Regulation of Hormone-Sensitive Lipase in Streptozotocin-Induced Diabetic Rats

Carole Sztalryd and Fredric B. Kraemer

Insulin deficiency as seen in insulin-dependent diabetes mellitus causes an activation of lipolysis in adipose tissue that results in hydrolysis of stored triglycerides and release of large amounts of fatty acids into the plasma, leading to diabetic ketoacidosis (DKA). Hormone-sensitive lipase (HSL) is thought to be the rate-limiting enzyme of lipolysis in adipose tissue. This study was designed to examine the effects of insulin deficiency on the regulation of HSL in isolated adipocytes. Insulin deficiency was induced by a single dose of streptozotocin. After 8 days, some animals were treated with insulin, and all animals were killed 10 days after induction of insulin deficiency. Compared with levels in control rats, 10 days of insulin deficiency increased HSL activity twofold ($P < .05$), as assayed for neutral cholesterol esterase activity, and insulin treatment returned HSL activity to normal. HSL protein was increased twofold ($P < .05$) in streptozotocin-induced diabetic rats, as estimated by immunoblotting, but remained elevated after insulin treatment. Levels of HSL mRNA assessed by Northern blot analysis also increased twofold ($P < .01$) in adipose cells isolated from streptozotocin-induced diabetic rats, and remained elevated after insulin treatment. In conclusion, our studies suggest that 10 days of insulin deficiency increases HSL expression via pretranslational mechanisms and short-term insulin treatment returns HSL activity to normal via posttranslational mechanisms in adipose tissue.

DIABETIC KETOACIDOSIS (DKA) is one of the most acute complications of diabetes mellitus, and if misdiagnosed or untreated, it carries a high mortality.[1,2] DKA is characterized by hypoinsulinemia and increased concentrations of counterregulatory hormones that cause an activation of lipolysis in adipose tissue, resulting in hydrolysis of stored triglycerides and release of large amounts of fatty acids into the plasma.[3] These free fatty acids (FFA) are delivered to the liver, where they are oxidized to ketoacids.[4,5] Although the release of FFA is a necessary first step leading to DKA, the exact mechanism leading to increased lipolysis remains to be established. The key enzyme in regulating lipolysis is hormone-sensitive lipase (HSL). HSL catalyzes the first and second step in the breakdown of triglycerides, releasing 2 mol fatty acid and 1 mol monoacylglycerol.[6] The final, third step in lipolysis, hydrolysis of monoacylglycerol to fatty acid and glycerol, is catalyzed by a separate, specific monoacylglycerol lipase.[7] Acute activation of HSL has been shown to be controlled by phosphorylation/dephosphorylation events, which are regulated by the respective activities of protein kinase A (via adenylate cyclase–generated cyclic adenosine monophosphate, [cAMP]) and protein phosphatase. Many of the counterregulatory hormones (catecholamines, corticotropin, glucagon, etc.) stimulate protein kinase A–mediated phosphorylation of HSL and activate the enzyme. Insulin is the most potent antilipolytic hormone.[8] The antilipolytic action of insulin is thought to be mediated by a shift in the steady-state distribution of phosphorylated and dephosphorylated forms of HSL toward the (inactive) dephosphorylated form by (1) decreasing the rate of phosphorylation of HSL by decreasing the amount of intracellular cAMP via activation of a cAMP phosphodiesterase,[9-11] and (2) increasing the rate of dephosphorylation of HSL via activation directly or indirectly of protein phosphatases.[12-14] Most studies investigating hormonal regulation of HSL have been performed in vitro and were conducted during short-term incubations that favor posttranslational control. However, mechanisms other than posttranslational events are probably important in regulating HSL, since HSL mRNA levels are known to vary during development[15] and among different fat depots.[16] The present study was designed to examine the effects of insulin deficiency on the regulation of HSL. For this purpose, streptozotocin-injected rats were studied and HSL activity, HSL immunoreactive protein, and HSL mRNA levels were measured in adipose tissue.

