Lisofylline, A Novel Anti-inflammatory Agent, Enhances Glucose-Stimulated Insulin Secretion In Vivo and In Vitro: Studies in Prediabetic and Normal Rats

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Previous studies from this laboratory have shown that the novel anti-inflammatory agent, lisofylline (LSF), improves oral glucose tolerance in streptozotocin (STZ) diabetic rats. Subsequent studies suggested that the improved glucose tolerance could be the result of enhanced β-cell functioning. The possibility that LSF enhancement of insulin release in these animals is the result of direct effects of this agent on a residual population of functionally normal β cells was further evaluated in these studies. In vivo studies: 6- to 8-week-old male rats were administered STZ (35 mg/kg body weight) intravenously. After 10 days, LSF administration (25 mg/kg body weight, twice daily) was initiated in the treated group (n = 11) for comparison with the vehicle-injected controls (n = 10). Body weight, food intake, and serum glucose and insulin levels were monitored weekly. Glucose and insulin responses to an oral glucose bolus were measured at 4 to 5 weeks as an index of LSF effects on impaired glucose tolerance. Glucose areas under curve (AUC) during the 2-hour tolerance tests in the LSF-treated rats (n = 11) were 23,390 ± 253 versus 29,390 ± 1,006 mg/dL × min ($P < .0001$) in the vehicle-injected rats (n = 10). Improved glucose tolerance was associated with increases in blood insulin levels in the LSF-treated rats, AUC (+LSF) = 6,564 ± 66 versus 5,127 ± 633 μU/mL × min in the vehicle-injected STZ-rats (not significant [NS]). These observations suggested that the improved glucose tolerance is the result of direct effects of LSF on glucose-induced release of insulin. In vitro studies: the validity of this hypothesis was subsequently tested using isolated perfused pancreas preparations from normal rats. In this series of experiments, 12-week-old animals were used, and pancreases were perfused in situ using single-pass technique. Three levels of LSF were directly infused into individual pancreas preparations and included 20 (n = 5), 40 (n = 4), and 60 (n = 4) μmol/L. First (minutes 3 to 10) and second (minutes 13 to 35) phase glucose-stimulated (300 mg/dL) insulin response areas (AUC) for the 2 phases measured in the LSF-infused pancreases were compared with AUC in vehicle-infused pancreases (n = 4). At LSF concentrations of 20 and 40 μmol/L, total insulin released during the first phase of glucose stimulation was more than twice that of the controls (3,919 ± 739 and 3,643 ± 630 μU, respectively *v* 1,481 ± 269 μU, $P < .03$). A total of 60 μmol/L LSF did not significantly enhance first phase glucose-induced insulin secretion. Second phase comparisons of total insulin released in the LSF-infused versus the controls showed differences of comparable magnitude (about 2-fold) with statistical significance ($P < .03$) observed at all 3 levels of LSF. These findings demonstrate that LSF enhances glucose-stimulated insulin release in vitro. Enhanced β-cell functioning by LSF likely represents an important factor underlying improved glucose tolerance in vivo. In addition, the in vitro observations in normal rat pancreas indicate that the LSF effect is not limited to β-cell dysfunction per se. These results support the conclusion that agents, such as LSF, may have therapeutic benefits in type 2 diabetes.

LISOFYLLINE (LSF) is a novel anti-inflammatory compound that was originally developed to reduce cellular damage due to ischemic reperfusion, hypoxia, or autoimmune disease.[1,2] LSF is several hundred-fold more effective than its parent compound, pentoxyfylline, at inhibiting responses to treatment with inflammatory cytokines.[2] These actions of LSF appear to be the result of modulatory effects of this agent on intracellular lipid signaling, which includes oxidative modification of fatty acids and resultant reduction of free radicals. Specifically, LSF has been shown to block actions of cytokines, such as interleukin (IL)-12, which exerts these effects through a common lipid intracellular signaling pathway. Our group has previously shown that LSF can decrease dysfunction caused by IL-1β in rat pancreatic islets.[3] In relation to these protective effects of LSF, we demonstrated that this agent prevented the ability of IL-1β to decrease insulin secretion in rat islets exposed to IL-1β and LSF over a 24-hour period of incubation. In more recent studies, we have measured LSF effects on both insulin secretion and mitochondrial metabolism in an insulin secreting β-cell line, INS-1 cells, exposed to a combination of proinflammatory cytokines, including IL-1β, interferon-γ, and tumor necrosis factor-α.[4] In these studies, we observed that when LSF was applied to INS-1 cells simultaneously with these cytokines, glucose-stimulated insulin secretion and mitochondrial metabolism were restored to control levels. Because the production and release of insulin are energy requiring processes, the observations in the INS-1 cells suggest that LSF exerts potentiating effects on insulin secretion by stimulation of mitochondrial metabolism in β cells.

