of SAA3 cDNA can be observed in adipose tissue from streptozotocin-treated mice compared with untreated mice. Levels in liver do not change in response to streptozotocin treatment. *F*, analysis of mRNA levels of SAA3 in liver and adipose tissue from streptozotocin-treated *versus* untreated mice by Northern blot analysis. SAA3 mRNA levels are induced in adipose tissue upon streptozotocin treatment, whereas SAA3 levels in liver are slightly decreased upon treatment.

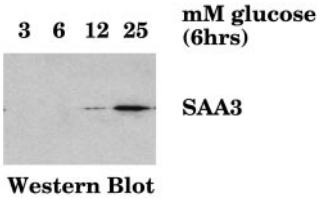

FIG. 6. **Hyperglycemia induces SAA3 protein in 3T3-L1 adipocytes.** 3T3-L1 adipocytes were treated for 6 h with Dulbecco's modified Eagle's medium containing 5 mM glucose and then exposed for an additional 6 h to increasing levels of glucose (3 to 25 mM). Cells were subsequently lysed and analyzed by Western blot analysis for the presence of SAA3.

increased in Type II diabetes (21, 22). Furthermore, elevated levels of complement factor C3 have also been reported in diabetic patients (23) as well as elevated levels of PAI-1 (24). Yokoyama *et al.* (25) find that acute phase reactants are elevated in Type I diabetes, especially in albuminuric patients. Serum leptin concentrations are responsive to an acute phase or stress response, independent of body mass index (26).

It is theoretically possible that the increase in acute phase reactant proteins, some of which have shown to directly affect lipid metabolism, may contribute to the dyslipidemia associated with diabetes. For example, serum amyloid A displaces apolipoprotein A1 from HDL_3, thereby increasing HDL binding to macrophages with the net effect of redirecting HDL cholesterol from the liver to the macrophage for tissue repair (27, 28). A series of prospective cohort studies demonstrate that inflammatory parameters (such as serum amyloid A) and cytokines (such as interleukin-6) are all elevated at base line among patients at risk for future coronary occlusion. Furthermore, data derived from randomized clinical trials suggest that the efficacy of common preventive agents for coronary heart disease, such as aspirin and hydroxymethylglutaryl-CoA reductase inhibitors, may be derived in part from interactions with the inflammatory system (for review, see Ref. 29). A more recent study determined that elevated levels of C-reactive protein, even in the absence of hyperlipidemia, are associated with an increased risk of coronary events (30). Statin therapy reduces the level of C-reactive protein independent of its effect on

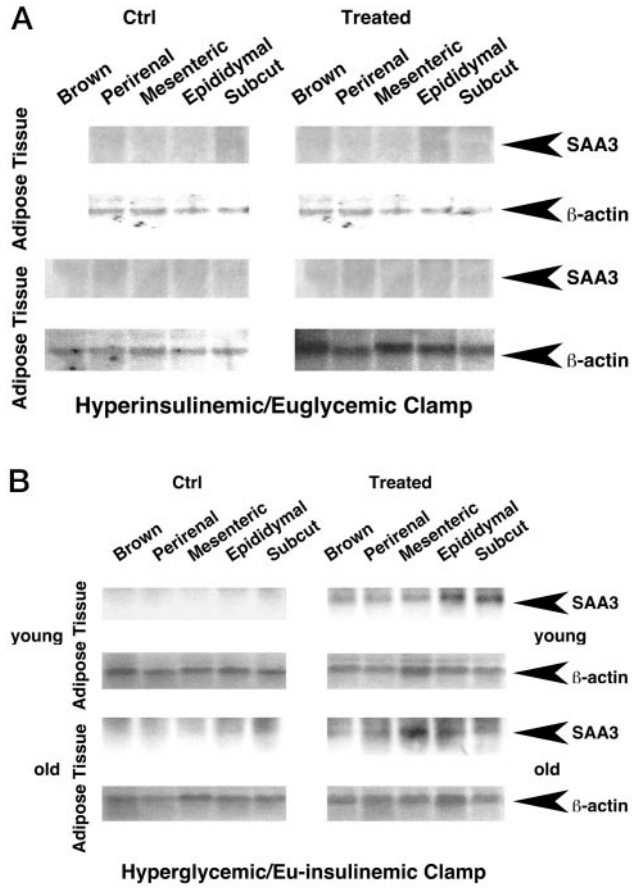

FIG. 7. **Hyperinsulinemic and hyperglycemic clamp studies.** *A*, hyperinsulinemic/euglycemic clamp studies were performed as indicated under "Experimental Procedures." At the end of the study, total RNA from various adipose depots was isolated and analyzed by Northern blot analysis for the presence of SAA3 and β-actin (for normalization). This analysis was performed independently on two separate rats, and data for both rats are shown. *B*, hyperglycemic/eu-insulinemic clamp. This study was similar to the one described in *A* except glucose levels were kept around 300 mg/dl, whereas the insulin levels were kept in the basal state. Two animals are shown (young and old).

