Lisofylline, a Novel Antiinflammatory Agent, Protects Pancreatic β-Cells from Proinflammatory Cytokine Damage by Promoting Mitochondrial Metabolism

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Proinflammatory cytokine-mediated pancreatic β-cell dysfunction is a key pathological event in type 1 diabetes mellitus. Lisofylline (LSF), an anti-inflammatory agent, has been shown to protect pancreatic islets from IL-1β-induced inhibitory effects on insulin release. However, the mechanism of LSF action is not known. Increasing evidence suggests that the mitochondria play an important role in regulating the β-cell insulin release capacity and the control of cellular viability.

To examine the direct effects of LSF on β-cells, insulin-secreting INS-1 cells were exposed to a combination of recombinant IL-1β, TNFα, and IFNγ with or without LSF for 18 h. Basal and glucose-stimulated static insulin release were measured using RIA. INS-1 cell viability was determined using in situ terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling and LIVE/DEAD dual fluorescence labeling. To evaluate INS-1 mitochondrial function, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolism, change in mitochondrial membrane potential, and intracellular ATP levels were assessed.

AUTOIMMUNE-MEDIATED mononuclear cell infiltration leading to the release of proinflammatory cytokines and subsequent destruction of insulin-producing β-cells constitute the major events in the development of type 1 diabetes mellitus. The release of IL-1β, TNFα, and IFNγ, is believed predominately to be a result of inflammatory Th1 lymphocytes as well as cytotoxic T cell and macrophage activation (1). These cytokines are cytotoxic to pancreatic β-cells by inducing the formation of oxygen free radicals, nitric oxide, and lipid peroxides within the β-cells.

The antiinflammatory compound lisofylline (LSF), 1-(5-R-hydroxyhexyl)-3,7-dimethylxanthine, was originally used to reduce the incidence of graft vs. host disease and to prevent the onset of experimental autoimmune encephalomyelitis by blocking IL-12-induced T helper 1 differentiation (2). It also protected hypoxia-induced lung injury and IL-1β-induced reduction in insulin secretion in pancreatic islets by modulating lipopid inflammatory mediator production (3, 4). In the islet study, LSF reduced IL-1β toxicity without modulating nitric oxide synthase expression. Therefore, the precise mechanism by which LSF protects the β-cell against cytokine injury has not been clarified. This study was designed to determine whether LSF has any direct effect to protect pancreatic β-cells from cytokine toxicity and to explore the possible underlying mechanism of LSF action.

The mitochondrion is a pivotal subcellular organelle in pancreatic β-cells. It governs the insulin secretory function in β-cells (5) as well as regulates cell viability in general (6). Glucose transported into β-cells eventually enters the tricarboxylic acid cycle in mitochondria to generate ATP. The increase in cytosolic ATP to ADP ratio leads to insulin exocytosis. On the other hand, several mitochondrial factors, such as Bcl-2 and cytochrome c, are directly involved in regulating cell death and survival. The current results indicate that mitochondria in insulin-secreting β-cells may be a potential target for LSF protective effects. By promoting mitochondrial metabolism, LSF may restore both the insulin secretory capability and cell viability in β-cells exposed to multiple proinflammatory cytokines.

Materials and Methods

Culture of rat insulin-secreting INS-1 cells

The cell line was originally developed in Dr. Claes Wollheim’s laboratory (7). The INS-1 cells maintained in our laboratory were a gift from Dr. Christopher Rhodes (Pacific Northwest Research Institute, Seattle, WA). Cells from passages 10–30 were maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% heat-inactivated FBS, 10 mM HEPES, 200 μM L-glutamine, 1 mM sodium pyruvate, 5 mM 2-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin at pH 7.4. The cells were cultured in a 37°C in a humidified incubator supplied with 5% carbon dioxide. Fresh medium was replaced...
every 2 d. Unless otherwise stated, the cells were plated at a density of 10^4/cm². Culture vessels (dishes and chamber slides) used for experiments were coated with poly-d-lysine and gelatin (Sigma, St. Louis, MO) to retain detached and dead cells so that seeding cell numbers reflect the actual cell numbers after all treatment conditions.