These in vitro observations are in accord with in vivo data demonstrating that chronic LSF treatment reduces the onset of diabetes in the non-obese diabetic (NOD) mouse model of type 1 diabetes.[5] In the current studies, we have provided new data on LSF effects in vivo using a streptozotocin (STZ) prediabetic animal model. In addition, we have also provided further validation for the hypothesis that LSF has direct effects on the endocrine pancreas by demonstrating that this agent enhances glucose-stimulated insulin secretion in isolated perfused rat pancreas preparations from normal rats. The potentiating effects of LSF on insulin secretion in perfused pancreas from normal rats shown here suggests that enhanced insulin secretion in the STZ rats may be unrelated to STZ-induced β-cell defects per se, but reflect beneficial effects of LSF on function-

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ing of a residual population of β cells.[6,7] The LSF enhancement of glucose-stimulated insulin secretion observed in vivo in STZ prediabetic rats and in vitro in perfused pancreas from normal rats provides support for the conclusion that agents, such as LSF, could have significant potential for use in treatment of patients with type 2 diabetes.

MATERIALS AND METHODS

In these experiments, 2 batches of 16 rats were studied separately. We performed preliminary studies to determine te dose of STZ required to produce mildly diabetic animals with type 2 characteristics.[8] These characteristics included significant impairment of glucose tolerance and decreased insulin responsiveness to an oral glucose load.[8] The prediabetic animals were then used to study the potential of the agent LSF to improve glycemic control.

STZ Administration Procedure

Male Sprague-Dawley rats purchased from Charles River (Wilmington, MA) were administered STZ at 6 to 8 weeks of age when their body weights were between 175 and 200 g. Freshly prepared STZ solution was injected intravenously at a dose of 35 mg/kg body weight in overnight fasted rats.[8] In these studies, the goal was to produce animals with ambient fed-state blood glucose levels near 200 mg/dL. Because a relatively low dose of STZ was used, the defining criterion for inclusion of animals was successful injection of STZ into the vascular compartment. The success rate was over 90% so that data from only a small number of rats was not included in the final analysis. Fed-state serum glucose levels were determined after 4 days using samples of blood collected by tail bleeding, and all animals were distributed into treated and untreated groups. Samples of blood for glucose and insulin determinations were also collected just prior to STZ administration (zero time) and at weekly intervals thereafter until the fourth week when oral glucose tolerance tests were performed. In addition, body weights and food intakes were monitored during the entire course of study.

Oral Glucose Tolerance Tests

Insulin and glucose responses during an oral glucose tolerance test (OGTT) were measured between 4 and 5 weeks after STZ administration and were used as an index of LSF effectiveness. In this procedure, all rats were fasted overnight and tolerance tests were performed between 8 and 11 AM. Following collection of a baseline blood sample, a prewarmed 75% glucose solution was administered (250 mg/kg body weight) orally, using a 1-mL plastic syringe. The syringe was filled with a predetermined volume of glucose solution. The syringe tip is then touched to the oral mucosa to initiate lapping. The entire solution is then delivered over an approximately 30-second interval. After glucose administration, postglucose blood samples were taken at 15, 30, 60, and 120 minutes. Tail bleeding was used for collection of blood samples and serum harvesting for measurement of glucose and insulin. Samples ($\sim$1 mL) were collected in microfuge tubes and stored on ice. Sera were separated by centrifugation (Eppendorf Microcentrifuge, Madison, WI) and stored frozed until assay.

LSF Treatment Procedure

Treatment with LSF was commenced 10 days after STZ administration. LSF (60 mg/mL in water) was injected intraperitoneally (IP) (25 mg/kg body weight) twice daily (7:30 AM/7:30 PM) in 6 to 8 rats, commencing after collection of baseline blood samples (5 to 7 days after STZ administration). Control STZ rats (6 to 8) and sham-operated normal rats (3 to 4) were injected IP twice daily with sterile saline as vehicle. The treatment period was 4 to 5 weeks.

In Vitro Studies on Effects of LSF on Glucose-Stimulated Insulin Secretion in Perfused Pancreases From Normal Rats

Pancreases of overnight fasted male Sprague-Dawley rats of body weight 250 to 350 g were surgically isolated for in situ perfusion as described previously.[9,10] Krebs-Ringer Bicarbonate medium containing 0.1% bovine serum albumin (BSA)/4% dextran was used as perfusate and was continuously gassed with 95%O_2/5%CO_2 and maintained at 37°C. Following a 15-minute stabilization period of cyclic perfusion, 55-minute single-pass perfusions with sequential sampling were performed. A 300-mg% glucose stimulus was infused from minutes 2 to 50. A stock solution of LSF in saline was infused into the portal input to produce concentrations of 20, 40, or 60 μmol/L. LSF was infused between minutes 3 and 10 of the first phase of insulin release and between minutes 16 and 35 of the second phase.