MATERIALS AND METHODS

Chemicals

Reagents were obtained from the following sources: collagenase from Worthington Biochemical (Freehold, NJ); Triton X-100, L-α-phosphatidylcholine, cholesterol oleate, leupeptin, and aprotinin from Sigma Chemical (St Louis, MO); bovine serum albumin (fraction V) from Intergen (Purchase, NY); cholesterol-[^{14}C]oleate from E.I. Dupont de Nemours (Boston, MA); ECL Western blotting detection reagents and horseradish peroxidase–linked whole antibody antirabbit IgG from Amersham (Arlington Heights, IL); nitrocellulose paper from Schleicher and Schuell (Keene, NH); and oligolabeling kit from Pharmacia LKB Biotechnology (Piscataway, NJ). All other chemicals were obtained from standard commercial sources.

Animals

Male Sprague-Dawley rats weighing 180 to 200 g were obtained from Bantin and Kingman (Fremont, CA) and maintained according to Stanford University guidelines on a 12-hour (6 AM to 6 PM) light-dark cycle. Rats were fed Purina Laboratory Chow (St Louis, MO) and had access to water ad libitum; groups of rats (10 to 15 per group) received a single dose of streptozotocin 45 mg/kg body weight by tail vein injection as described previously.[17] Eight days later, some streptozotocin-injected rats were treated with NPH insulin 5 U daily. All animals were killed 10 days after streptozoto-

From the Division of Endocrinology, Gerontology, and Metabolism, Department of Medicine, Stanford University School of Medicine, Stanford; and the Department of Veterans Affairs Medical Center, Palo Alto, CA.

Submitted April 6, 1994; accepted March 15, 1995.

Supported in part by research grants from the Research Service of the Department of Veterans Affairs and by Grant No. HL 42865 from the National Institutes of Health.

Address reprint requests to Fredric B. Kraemer, MD, Division of Endocrinology, S-005, Stanford University Medical Center, Stanford, CA 94305.

0026-0495/95/4411-0004$03.00/0

cin injection between 9 and 10 AM by decapitation, and serum was obtained and frozen for later analysis. Retroperitoneal fad pads were quickly removed from each rat and washed with phosphate-buffered saline (pH 7.4) for isolation of adipocytes. All animals gained weight during the 10-day experimental period, but at the time of death control animals weighed 286 ± 5 g while streptozotocin-induced diabetic and insulin-treated diabetic animals weighed only 249 ± 3 and 257 ± 4 g, respectively ($P < .001$ *v* control; diabetic and insulin-treated diabetic were not significantly different).

Preparation of Isolated Adipocytes

Adipose cells were isolated by collagenase digestion as previously described.[16] Retroperitoneal fat pads were pooled from three rats, separately minced with scissors, and placed in plastic flasks in Krebs-Ringer bicarbonate–HEPES buffer (120 mmol/L NaCl, 4 mmol/L, KH_2PO_4, 1 mmol/L $MgSO_4/7H_2O$, 1 mmol/L $CaCl_2$, 10 mmol/L $NaHCO_3$, and 27 mmol/L HEPES, pH 7.4) containing 3% bovine serum albumin. Collagenase digestion was performed at 37°C in a gyratory water bath shaker for 60 minutes. Cells were washed three times in phosphate buffer (pH 7.4), allowed to separate from the infranatant by flotation, and suspended in 1 mL washing buffer.

Measurement of HSL Activity

Measurement of HSL activity was performed on isolated adipocytes using a cholesterol-[^{14}C]oleate emulsion as described previously.[18] Isolated adipose cells were concentrated by centrifuging 0.5 mL suspended cells through 0.5 mL silicone oil. Packed cells were collected and placed into 500 μL 50-mmol/L Tris hydrochloride and 1 mmol/L EDTA, (3-[(3-cholamido-propyl)dimethylammonio]-1-propane-sulfunate) (CHAPS) 0.2% (wt/vol) containing leupeptin 1 U/mL. Samples were vortexed and briefly sonicated. After centrifuging the homogenates at 14,000 × *g* for 15 minutes, infranatants under the fat cake were carefully removed, and aliquots (50 to 100 μL) were assayed in duplicate for neutral cholesterol esterase activity.