Cytokine and lisofylline treatment of INS-1 cells

INS-1 cells were treated with vehicle alone or with the combination of recombinant rat IL-1α (5 ng/ml), IFNγ (100 ng/ml), and TNFα (10 ng/ml; R&D Systems, Inc., Minneapolis, MN) suspended in complete RPMI medium. LSF (provided by Cell Therapeutics, Inc., Seattle, WA) was supplied in 10 mM RPMI medium. LSF (provided by Cell Therapeutics, Inc., Seattle, WA) was incubated in the culture medium for 1 h. The treated cells were washed before labeling to eliminate serum esterase activity. This was followed by incubating the cells in PBS containing 1 μM calcein-AM and 6 μM ethidium homodimer (EthD-1) for 60 min at 37 C. The coverslips were washed and mounted onto a glass slide. The specimen was analyzed under an Olympus Corp. BH-2 fluorescence microscope. Calcein and EthD-1 were both excited with a conventional fluorescence excitation optical filter at 485 nm. The fluorescence emissions from both fluorophores were viewed simultaneously using a fluorescence longpass optical filter (Molecular Probes, Inc.). The images were recorded and analyzed using a CCD camera and Image Pro software.

Static insulin secretion measurement

At the end of treatment, cells were washed with Krebs-Ringer-bicarbonate-HEPES buffer (KRB) containing 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 1.0 mM CaCl2, 10 mM HEPES, and 0.1% BSA at 37 C, pH 7.4. They were preincubated in the same buffer for 30 min, followed by 60-min incubation in KRB supplemented with 15 mM d-glucose (J. T. Baker, Phillipsburg, NJ). The supernatant was harvested and subjected to centrifugation to eliminate residual cells. Insulin secreted into the supernatant was measured by RIA with rat insulin as a standard.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolism in INS-1 cells

After cell treatments, cells were washed and preincubated in KRB as described above. The cells were replaced with KRB containing 0.1 mg/ml MTT (Sigma) with or without 15 mM d-glucose. After a 60-min incubation, the supernatants were removed. The insoluble MTT metabolite within the cell monolayer was extracted with isopropanol. The absorbance of the extracts at 950 nm was measured using a standard microplate reader as an indication of the degree of mitochondrial metabolism in general (9).

In situ terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) of DNA breakage

To access the cytotoxic effect of multiple cytokines in INS-1 cells and resulting cell death, the early cell death characteristic of endonucleolysis was detected using TUNEL assay following the manufacturer’s instruction (Roche Molecular Biochemicals, Indianapolis, IN). After treatment, INS-1 cells grown on culture grade chamber slides with poly-d-lysine and gelatin (Sigma) coating were washed before preparation for confocal microscopy. Endogenous peroxidase was blocked upon mitochondrial membrane polarization forming an orange-red fluorescence. When ΔΨm is disrupted, the dye cannot access the transmembrane space of mitochondria and reverts to its green monomeric form. INS-1 cells treated with or without the cytokines and LSF were incubated with desiprier in the manufacturer-supplied reaction buffer for 20 min. The cells were then stabilized and viewed under a fluorescence microscope with the same excitation and emission wavelengths mentioned above.

Measurement of intracellular ATP concentration

The cytosolic ATP level was assessed in INS-1 cells as another indication of mitochondrial metabolism. INS-1 cells (2 × 10^5) from each treatment condition were pelleted and immediately extracted by addition of 30 μl 0.1 N NaOH/0.5 mM EDTA and incubated at 60 C for 20 min. The cell extracts were either assayed immediately or stored at −20 C (10). It has been demonstrated that the ATP content remains stable in this condition for 1 yr (10). ATP content was determined with an ATP-dependent oxidation of luciferin catalyzed by a highly purified firefly luciferase (11) coupled with scintillation counting (12). Five microliters of cell extract (containing up to 8 μM ATP/sample) were diluted into 1 ml 10 mM HEPES buffer (pH 7.8). Luciferase reagent (100 μl; Roche Molecular Biochemicals) was added to the diluted cell extracts. All samples were immediately counted for 30 sec in an LS 6900 scintillation counter (Beckman Coulter, Inc., Fullerton, CA) with the isotope window wide open. The ATP concentration per cell was calculated against a standard curve made with 1-7 × 10^-14 mol ATP (Roche Molecular Biochemicals).