First phase. Two baseline (preglucose) samples were collected prior to initiation of glucose infusion. Eleven ½- or 1-minute increments were taken thereafter between minutes 3 and 10 for measurement of first phase insulin release. First phase insulin response areas were computed from rates of insulin release between the third and tenth minutes. LSF effects on the first phase were determined by comparing insulin response areas from minutes 3 to 10 in the LSF-infused pancreases with response areas in the controls using the Student's *t* test.

Second phase. Insulin release rates during the second phase were measured in 8 samples of total effluent (collected in either 3- or 5-minute increments) taken between minutes 13 and 35. Second phase insulin areas were computed from the rates of insulin release. LSF effects on the second phase were determined by comparing insulin areas in the LSF-infused pancreases with areas in the controls using the Student's *t* test. Two perfusions were performed each day and included 1 saline-infused control and 1 LSF-infused preparation.

Rats used in these studies were fed commercial chow (Harlan Teklad 7012, Madison, WI) and water was available ad libitum. Animals were housed in the University of Virginia vivarium and maintained under constant conditions of temperature and humidity and a regular light (7:30 AM to 7:30 PM)/dark cycle. All procedures were performed using protocols approved by the university's Animal Care and Utilization Committee.

Assay Methods

Glucose. Serum glucose levels were measured using a Beckman Glucose Analyzer (Model 2, Fullerton, CA).

Insulin. Serum and perfusate insulin concentrations were assayed in duplicate using the method of Herbert et al[11] and rat insulin standards.

Data Analysis

Values shown in figures, tables, and text are reported as mean $\pm$ SEM. The Student's *t* test for unpaired observations was used for all data comparisons for statistical significance. Area under curve (AUC) values are reported as total areas and were calculated using a modification of the trapezoidal rule.

RESULTS

Studies in the STZ Rat Model

Body weight gain in the LSF-treated and vehicle-injected STZ rats was monitored weekly and compared with weight gain in normal rats over the 6-week course of study (Table 1). Weight gain was similar in all 3 groups of rats and was not influenced by LSF administration. Food consumption in the STZ rats was essentially the same as food consumption in the normal rats. In accord with the lack of any effect on body weight gain, no effects of LSF treatment on food intake were

Table 1. Body Weight Gain and Food Intake in Normal Rats and in LSF-Treated and Untreated Streptozotocin Diabetic Rats

Time (days)	Fed Body Weight, g (mean ± SEM)			Food Intake, g/48 h (mean ± SEM)		
	Normal (n = 6)	LSF Treated (n = 12)	VEH Treated (n = 11)	Normal (n = 6)	LSF Treated (n = 12)	VEH Treated (n = 11)
0	119 ± 2	130 ± 4	128 ± 5	—	—	—
7	182 ± 6	182 ± 4	178 ± 5	62 ± 3	61 ± 2	63 ± 2
14	217 ± 5	221 ± 4	219 ± 3	—	—	—
21	277 ± 9	271 ± 5	266 ± 5	63 ± 3	65 ± 4	66 ± 4
28	333 ± 17	319 ± 6	314 ± 6	64 ± 3	64 ± 5	67 ± 4
35	374 ± 14	360 ± 10	350 ± 10	64 ± 4	61 ± 7	66 ± 4
42	411 ± 18	389 ± 12	381 ± 11	63 ± 10	61 ± 7	69 ± 4
52	457 ± 23	431 ± 16	419 ± 15	66 ± 6	60 ± 3	63 ± 2

NOTE. Streptozotocin (35 mg/kg body weight) was administered intravenously on day 14 in the diabetic rats. Lisofylline (25 mg/kg body weight, IP, twice daily) administration was commenced on day 21 in the treated rats. Untreated diabetic and normal rats were injected with vehicle (normal saline) twice daily.

observed in the treated STZ rats (Table 1). The animals used in these studies had a baseline (pre-STZ) glucose level of 141 ± 4mg/dL measured in 8 animals. Seven days after STZ administration, the pooled serum glucose level for all STZ rats was 227 ± 30 mg/dL (n = 23). Prior to commencing LSF treatment, the mean serum glucose level was 226 ± 32 mg/dL in the designated LSF treatment group (n = 12) and 228 ± 33 mg/dL in the vehicle-injected STZ group (n = 11).