TABLE I

Biochemical characteristics and glucose fluxes during hyperglycemia (Glc), hyperinsulinemia (Ins), and saline infusions in lean rats

Plasma glucose and insulin levels, total glucose uptake (TGU; determined by [3-^{3}H]glucose in mg/kg/min), and adipose glucose uptake (AGU; determined by 2-deoxyglucose) in subcutaneous tissue (expressed in mg/kg tissue/min) during the last hour of the clamp are shown. All values are expressed as mean ± S.E.

	Glc	Ins	Saline
Glucose (mM)	17.6 ± 0.3[a]	6.9 ± 0.1	7.0 ± 0.4
Insulin (microunits/ml)	13 ± 4[a]	58 ± 4	11 ± 3
TGU (mg/kg tissue/min)	28 ± 4[b]	25 ± 4[b]	12 ± 3
AGU (mg/kg tissue/min)	23 ± 4[b]	20 ± 2[b]	4 ± 1

[a] $p < 0.001$ *vs.* others.
[b] $p < 0.001$ *vs.* saline.

lipid levels, potentially through an anti-inflammatory mechanism that these compounds can also exert. Statin therapy may therefore be effective in the primary prevention of coronary events among subjects with relatively low lipid levels but with elevated levels of the acute reactant C-reactive protein.

Very little is known about the potential diagnostic and therapeutic significance of the subclinical inflammatory state and its relation to hyperglycemia. However, taken together, our results show that therapies targeting chronic low grade inflammation in the hyperglycemic state for cardiovascular disease prevention should evaluate their effects on the adipocyte as well as the liver.

Although the liver is conventionally viewed as the major site of synthesis of many acute phase reactants, it has become increasingly apparent that adipose tissue may play a significant role in the overall systemic levels of these proteins in response to appropriate stimuli. In addition to our work here focusing on SAA3, adipocytes have been proven to express large amounts of complement factor C3 (3) and PAI-1 (for review, see Ref. 31).

This is the first report that systematically looks at the presence of acute phase reactant proteins in adipose tissue and describes the transcriptional phenomena associated with pro-inflammatory stimuli on these cells. Our data indicate that α1-acid glycoprotein is abundantly expressed in adipocytes, as judged by the high abundance of the mRNA (this paper) and protein.[2] AGP, also known as orosomucoid (ORM), is a 41–43-kDa glycoprotein and is one of the major acute phase proteins in humans and mice. As with most acute phase proteins, its serum concentration increases in response to systemic tissue injury, inflammation, or infection, and these changes in serum protein concentrations have been correlated with increases in hepatic synthesis (for review, see Ref. 32). Even though the levels of α1-acid glycoprotein and serum amyloid A are mostly governed by the same factors in liver, we found strikingly different regulation in adipose tissue. Although α1-acid glycoprotein is expressed at high levels, its synthesis cannot be further induced by pro-inflammatory stimuli, and its expression is repressed in the diabetic state. This demonstrates a different regulation from SAA3 and may indicate a need for constitutive high level expression in adipocytes. Future studies will have to address which, if any, additional proteinaceous and non-proteinaceous factors may be associated with α1-acid glycoprotein released from adipocytes. Interestingly, a very recent report by Cierniewski and co-workers (33) demonstrates that AGP interacts with plasminogen activator inhibitor type 1 and stabilizes its inhibitory activity. The authors propose that the complex of PAI-1 with AGP could play a role as an alternative reservoir of the physiologically active form of the inhibitor, particularly during inflammation or other acute phase reactions. The fact that both AGP and PAI-1 are co-expressed in adipose tissue is therefore not surprising, and it seems likely that AGP would associate with PAI-1 during biogenesis and potentially influence PAI-1 activity.