Immunoblotting

Isolated adipocytes were vortexed and homogenized briefly with a polytron in 1 mL ice-cold lysis buffer containing 0.15 mol/L NaCl, 3% Triton X-100, 0.1% lauryl sarcosyl, and leupeptin 1 U/mL. All homogenates were centrifuged at 14,000 × *g* for 15 minutes. The infranatant below the fat cake was removed and kept frozen at −80°C. Samples were electrophoresed on 10% polyacrylamide gels under reducing conditions as described previously.[16,18] Immunoblotting was performed using rabbit polyclonal antirat HSL fusion-protein antibodies (1:20,000 dilution) as published previously.[18] Immunoblots were visualized using ECL, and the relative amounts of immunodetectable HSL contained in each lane were determined by scanning with an LKB Ultra scan XL enhancer laser densitometer and Gel scan XL software (Pharmacia LKB Biotechnology) on a NEC computer (Boxborough, MA).

RNA Isolation and Measurement

Total cellular RNA was extracted from freshly isolated adipocytes by $CHCl_3$: phenol extraction as previously described.[15,16] Hybridizations were performed using a rat HSL cDNA (a kind gift of Dr M. Schotz, University of California, Los Angeles) as described previously.[15,16] Autoradiographs were obtained and analyzed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Other Assays

Glucose, triglycerides, cholesterol, and FFA levels were measured as previously described.[17] Protein level was measured with a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL).

Statistical Analysis

Data are expressed as the mean ± SEM. Statistical analyses were performed by ANOVA using StatView software (ABACUS Concepts, Berkeley, CA) on a Macintosh II computer (Apple Computer, Cupertino, CA).

RESULTS

Serum Concentrations

As expected, streptozotocin-induced diabetes caused significant alterations in serum glucose and lipid levels (Table 1). Serum glucose concentrations 10 days after streptozotocin injection were increased 3.5-fold ($P < .0001$) and FFA increased approximately 70% ($P < .02$). Serum triglyceride increased approximately 30%, but this increase failed to reach statistical significance. Two days of insulin therapy was sufficient to return glucose, FFA, and triglyceride concentrations to control values.

HSL Activity

The increase in serum concentration of FFA is consistent with an increased rate of lipolysis occurring in adipose tissue. When HSL activity was measured in extracts of adipose cells isolated from retroperitoneal fat pads from control and diabetic animals (Fig 1), diabetes was associated with a 100% increase in activity ($P < .0001$), determined as neutral cholesterol esterase activity. As observed with glucose and FFA concentrations, 2 days of insulin therapy returned HSL activity to control levels.

Quantity of HSL

Because the increase in HSL activity observed during streptozotocin-induced diabetes could be due to either an increased activation of the enzyme caused by phosphorylation of HSL or a greater amount of HSL protein, immunoreactive HSL protein levels in isolated adipose cells from the experimental groups were determined using rabbit polyclonal antirat HSL protein-fusion antibodies that recognize intact HSL.[18] Streptozotocin-induced diabetes caused

Table 1. Effects of Streptozotocin-Induced Diabetes on Serum Glucose and Lipid Concentrations

Group	Glucose (mmol/L)	FFA (μmol/L)	Triglycerides (mmol/L)
Control (n = 10)	7.2 ± 0.3	563 ± 90	1.59 ± 0.27
Streptozotocin-induced diabetic (n = 20)	27.2 ± 0.9*	959 ± 108†	2.09 ± 0.19
Streptozotocin-induced diabetic treated with insulin (n = 9)	8.8 ± 1.7	432 ± 32	1.69 ± 0.27

NOTE. Results are the mean ± SEM.
*$P < .0001$ *v* control.
†$P < .02$ *v* control.