Figure 1 compares serum glucose responses to an oral glucose load in the LSF-treated and vehicle-injected STZ rats with responses in the normal animals. As expected, serum glucose levels in the untreated rats were significantly elevated at all time points as compared with the normal rats. In contrast, mean glucose concentrations in the treated STZ rats were not significantly elevated relative to glucose levels in the normal rats and were 20% to 25% lower than levels in the vehicle STZ rats ($P < .05$). Similar findings are also recorded in Fig 2 where the area under the glucose curve shows that LSF normalized the response in the treated animals.

Figure 3 compares the associated serum insulin responses to oral glucose in the LSF-treated and vehicle-injected STZ rats with insulin responses in the normal rats. Insulin responses in the vehicle-injected STZ rats were nearly 40% lower than responses in the normal rats with these differences being significant starting at the first hour after oral glucose administration. In contrast, serum insulin responses in the LSF-treated rats were similar to responses measured in the normal rats at all points shown. Also, the late insulin response levels measured at 2 hours in the treated rats were comparable to insulin levels in the normal animals.

Two-hour insulin response areas computed from the data shown in Fig 3 for the 3 groups of rats are compared in Fig 4. As seen in Fig 4, insulin response areas in the vehicle-injected STZ rats were significantly lower than areas in the normal animals. As suggested by data shown in Fig 3, insulin response area in the LSF-treated group was not statistically different from the insulin area in the normal group.

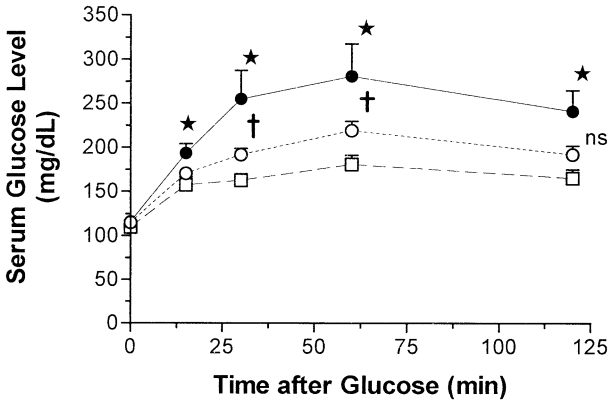

Fig 1. Effect of LSF on serum glucose responses during a 2-hour OGTTs in treated and untreated diabetic rats and in normal rats. Glucose (250 mg/100 g body weight) was administered orally at zero time, and postglucose blood samples were taken at the time points indicated. Symbols designate rat groups as follows. - -○- -, LSF injected (n = 12); —●—, vehicle injected (n = 11); – –□– –, normal (n = 6). *Indicates significant difference ($P \leq .05$) relative to glucose level in the normal rats; †indicates significant difference ($P < .05$) relative to glucose level in vehicle diabetic rats.

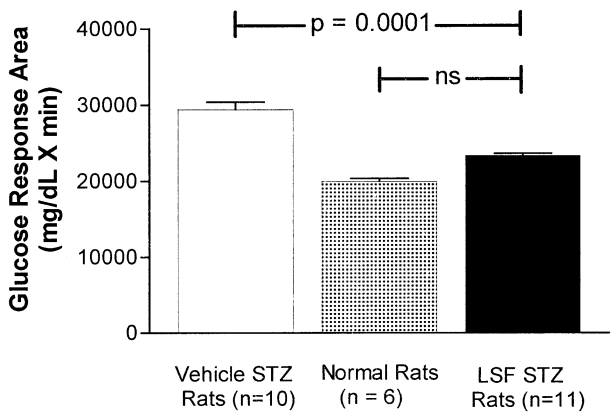

Fig 2. Effect of LSF on glucose response areas during 2-hour OGTTs in treated and untreated diabetic rats and in normal rats. Total areas under glucose response curves were calculated for each group of rats as indicated in Fig 1.

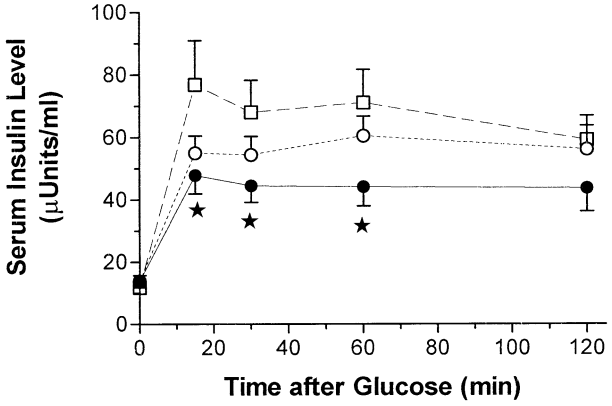

Fig 3. Effect of LSF on serum insulin responses during a 2-hour OGTT in treated and untreated diabetic rats and in normal rats. Glucose (250 mg/100 g body weight) was administered orally at zero time, and postglucose blood samples were taken at the time points indicated. Symbols designate rat groups as indicated previously (see Fig 1 legend). *Indicates significant difference ($P \leq .05$) relative to insulin level in the normal rats.