We found that the lipocalin 24p3 is also abundantly expressed in adipose tissue and repressed in the diabetic state. In contrast to α1-acid glycoprotein, basal levels of 24p3 are very low in 3T3-L1 adipocytes. Similar to SAA3, 24p3 can be induced by LPS. However, unlike SAA3, 24p3 is not induced by TNFα in 3T3-L1 adipocytes. 24p3 has many postulated functions (16, 34–38), among them the potential to serve as a carrier protein, similar to the function proposed for AGP, since the protein has been shown to have binding sites for hydrophobic ligands (35).

Interestingly, Friedman and co-workers (39) report microarray data showing an increase in SAA3 levels in the white adipose tissue of *ob*/*ob* mice when compared with their lean littermates (39). In addition, they also report 24p3 to be up-regulated to approximately the same extent as SAA3. Although our data fully support the reported up-regulation in SAA3, we failed to observe the up-regulation of 24p3 but actually observed a significant decrease in 24p3 in the diabetic model. Although we cannot explain the observed differences with respect to this specific EST comprising 24p3, there are many

[2] Y. Lin and P. E. Scherer, unpublished observations.

interesting conclusions that can be drawn from Friedman's comprehensive microarray study. As the authors point out, many of the most highly up-regulated mRNAs in adipose tissue in the diabetic state fall into the category of inflammatory molecules, including the induction of acute phase reactants and several macrophage marker proteins that are induced within the adipocytes and not within the stromal cell fraction. Additional mRNAs that are highly induced correspond to messages encoding proteins such as heme oxygenase and superoxide dismutase that help cells cope with the increased oxidative stress seen under hyperglycemic conditions. In agreement with the notion that reactive oxygen species are present at elevated levels during hyperglycemia, we found that hyperglycemia *per se* without the concomitant increase of insulin is at the core of the observed increase of SAA3 in white adipose tissue.

Another perhaps unexpected finding is that in adipocytes, not all members of the acute phase reactant family of proteins are regulated in the same manner as they are in hepatocytes. It is clear, however, that the constitutive expression of some of these proteins reflects a functional requirement for their presence under normal conditions to sustain homeostasis as part of an autocrine, paracrine, or endocrine loop. Future studies will have to address whether any bioactive molecules are associated with these proteins as part of their well described carrier functions. Furthermore, the signal transduction pathways that lead to the highly specific transcriptional induction patterns observed for the various acute phase reactants described in this paper will have to be worked out.

Acknowledgments—We thank members of the Scherer laboratory, especially Dr. Terry Combs for valuable assistance with the *in vivo* experiments. We also thank Dr. Michael Brownlee for helpful discussions, Dr. David Cohen and Sonya VanPatten for assistance with the diabetic mouse models, and Xiao Man Yang and Marie Thompson for technical assistance.