Statistics and calculations

All numerical data are expressed as the mean ± SE. The listed n values represent the number of experiments performed using INS-1 cultured from different passages. Statistical significance was determined by one- or two-way ANOVA (depending upon experimental design) coupled with Bonferroni posttests to compare replicate means.

Results

Effects of LSF on cytokine-imposed inhibition of insulin secretion in INS-1 cells

Figure 1 shows that both basal (0 mm) and glucose-stimulated (15 mm) insulin secretion were retarded by an 18-h multiple-cytokine treatment [basal, 7.8 ± 0.30 vs. 10.0 ± 0.46 ng/mL (P < 0.005); glucose-stimulated insulin secretion, 11.6 ± 0.86 vs. 17.4 ± 1.86 ng/mL (P < 0.005)]. When 20 μM LSF...
LSF was added simultaneously with the cytokines, the inhibitory effects of these cytokines on insulin release were prevented. An increase in both basal and glucose-stimulated insulin secretion by 20 μM LSF alone was consistently observed. The inactive analog of LSF CT 1501S did not block the cytokine effects or alter insulin secretion (data not shown).

**Effect of cytokines and LSF on mitochondrial MTT metabolism**

MTT metabolism was assessed during the course of static insulin secretion measurement. At a concentration of 0.1 mg/ml, MTT does not interfere with insulin release in INS-1 cells or other β-cell lines (8, 9). Figure 2 indicates that cytokine treatment produced a reduction in basal (33.6%; n = 12; P < 0.001) and glucose-stimulated (44.4%; n = 12; P < 0.001) MTT metabolism. LSF at 20 μM stimulated MTT metabolism in both basal (17.3%) and glucose-stimulated (16.2%) INS-1 cells and prevented the cytokine-induced reduction of MTT metabolism. LSF had no effect on cell growth and proliferation by cell counting and [3H]thymidine incorporation (data not shown). These results suggest that LSF may have a direct effect on promoting INS-1 cell mitochondrial metabolism.

**LSF protects INS-1 cells from cytokine-mediated cytotoxicity**

Multiple proinflammatory cytokines are known to have cytotoxic effects on pancreatic β-cells and islets of the Langhans (1, 13, 14). We also previously demonstrated in a titration and time-course study that a combination of recombinant IL-1β, IFNγ, and TNFα could induce apoptotic cell death in insulin-secreting β-cells (8) starting as early as 6 h. We have demonstrated in Figs. 3 and 4 that LSF prevented multiple cytokine-induced cell death. Both in situ TUNEL assay and LIVE/DEAD dual fluorescent labeling were used to assess the cell death rate and the nature of cell death. This protective effect is specific to LSF based on the fact that an inactive analog of LSF, 1501S, did not prevent cytokine-induced apoptosis (data not shown). Figure 3a shows the in situ TUNEL stain in control (A), cytokine-treated (B), LSF-treated (C), and cytokine- and LSF-treated (D) INS-1 cells. TUNEL-positive cells were clearly present (dark brown-stained, represented by white arrows) in the cytokine-treated group, with a minor reduction in cellularity at the end of the 18-h treatment. These changes were not found in control or LSF alone-treated cells. In cells treated with both LSF and cytokines, no TUNEL-positive cells were seen.

By quantifying TUNEL-positive INS-1 cells under each experimental condition and subsequent statistical analysis, Fig. 3b shows that multiple cytokine treatment resulted in 49% cell death (n = 8; P < 0.001), whereas there was a dose-dependent LSF effect to restore cell viability (EC50 9.2 μM). LSF (50 μM) restored cell viability to the control level (4.9% vs. 5.0% control). LSF alone (0–100 μM) did not alter INS-1 cell viability compared with vehicle control. An optimal concentration of 30 μM LSF was chosen based on the dose effects on cell viability and insulin secretion.