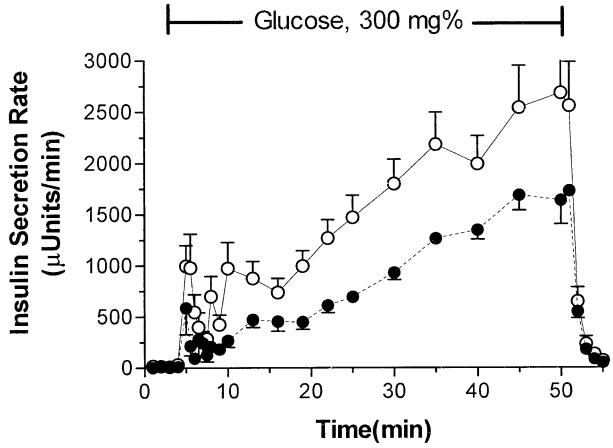

Fig 5. Effect of 20 μmol/L LSF on glucose-induced insulin secretion rates in perfused pancreases from normal rats. A 300-mg % glucose stimulus was infused from minute 2 to minute 50. LSF was infused from minutes 3 to 10 of the first phase and from minutes 16 to 35 of the second phase. —○—, LSF infused (n = 5); - -●- -, vehicle infused (n = 4).

Studies in Isolated Perfused Pancreas From Normal Rats

To evaluate whether LSF could directly modulate glucose-induced insulin secretion, we utilized isolated perfused pancreases infused with LSF during glucose stimulation. The effects of LSF on glucose-stimulated insulin secretion in perfused pancreases from normal rats are recorded in Figs 5 and 6 and are summarized in Table 2. Three levels of LSF (20, 40, and 60 μmol/L) were directly infused during the first and second phases of insulin release at the times indicated (see legend to Fig 5) and compared with vehicle (saline)-infused control pancreases. As seen in Fig 5, exposure of β cells to 20 μmol/L LSF resulted in enhanced rates of insulin secretion for both phases of glucose-stimulated insulin release. As indicated in Table 2, during the first phase, the total insulin released by the LSF-infused pancreases was 3,919 ± 739 μU, which was nearly 3-fold greater than that released by the saline-infused controls, 1,481 ± 269 μU ($P = .03$). As compared with the first phase difference, the difference between total insulin released by the LSF-infused pancreases relative to the vehicle controls during the second phase increase was significant, but smaller, being about 2-fold; 30,240 ± 4,959 versus 16,760 ± 1,174 μU ($P = .03$), respectively. It should be noted that the modulatory effect of LSF on first phase insulin secretion was maintained after infusion of the agent was stopped at minute 10 (see Fig 5). Similarly, enhancement of insulin secretion continued to be observed after LSF infusion was stopped at minute 35 of the second phase (Fig 5). As seen in Fig 6, a significant enhance-

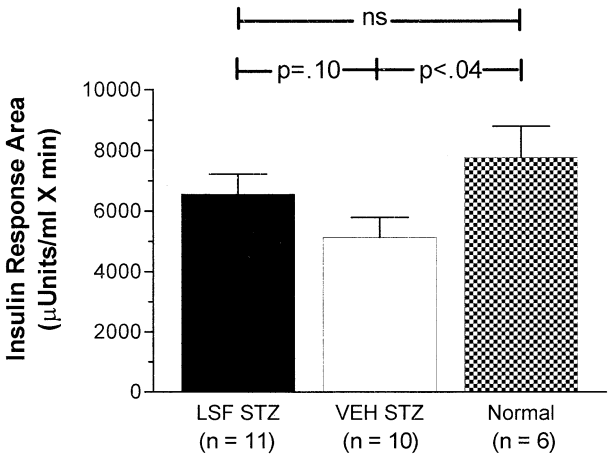

Fig 4. Effect of LSF on insulin response areas during 2-hour OGTTs in treated and untreated diabetic rats and in normal rats. Total areas under insulin response curves were calculated for each group of rats as indicated in Fig 3.