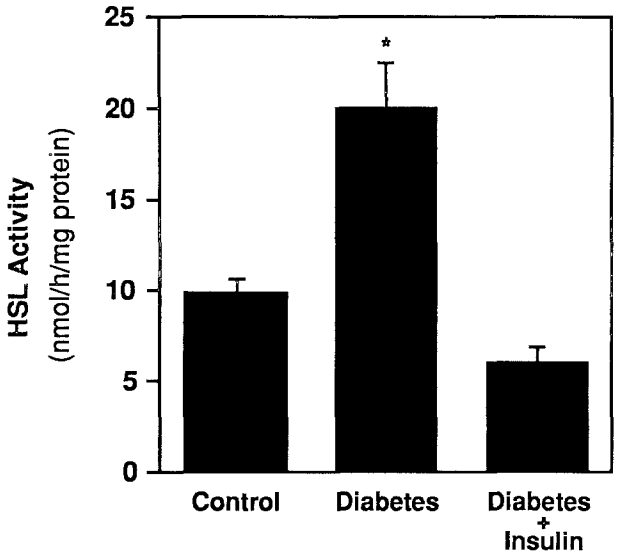

Fig 1. Effects of streptozotocin-induced diabetes on HSL activity in retroperitoneal fat. Results are the mean ± SEM of 8 separate experiments. *$P < .0001$ *v* control.

approximately a twofold increase in the expression of immunoreactive HSL protein in extracts of isolated adipose cells ($P < .05$; Fig 2). However, in contrast to the decrease in HSL activity to normal with 2 days of insulin therapy, HSL immunoreactive protein remained approximately twofold higher than control levels after 2 days of insulin treatment.

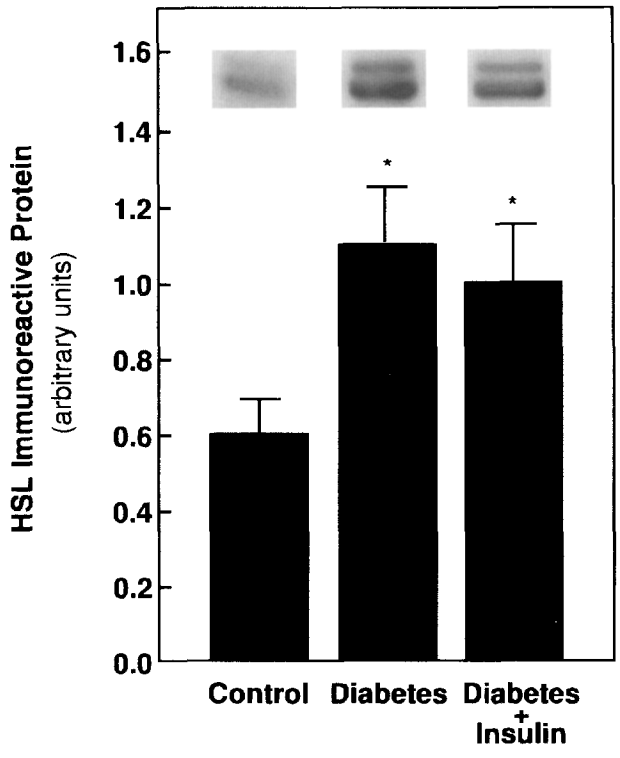

Fig 2. Effects of streptozotocin-induced diabetes on immunoreactive HSL protein in retroperitoneal fat. Results are the mean ± SEM of 8 separate densitometric scans of immunoblots of HSL in isolated adipose cells. The inset above each bar is a representative example of an immunoblot of HSL from control or experimental groups. *$P < .05$ *v* control.

Levels of HSL mRNA

Since streptozotocin-induced diabetes increased the activity and amount of immunoreactive HSL in adipose tissue in parallel, levels of HSL mRNA were assessed by Northern blot analysis in retroperitoneal adipose cells isolated from the experimental groups (Fig 3). HSL mRNA levels were increased approximately twofold in isolated adipocytes from streptozotocin-induced diabetic rats ($P < .005$), whether untreated or treated with insulin for 2 days.

DISCUSSION

Insulin deficiency is associated with a marked increase in FFA flux secondary to the stimulation of lipolysis in adipocytes that is mediated through an increase in lipolytic hormones (catecholamines, corticotropin, glucagon, etc.) and a decrease in antilipolytic (insulin) hormones. To explore the mechanisms for this lipolytic response, previous studies have measured basal and stimulated lipolytic activity assessed as glycerol release in adipocytes isolated from control and streptozotocin-induced diabetic rats.[19-23] Results of these studies showed an increased sensitivity to fast-acting lipolytic hormones in diabetic rats.[19-21] In addi-