**LIVE/DEAD dual fluorescent labeling revealed similar information as the TUNEL assay**

Cytokine treatment resulted in compromised plasma membrane permeability, as reflected by ethidium homodimer (red dye) gaining entry to intracellular space, which would be excluded due to its negative charge under normal circumstances (Fig. 4B). Cellular nonspecific ester hydrolase activity and plasma membrane integrity were also compromised, thus reducing green fluorescence emission from free calcein in cytokine-treated cells. The ethidium-stained (red) nuclei in cytokine-treated INS-1 cells were disintegrated into several condensed entities, which are consistent with apoptotic cells. In contrast, there was no detectable staining of nuclei and no reduction in cellular
esterase activities in cytokine and LSF-treated INS-1 cells (Fig. 4D). Instead of showing the normal spreading cell morphology, these cells were rounded, indicating that cell morphology was not completely restored. LSF added alone led to no visible changes compared with the control INS-1 cells (Fig. 4C).

**LSF normalized mitochondrial membrane potential in cytokine-treated INS-1 cells**

The $\Delta \Psi_m$ is negative inside energized mitochondria under physiological conditions (15). Glucose uptake and transport into the tricarboxylic acid cycle in pancreatic $\beta$-cells results in generation and transfer of ATP into the cytoplasm and mitochondrial membrane hyperpolarization due to electron transfer along the electron transport chain situated on the inner membrane of the mitochondria (5). The uptake of depsipher, a lipophilic carbocyanide, is driven by $\Delta \Psi_m$. Depending on $\Delta \Psi_m$, the dye forms aggregates and undergoes a reverse shift in emission from green fluorescence (527 nm) to red fluorescence (590 nm) as more aggregates form at increasingly hyperpolarized $\Delta \Psi_m$. These red aggregates were consistently found in control INS-1 cells (Fig. 5A), whereas the red aggregates reversed to more homogenous green fluorescence in multiple cytokine-treated cells, indicating mitochondrial membrane depolarization and reduced $\Delta \Psi_m$ (Fig. 5B). LSF at 30 $\mu$M restored the $\Delta \Psi_m$ as reflected by the reappearance of red depsipher aggregates after cytokine treatment (Fig. 5D). There was no $\Delta \Psi_m$ disruption in LSF alone-treated INS-1 cells (Fig. 5C).

These results demonstrate that IL-1$\beta$, TNF$\alpha$, and IFN$\gamma$ at the given concentrations disrupted $\Delta \Psi_m$ in INS-1 cells, as observed at the end of the 18-h treatment period. Simultaneous addition of LSF with the cytokines maintained $\Delta \Psi_m$ at normal levels.

**Effect of LSF on cellular ATP levels in cytokine-treated INS-1 cells**

Due to the low level of lactate dehydrogenase, pyruvate generated from the glycolytic pathway is channeled to the mitochondria, resulting in more than 90% of carbons from glucose entering the $\beta$-cell being converted to carbon dioxide in mitochondria (5). Thus, ATP production or the intracel-
lular ATP level becomes an indicator of the mitochondrial metabolic state. The intracellular ATP concentration was assessed in INS-1 cells under all experimental conditions. At the end of 18 h of treatment, cells were subjected to KRB solution with or without 15 mM glucose for 60 min. ATP was then extracted and measured using a luciferase assay coupled with scintillation counting. The ATP concentration was normalized by the plating cell number, as the experiment was finished within the period when no cell doubling occurred. ATP levels in INS-1 cells were comparable to available data from other insulin-producing β-cells (10). Reductions in basal (43.5%) and glucose-stimulated (30.8%) cytosolic ATP levels were observed in cytokine-treated INS-1 cells (Fig. 6). Lisofylline (30 μM) brought the cytokine-reduced ATP concentration to near-control levels (basal, 96.7%; glucose-stimulated, 93.2% of controls). Interestingly, LSF added alone to INS-1 cells resulted in a consistent elevation of intracellular ATP concentrations. This suggests that LSF’s protective effect on cytokine-treated INS-1 cells may be at least in part due to its effect on mitochondrial energy production.

**LSF independently increased glucose responsiveness, MTT metabolism, and insulin release in INS-1 cells**

We examined the effects of LSF alone on pancreatic β-cells to provide additional evidence that LSF protected cells from cytokine effects (Fig. 7). INS-1 cells maintained in complete RPMI medium were treated with 0–100 μM LSF for 18 h (to be consistent with the cytokine regimen). At the end of the treatment period, MTT metabolism (Fig. 7A) and static insulin secretion (Fig. 7B) were measured. At concentrations of 10 and 50 μM, LSF produced increases in basal and glucose-stimulated MTT metabolism as well as insulin secretion. There were no further increases in either MTT metabolism or insulin release when the LSF concentration was increased from 50 to 100 μM. No toxic effect of LSF was observed at a concentration of 100 μM, as judged by the morphological appearance of INS-1 cells and by the above-described cytotoxicity assays (TUNEL, LIVE/DEAD, and mitochondrial metabolism).