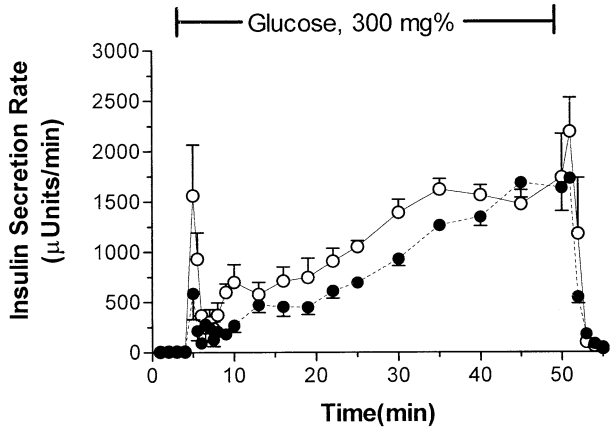

Fig 6. Effect of 40 μmol/L LSF on glucose-induced insulin secretion rates in perfused pancreases from normal rats. A 300-mg% glucose stimulus was infused from minute 2 to minute 50. LSF was infused from minutes 3 to 10 of the first phase and from minutes 16 to 35 of the second phase. —○—, LSF infused (n = 5); - -●- -, vehicle infused (n = 4).

Table 2. Effect of LSF on First and Second Phase Insulin Response Areas in Perfused Rat Pancreas

	First Phase* (minutes 3 to 10)	Second Phase* (minutes 13 to 35)
Controls (4)	1,481 ± 269	16,760 ± 1,174
LSF (20 μmol/L) (5)	3,919 ± 739 (P = .03)	30,240 ± 4,959 (P = .03)
LSF (40 μmol/L) (4)	3,643 ± 630 (P = .02)	24,320 ± 2,515 (P = .03)
LSF (60 μmol/L) (4)	2,562 ± 559 (P = NS)	29,230 ± 2,103 (P = .002)

NOTE. P values: Significance of differences compared with controls using Student's t test for unpaired observations.
Abbreviation: NS, not significant.
*Total insulin released (mean ± SEM) in μU.

ment of rates of insulin secretion by LSF during both phases of insulin release was also observed when the infused concentration was 40 μmol/L. The first phase area for total insulin released (Table 2) was 3,643 ± 630 μU, which was 2.5-fold greater than mean area in the saline controls (P = .02). A significant effect (increase) of 40 μmol/L LSF on total insulin release during the second phase was also measured: 24,320 ± 2,515 as compared with 16,760 ± 1,174 μU in the controls (P = .03). As shown in Table 2, insulin responses of pancreases infused with 40 μmol/L LSF were of smaller magnitude as compared with responses to 20 μmol/L LSF. As was the case with the lower LSF concentrations, enhanced insulin secretory responses during both phases of insulin release were also observed at 60 μmol/L LSF (Table 2). However, as compared with f irst phase insulin release at 20 and 40 μmol/L, the response at 60 μmol/L was of smaller magnitude, 2,562 ± 559 μU, being 2-fold greater than that of the controls. The first phase effect of LSF was, however, not statistically different from that of the vehicle-infused controls. The total insulin released during the second phase (29,230 ± 2,103 μU) by pancreases infused with 60 μmol/L LSF was comparable to that observed in pancreases infused with the lower concentrations of LSF.

DISCUSSION

In these studies a novel anti-inflammatory agent, LSF, was evaluated in vivo and in vitro to determine its effects on glucose tolerance and insulin secretion. Similar to the intermediate STZ dose rats characterized previously,[8] the STZ prediabetic rats used in these studies showed essentially normal body weight gain. In addition, food intake in the untreated STZ animals was increased as compared with food intake in the normal animals, indicating presence of mild hyperphagia in the STZ animals. Interestingly, hyperphagia was not present in the LSF-treated rats; although these animals gained weight at rates comparable to the untreated STZ rats. These differences in food consumption and utilization efficiency are likely due to improved β-cell functioning and maintenance of higher postprandial insulin levels during feeding in the treated rats.

Concerning the mechanism of action of LSF, studies pointing to the existence of an intimate connection between insulin secretion, β-cell glucose metabolism, and intracellular disposition of long-chain fatty acids are of note.

Thus, stimulatory levels of glucose are known to promote generation of malonyl CoA, an inhibitor of carnatine palmitoyl transferase I (CPT-1). The resulting accumulation of acyl CoA in the cytosol may then act to potentiate insulin secretion.