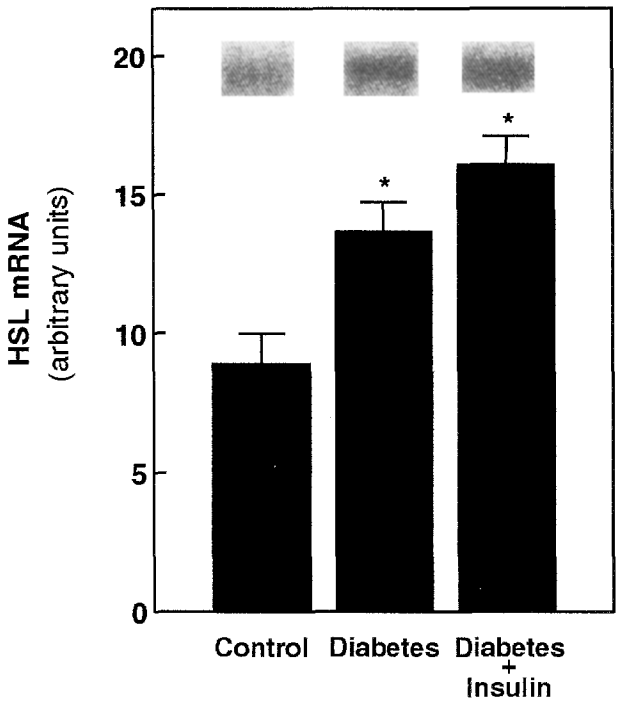

Fig 3. Effects of streptozotocin-induced diabetes on HSL mRNA levels in retroperitoneal fat. Results are the mean ± SEM of 8 separate densitometric scans of HSL mRNA from adipocytes isolated from retroperitoneal fat pads from control, streptozotocin-induced diabetic, or streptozotocin-induced diabetic rats treated with insulin daily for 2 days. Ethidium bromide staining demonstrated equivalent amounts of RNA in each lane (not shown). The inset above each bar is a representative example of HSL mRNA from control or experimental groups. †$P < .005$ *v* control.

tion, an increased sensitivity to the antilipolytic effects of adenosine was observed, with a resultant twofold increase in the rate of basal glycerol release in adipocytes isolated from streptozotocin-induced diabetic rats.[22,23] Since HSL is thought to be the rate-limiting enzyme in lipolysis,[24] these results suggested that there is a twofold increase in HSL activity in streptozotocin-induced insulin deficiency. However, measurement of glycerol release from adipocytes is an indirect, albeit fairly reliable, assessment of HSL activity and does not allow inferences to be drawn regarding cellular mechanisms leading to the alterations in HSL activity. HSL activity is thought to be regulated primarily via posttranslational mechanisms involving phosphorylation-induced activation of the enzyme and dephosphorylation-induced inactivation.[25] However, recent evidence suggests that pretranslational mechanisms are also important in regulating HSL activity. For instance, we have observed that expression of HSL mRNA levels varies with development in several different tissues in the rat.[15] In addition, we have shown that the variance in lipolytic response among different adipose depots in the rat is due in part to differences in levels of HSL mRNA and HSL immunoreactive protein.[16] Moreover, dexamethasone has recently been shown to increase lipolysis and HSL mRNA levels in isolated rat adipocytes.[26]