We also performed an additional experiment to address the question of whether LSF could increase glucose-induced insulin secretion in INS-1 cells in light of its effect on mitochondria and static insulin secretion. INS-1 cells treated with or without 30 μM LSF for 18 h were exposed to different concentrations of glucose (0–25 mM) for 60 min. The insulin released into the supernatant was measured by RIA. LSF increased insulin release in response to glucose (Fig. 7C), indicating a net increase in insulin secretion in LSF-treated INS-1 cells. The LSF stimulatory effects on MTT metabolism and insulin secretion and on β-cell protection are specific, in that the inactive isomer of LSF (CT1501S) produced no alterations in MTT metabolism or insulin secretion. It also failed to protect INS-1 cells from toxic effects of cytokines (data not shown). It is noted that LSF at low concentrations caused the suppression of insulin secretion and glucose-stimulated MTT metabolism.

**Discussion**

LSF is an anti-inflammatory agent that can reduce IL-12 signaling. Most studies with LSF have focused on its effects in modulating T helper 1 lymphocyte development, inhibiting proinflammatory cytokine production (2, 16), and regulating inflammatory lipid mediator release (17–19). LSF has been shown to have beneficial effects in several inflammatory disorders, such as sepsis (19, 20), hypoxia, and hemorrhagic organ injury (4, 21, 22), as well as in autoimmune diseases (23, 24). The effect of LSF to maintain insulin secretion in IL-1β-treated rat islets was also previously reported (3). In this study we examined the direct effects of LSF on multiple proinflammatory cytokine actions in INS-1 cells. The major and novel finding is that LSF protected β-cells from multiple inflammatory cytokine-mediated injury, as indicated by its ability to maintain insulin secretory capability and cell viability. The protective effects of LSF occur at least in part by promoting β-cell mitochondrial metabolism, normalizing mitochondrial membrane potential, and stimulating energy production.

The mitochondrion is a critical subcellular organelle. In addition to energy manufacture, it participates in regulating and controlling cell survival and cell death (6, 15, 25, 26). Before the manifestation of classical cell death, a change in mitochondrial membrane integrity is seen, resulting in the dissipation of its transmembrane ΔΨm, and the release of membrane proteins such as caspases and caspase activators (cytochrome c and heat shock protein 10) (15). These proteins, in turn, activate downstream pathways leading to apoptosis. Effectors such as cytokines could directly target the mitochondrial membrane to trigger permeabilization. In this study we also observed changes in ΔΨm, reduced mitochon-
drial metabolism, and ATP levels in multiple cytokine-treated INS-1 cells. In pancreatic β-cells, the mitochondria constitutes an important component of nutrient-induced stimulation-secretion coupling (27–29). Severe mitochondrial DNA mutations are linked to certain types of diabetes mellitus (5). Mitochondrial inhibition by protonophore or mitochondrial depletion results in failed glucose- and methylsuccinate-stimulated insulin secretion, while KCl-potentiated exocytosis remained intact in INS-1 cells (5). A very recent study (30) showed that circulating adrenal steroids directly enhanced glucose-stimulated insulin secretion by increasing the expression of mitochondrial and peroxisomal lipid metabolic enzymes. In INS-1 cells treated with the combination of IL-1β, IFNγ, and TNFα, there appeared to be an inhibition of glucose-stimulated insulin release independent from the reduction in cell number by apoptosis. Although cell death could have contributed to this MTT reduction, the cytokine inhibition of MTT metabolism in glucose-stimulated INS-1 cells, where cell numbers remained the same as those with no glucose treatment, suggests a functional mitochondrial inhibition in addition to cytotoxicity induced by cytokine treatment. The mechanism for this effect will require additional studies. Previous studies have clearly shown an important role of inducible nitric oxide (NO) synthase (iNOS) in reduced mitochondrial function in β-cells and islets (1, 11, 31). We investigated whether the LSF protective action was due to direct inhibition of iNOS expression and activity in response to cytokines. LSF at the same concentration that protected INS-1 cells did not impair cytokine-induced iNOS protein expression or NO formation (data not shown). Although multiple cytokines can still induce β-cell apoptosis in iNOS-null mice (32), it is likely that iNOS plays an important role in β-cell damage in response to multiple inflammatory cytokines. Therefore, it is still possible that although LSF did not affect iNOS expression or NO levels, it could act by preventing the damaging effects of NO on mitochondrial function. Additional studies will be needed to address this issue.