Increased levels of acyl CoA in the cytosol and consequential buildup of certain long-chain acyl CoA species capable of activating enzymes in the glycolytic pathway and/or tricarboxylic acid cycle could lead to increased glucose metabolism.[12]In relation to this, it is generally accepted that insulin secretion requires enhanced rates of glycolysis in β cells.[13] Experimental support for this hypothesis has derived from studies using isolated perfused rat pancreas preparations.[14] Using these perfusion preparations, it has been observed that hydroxycitrate, an inhibitor of the production of malonyl CoA from glucose, also prevents insulin secretion. Similarly, these investigators observed that addition of palmitic acid, which favors accumulation of palmitoyl CoA, enhances insulin secretion and reverses the negative effects of hydroxycitrate. In previous studies, we observed that LSF could prevent accumulation of a specific phosphatidic acid, presumably by inhibiting certain acyl transferases, which would also lead to accumulation of acyl CoA in the cytosol.[3] Thus, a possible mechanism of LSF action could involve inhibition of acyl transferase activity and reacylation of fatty acids, such as arachidonic acid and/or eicosanoids into membrane phospholipids. In relation to this, we observed that LSF is able to ameliorate IL-1β-induced dysfunction in cultured rat islets exposed to both IL-1β and LSF over a 24-hour period. However, in the studies shown here, we have also observed direct effects of LSF to significantly enhance glucose-induced insulin secretion in perfused pancreas preparations with this response occurring within minutes after exposure to low concentrations (20 μmol/L) of LSF that can be obtained in vivo.

Many studies support the conclusion that a signal for insulin secretion is generated in the mitochondria.[13-16] Important sites of control of mitochondrial metabolism and thus possible sites of LSF action include activation of the L-glycerol-3-phosphate shuttle[14,15] and glucose-mediated changes in catabolism of endogenous fuels, specifically fatty acids.[16-18]

Similarly, a family of proteins located in the inner mitochondrial membrane that act as proton channels has also been identified as a regulatory site of insulin secretion[19] and thus, a possible site of LSF action. Pancreatic islets isolated from mice deficient in one of these uncoupling proteins (UCP2) secrete excessive amounts of insulin in response to intravenous glucose and have higher levels of adenosine triphosphate (ATP) as compared with normal control mice.[19] In relation to this, in more recent studies from this laboratory, we tested LSF for its effects on mitochondrial metabolism and insulin secretion.[4] In these experiments, LSF effects were evaluated in the derived

rat β-cell line, INS-1 cells. LSF exposure increased glucose-stimulated ATP generation in the INS cells.

These responses were associated with correlated increases in rates of insulin secretion suggesting that LSF exerts modulatory effects on glucose-induced insulin secretion by increasing oxidative phosphorylation and ATP production in β-cell mitochondria.

In the studies shown here, which used unperturbed intact whole pancreas preparations, we also observed potentiation of glucose-stimulated insulin secretion. In addition, we observed no clear differences between first and second phase insulin secretory responses to infused LSF, although enhancement of the first phase release responses were greater at 20 and 40 μmol/L LSF as compared with 60 μmol/L. The absence of direct relationship between first phase release responses and LSF concentration suggests that at the concentrations studied, LSF may not directly affect β-cell sensitivity to glucose. These in vitro observations are in accord with the in vivo data indicating absence of LSF effects on the early insulin response after oral glucose administration.

All of these observations suggest that, in accord with our previous findings,[4] LSF enhancement of insulin secretion is due to increased energy metabolism in β cells. This conclusion is compatible with the presence of LSF effects on both the first and the second phase of insulin release, which represent the insulin storage and synthesis compartments, respectively,[20] because functioning of both compartments require energy.[15,19,20] Similarly in relation to the LSF effect on mitochondrial metabolism, it was observed that enhanced insulin secretion continued to exist after LSF infusion was terminated. The presence of this delayed off response pattern is also in accord with our observations that LSF may act by stimulating mitochondrial metabolism. Although it should be noted that the lack of consistent effects of LSF on the first phase also indicates that the LSF effects observed here are not restricted to enhanced energy production. This conclusion is in accord with observations in various in vitro systems suggesting that LSF has multiple effects.[2] The sustained effects of LSF to enhance insulin secretion observed in the perfusion studies is similarly in accord with this. LSF was infused in 2 increments, which consisted of a 7-minute infusion during the first phase of insulin release and a 16-minute infusion during the second phase. This format was used to compare β-cell secretory responses during each phase of insulin release separately. The LSF dose-response insulin secretion characteristics do not appear to be the same for each phase of insulin release. The observations also suggest that the persistent effects of LSF are transient indicating that β-cell uptake of LSF is reversible. Additional studies will be necessary to fully clarify the mechanism of LSF action in the pancreas.