REFERENCES

1. Lin, Y., Lee, H., Berg, A. H., Lisanti, M. P., Shapiro, L., and Scherer, P. E. (2000) *J. Biol. Chem.* **275,** 24255–24263
2. White, R. T., Damm, D., Hancock, N., Rosen, B. S., Lowell, B. B., Usher, P., Flier, J. S., and Spiegelman, B. M. (1992) *J. Biol. Chem.* **267,** 9210–9213
3. Choy, L. N., Rosen, B. S., and Spiegelman, B. M. (1992) *J. Biol. Chem.* **267,** 12736–12741
4. Cook, K. S., Min, H. Y., Johnson, D., Chaplinsky, R. J., Flier, J. S., Hunt, C. R., and Spiegelman, B. M. (1987) *Science* **237,** 402–405
5. Scherer, P. E., Bickel, P. E., Kotler, M., and Lodish, H. F. (1998) *Nat. Biotechnol.* **16,** 581–586
6. Hotamisligil, G. S., Shargill, N. S., and Spiegelman, B. M. (1993) *Science* **259,** 87–91
7. Mattacks, C. A., and Pond, C. M. (1999) *Cytokine* **11,** 334–346
8. Mohamed-Ali, V., Pinkney, J. H., and Coppack, S. W. (1998) *Int. J. Obes. Relat. Metab. Disord.* **22,** 1145–1158
9. Burgess-Beusse, B. L., and Darlington, G. J. (1998) *Mol. Cell. Biol.* **18,** 7269–7277
10. Juan, T. S., Wilson, D. R., Wilde, M. D., and Darlington, G. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2584–2588
11. Engelman, J. A., Lisanti, M. P., and Scherer, P. E. (1998) *J. Biol. Chem.* **273,** 32111–32120
12. Rodbell, M. (1964) *J. Biol. Chem.* **239,** 375–380
13. Baldini, G., Hohl, T., Lin, H. Y., and Lodish, H. F. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 5049–5052
14. Scherer, P. E., Lisanti, M. P., Baldini, G., Sargiacomo, M., Corley-Mastick, C., and Lodish, H. F. (1994) *J. Cell Biol.* **127,** 1233–1243
15. Berger, J., Tanen, M., Elbrecht, A., Hermanowski-Vosatka, A., Moller, D. E., Wright, S. D., and Thieringer, R. (2001) *J. Biol. Chem.* **276,** 12629–12635
16. Flower, D. R., North, A. C., and Attwood, T. K. (1991) *Biochem. Biophys. Res. Commun.* **180,** 69–74
17. Zerega, B., Cermelli, S., Michelis, B., Cancedda, R., and Cancedda, F. D. (2000) *Eur. J. Cell Biol.* **79,** 165–172
18. Baumann, H., and Gauldie, J. (1994) *Immunol. Today* **15,** 74–80
19. Hotamisligil, G. S. (1999) *J. Intern. Med.* **245,** 621–625
20. Pickup, J. C., Mattock, M. B., Chusney, G. D., and Burt, D. (1997) *Diabetologia* **40,** 1286–1292
21. Kannel, W. B., D'Agostino, R. B., Wilson, P. W., Belanger, A. J., and Gagnon, D. R. (1990) *Am. Heart J.* **120,** 672–676
22. Ganda, O. P., and Arkin, C. F. (1992) *Diabetes Care* **15,** 1245–1250
23. Figueredo, A., Ibarra, J. L., Bagazgoitia, J., Rodriguez, A., Molino, A. M., Fernandez-Cruz, A., and Patino, R. (1993) *Diabetes Care* **16,** 445–449
24. Yudkin, J. S. (1995) *J. Intern. Med.* **238,** 21–30
25. Yokoyama, H., Jensen, J. S., Jensen, T., and Deckert, T. (1995) *J. Intern. Med.* **237,** 519–523
26. Pickup, J. C., Chusney, G. D., and Mattock, M. B. (2000) *Clin. Endocrinol. (Oxf)* **52,** 107–112
27. Cabana, V. G., Siegel, J. N., and Sabesin, S. M. (1989) *J. Lipid Res.* **30,** 39–49
28. Steel, D. M., and Whitehead, A. S. (1994) *Immunol. Today* **15,** 81–88
29. Ridker, P. M. (1999) *Blood Coagul. Fibrinolysis* **10,** Suppl. 1, 9–12
30. Ridker, P. M., Rifai, N., Clearfield, M., Downs, J. R., Weis, S. E., Miles, J. S., and Gotto, A. M., Jr. (2001) *N. Engl. J. Med.* **344,** 1959–1965
31. Loskutoff, D. J., and Samad, F. (1998) *Arterioscler. Thromb. Vasc. Biol.* **18,** 1–6
32. Fournier, T., Medjoubi, N. N., and Porquet, D. (2000) *Biochim. Biophys. Acta* **1482,** 157–171
33. Boncela, J., Papiewska, I., Fijalkowska, I., Walkowiak, B., and Cierniewski, C. (2001) *J. Biol. Chem.* **276,** 35305–35311
34. Chu, S. T., Huang, H. L., Chen, J. M., and Chen, Y. H. (1996) *Biochem. J.* **316,** 545–550
35. Chu, S. T., Lin, H. J., Huang, H. L., and Chen, Y. H. (1998) *J. Pept. Res.* **52,** 390–397
36. Chu, S. T., Lee, Y. C., Nein, K. M., and Chen, Y. H. (2000) *Mol. Reprod Dev.* **57,** 26–36
37. Garay-Rojas, E., Harper, M., Hraba-Renevey, S., and Kress, M. (1996) *Gene* **170,** 173–180
38. Orabona, C., Dumoutier, L., and Renauld, J. C. (2001) *Eur. Cytokine Netw.* **12,** 154–161
39. Soukas, A., Cohen, P., Socci, N. D., and Friedman, J. M. (2000) *Genes Dev.* **14,** 963–980

Hyperglycemia-induced Production of Acute Phase Reactants in Adipose Tissue

Ying Lin, Michael W. Rajala, Joel P. Berger, David E. Moller, Nir Barzilai and Philipp E. Scherer

J. Biol. Chem. 2001, 276:42077-42083.
doi: 10.1074/jbc.M107101200 originally published online September 6, 2001