MTT, a tetrazolium salt, forms insoluble, purple-colored formazan upon reduction. In addition to succinate in mitochondria, there are other intracellular substrates, e.g. NADH and NADPH, that support MTT reduction, as shown in neurons and other cell types (33, 34). This indicates that MTT reduction reflects not only the mitochondrial metabolic state, but also the cellular redox potential. However, over 90% glucose carbons entering the β-cell are channeled to the mitochondria and are converted to CO₂ due to the extremely low level of lactate dehydrogenase in this unique cell type (5). Thus, the majority of reducing equivalents, NADH and FADH₂, in β-cells are produced in mitochondria. This indicates that MTT reduction in β-cells reflects cellular reducing equivalent levels as well as the mitochondrial metabolic state.

This study is the first to show that LSF exerts a direct effect to protect insulin-secreting pancreatic β-cells from proinflammatory cytokine-induced cytotoxicity. This direct protection may be accomplished in part by restoration of glucose stimulation-insulin secretion coupling and preservation of cell viability. The fact that LSF independently increased mi-
tochondrial metabolism, insulin release, glucose responsiveness, and intracellular ATP levels in \( \beta \)-cells suggests that promoting mitochondrial metabolism may be the underlying mechanism for its protective effect. Interestingly, an increase in ATP concentration in LSF-treated intestinal and hepatic tissue was also found to be associated with its protective effects caused by hemorrhagic injury (22). In additional experimental studies LSF is effective when applied before or simultaneously with cytokine addition to INS-1, suggesting that it acts at an early stage of cytokine-mediated \( \beta \)-cell toxicity (Chen, M., and J. L. Nadler, unpublished observations). LSF failed to rescue protonophore carbonyl cyanide \( p \)-trifluoromethoxyphenylhydrazone-induced mitochondria inhibition, indicating that LSF may not directly target the electron transport chain (data not shown). Ongoing studies in our laboratory are focusing on defining the potential molecular target of LSF in cytokine signaling pathways and glucose-mediated stimulation-secretion coupling pathways.

Proinflammatory cytokine-induced \( \beta \)-cell death and DNA disintegration are thought to be early events in type I diabetes (35). By overexpressing antiapoptotic mitochondrial proteins Bcl-2 and Bcl-x\(_L\), one can prevent pancreatic islet cells death induced by cytokines (36, 37). However, the impairment in nutrient-stimulated insulin release was not restored (37), suggesting that the two important functions of \( \beta \)-cell mitochondria, controlling cell viability and mediating stimulation-secretion coupling, may not be accomplished by the same set(s) of molecular pathways within the mitochondria. The current results provide evidence that LSF can restore both viability and secretory capability in cytokine-treated INS-1 cells. Additional studies will be needed to determine the \textit{in vivo} protective effects of LSF in autoimmunity-mediated \( \beta \)-cell damage. However, preliminary results suggest that LSF can maintain \( \beta \)-cell function and integrity in the nonobese diabetic mouse model of type I diabetes (38).

Therefore, agents such as LSF could potentially be useful for

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

\textbf{Fig. 7}. The independent effects of LSF on INS-1 cell MTT metabolism (A; \( n = 4; *; P < 0.01 \) compared with controls), static insulin secretion (B; \( n = 4; *; P < 0.05 \) compared with controls), and glucose responsiveness (C; \( n = 6; P < 0.001 \)). LSF (0–100 \( \mu \)M) was incubated with INS-1 cells for 18 h. The cells were then subjected to various concentrations of glucose for 60 min in KRB.
early intervention in type I diabetes and for defining the molecular targets in β-cells for protective interventions.

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