In addition, to the effect of increasing substrate concentration, ie, availability of fatty acid substrate, to enhance mitochondrial metabolism, the molecular action of fuel molecules to alter activity of regulatory enzymes represents another possible site of control of insulin secretion.[13,16] Such actions related to molecular structure could also include modulatory effects of LSF on insulin secretion. Examples of fuel molecules include 2-ketoisocaproic acid, a fatty acid which is metabolized in the mitochondria and stimulates insulin secretion.[13,21] In addition, the compound b (-) 2 amino-bicyclo [2, 2, 1] heptane-2-carboxylic acid, a nonmetabolizable analogue of leucine, stimulates insulin secretion by activating glutamate dehydrogenase, resulting in increased metabolism of endogenous amino acids.[15] The chemical structure of LSF is similar to the drug theophylline, a dimethylxanthine, although LSF also contains a 6-carbon side chain at the N-1 position of the dimethylxanthine core.[2] It has been demonstrated that at millimolar concentrations, both theophylline[23] and the xanthine-containing compound, pentoxyfylline,[24] stimulate glucose-induced insulin secretion in perfused rat pancreas preparations. Both of these drugs exert their insulin stimulatory effects by inhibiting cyclic adenosine monophosphate (cAMP) phosphodiesterase, which results in accumulation of cAMP, a known modulator of insulin secretion.[25,26] In the studies shown here, we observed that at 20 and 40 μmol/L concentrations, LSF caused a significant nearly 3-fold enhancement of first phase insulin release. In contrast, the first phase insulin response to 60 μmol/L LSF was of smaller magnitude and was not significantly different from the saline-infused control response. If the enhanced insulin responses at 20 and 40 μmol/L LSF were due to inhibition of cAMP phosphodiesterase, we would expect to observe enhancement of insulin release of greater magnitude at 60 μmol/L. LSF, at the concentrations used in this study, does not inhibit phosphodiesterase; only at high (millimolar) concentrations does LSF have inhibitory effects on cAMP phosphodiesterase. The conclusion that the LSF-mediated increase in insulin secretion observed here is not due to cAMP phosphodiesterase inhibition is similarly supported by studies using various in vitro preparations and demonstrating that many LSF-mediated effects on cellular metabolism are observed in the absence of any increases in cAMP level.[2]

In studies using mice, it has been noted that administration of multiple subdiabetogenic doses of STZ, resulting in partial damage to the islets, acts to trigger an inflammatory process with simultaneous STZ-invoked activation of the immune system.[27] Similarly, in support of this conceptualization, is the demonstration that administration of macrophage toxic particles almost completely prevents diabetes development in mice given multiple doses of STZ.[27] In relation to this, our previous studies examining effects of LSF in the NOD mouse model of type 1 diabetes mellitus should also be noted.

NOD mice spontaneously develop a form of insulin-dependent diabetes similar to the human disease and caused by an autoimmune T-cell–dependent destruction of islet cells.[28] In our studies using this model,[5] LSF administration at the same dose level as used here (25 mg/kg body weight, twice daily) significantly reduced the onset of diabetes in the NOD mice. In accord with our in vitro data[3,4] demonstrating LSF amelioration of cytokine-mediated β-cell dysfunction, the in vivo effects of LSF in NOD mice and in the STZ rats used here indicate that LSF may also enhance glucose-induced insulin secretion through its anti-inflammatory actions; either by inhibition of macrophage activation and release of proinflammatory cytokines or by preventing cytokine-induced loss of β-cell function. It is apparent, however, that the LSF-mediated enhancement of insulin secretion involving anti-inflammatory effects of LSF cannot explain the direct effect of this agent in the isolated

perfused normal rat pancreas. In this study, the protective effect of LSF was seen almost 2 weeks after injection of a single dose of STZ, which is probably beyond occurrence of insulitus, suggesting that only a minor part of the LSF effect involved its anti-inflammatory action. It is noteworthy that in mice injected with multiple doses of STZ, lymphocytic infiltration and insulitus peak within 7 days after the final injection of STZ.[27]

In conclusion, in these studies we have utilized chemically diabetic rats as a model of type 2 diabetes to assess the effect of the agent, LSF, on diabetic pathology. Increased insulin responses to oral glucose measured in the STZ rats chronically treated with LSF correlated with significant potentiation of glucose-induced insulin release by LSF directly infused into perfused pancreas of normal rats. The in vitro observations support the conclusion that improved insulin responses measured in vivo in the LSF-treated rats reflect improved functioning of the residual population of β cells present in the STZ rat pancreas. These results provide support for the use of agents, such as LSF, for improving insulin secretion and glycemic control in type 2 diabetes.

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