The current studies were undertaken to address the potential mechanisms responsible for the increased lipolysis observed with insulin deficiency in the rat. Our results show that streptozotocin-induced diabetes causes a twofold increase in HSL activity, a finding similar to the increase in basal glycerol release in adipocytes isolated from streptozotocin-induced diabetic rats noted previously.[22,23] This increase in HSL activity was paralleled by increases in the amount of immunoreactive HSL protein and levels of HSL mRNA, suggesting that control of HSL activity is mediated via pretranslational mechanisms under the present conditions. These results are similar to our recent observation on the regulation of HSL expression during fasting in rats.[27] Prolonged fasting in rats is associated with many of the same metabolic changes seen in insulin-deficient diabetes. Thus, in both fasting and insulin-deficient diabetes, there is an increase in FFA flux due to increased lipolysis secondary to increases in lipolytic hormones and decreases in insulin. Although these hormonal changes result in a decrease in adipose tissue mass, ie, weight loss, in both conditions, this is brought about by food deprivation in fasting, whereas caloric intake is actually increased in insulin-deficient rats.[28] In our fasting studies, 3 to 5 days of food deprivation was associated with an approximately twofold increase in HSL activity, HSL immunoreactive protein, and HSL mRNA levels whether assessed in intact fat pads or isolated adipose cells.[27] Hence, streptozotocin-induced insulin deficiency and prolonged fasting appear to regulate HSL activity similarly through probable increases in the rate of transcription of the HSL gene that result in greater quantities of the enzyme. This conclusion should not be interpreted to mean that posttranslational mechanisms are not important in regulating HSL activity. Indeed, in the current studies, treatment of streptozotocin-induced diabetic rats with insulin for 2 days returned HSL activity to normal; yet HSL immunoreactive protein and HSL mRNA levels remained elevated to the same extent as observed in diabetic animals not treated with insulin. Therefore, 2 days of insulin therapy reduced HSL activity from the increased levels of diabetic animals via posttranslational mechanisms, presumably through insulin's actions on the phosphorylation state of HSL (although phosphorylation of HSL was not directly evaluated). Furthermore, these results, along with our observation that HSL immunoreactive protein and HSL mRNA levels do not increase until after 3 to 5 days of fasting,[27] suggest that more than 2 days are required under these conditions to detect pretranslational alterations in HSL.

Although our results demonstrate that streptozotocin-induced insulin deficiency regulates HSL expression, at least in part, via pretranslational mechanisms, it is not possible to distinguish whether the probable increased transcription of the HSL gene is solely due to the absence of insulin or to the increase in lipolytic hormones. In addition, it is possible that the reductions in adipose cell size associated with streptozotocin-induced insulin deficiency and fasting somehow cause HSL expression to be increased. However, this is unlikely, since body weight and adiposity were not related to HSL expression in adipose tissue in rats during aging, in which no changes in HSL mRNA were noted from 3 weeks to 2 years of age while body weight increased from 60 to greater than 600 g.[15] Moreover, treatment of streptozotocin-induced diabetic rats with insulin for 2 days returned HSL activity to normal without affecting body weights of the experimental groups, although HSL immunoreactive protein and mRNA levels remained elevated. It has recently been reported that exposure of isolated rat adipocytes to insulin for 2 to 4 hours increases the activity of HSL when measured after maximal in vitro phosphorylation.[29] Although this result implies that hyperinsulinemia increases HSL expression and, by extension, that hypoinsulinemia should decrease HSL expression, it is just as likely that the changes observed with insulin exposure in vitro are due to alterations in phosphorylation[30,31] of HSL. Clearly, the rapid effects of insulin and fast-acting lipolytic hormones on lipolysis and HSL activity are mediated posttranslationally through phosphorylation of the enzyme by protein kinase A, causing activation, and dephosphorylation of the enzyme decreasing HSL activity.[12,25] It has been suggested that phosphorylation of HSL not only increases intrinsic activity of the enzyme, but also causes HSL to translocate from an aqueous cytosolic compartment to become associated with the lipid droplet.[32] Moreover, this translocation may occur through interaction of HSL with perilipin, an adipocyte-specific protein located on the surface of the lipid droplet and the major substrate of protein kinase A in adipose cells.[33,34] Further studies will be required to determine whether insulin, fast-acting lipolytic

hormones, or both have direct effects on the regulation of HSL transcription in addition to their effects on posttranslational control.

In conclusion, HSL activity in adipose tissue in the rat is increased in response to streptozotocin-induced insulin deficiency; the change in activity parallels increases in the amount of HSL immunoreactive protein and HSL mRNA levels. Furthermore, short-term treatment with insulin returns HSL activity to normal without altering the increased amounts of HSL immunoreactive protein and HSL mRNA. Thus, HSL activity appears to be regulated under prolonged conditions of insulin deficiency by pretranslational mechanisms, whereas short-term treatment with insulin controls HSL by posttranslational mechanisms.

ACKNOWLEDGMENT

We thank Dr Y.-D.I. Chen for assaying serum samples and S. Patel and V. Natu for excellent technical assistance.

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