Liposomal Delivery of Purified Heat Shock Protein hsp70 Into Rat Pancreatic Islets as Protection Against Interleukin 1β–Induced Impaired β-Cell Function

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Recently it has been demonstrated that heat shock protein 70 (hsp70) is induced in pancreatic islet cells during prolonged exposure to interleukin 1β (IL-1β). It is unclear whether this represents a cellular defense against the noxious action of IL-1β or whether hsp70 is involved in the suppressive action of the cytokine. To assess the role for hsp70 in isolated islets exposed to IL-1β, hsp70 was purified and introduced into cells of isolated rat pancreatic islets via the liposome technique. Delivery of hsp70 was efficient according to immunoblot analysis, but delivered hsp70 disappeared within 16 h. Hsp70-containing liposomes did not affect protein synthesis, insulin secretion, or islet insulin mRNA content. However, when hsp70 liposome–incubated islets were further exposed to IL-1β (25 U/ml) for 16 h, these islets released more insulin in response to glucose stimulation and contained more insulin mRNA than islets incubated with control liposomes and subsequently exposed to the cytokine. No protective effect of liposomes containing bovine serum albumin or ovalbumin were observed. We conclude that hsp70 may protect against IL-1β–induced impairment of pancreatic β-cell function.
***Diabetes* 40:1418–22**

Interleukin 1β (IL-1β) is a cytokine that, during prolonged exposure, severely suppresses β-cell function and may even be cytotoxic to β-cells (1–10). The mechanism for the action of the cytokine is not clear, but induction of free O_2 radicals (11,12), stimulation of phosphoinositide hydrolysis (13), eicosanoid formation (14,15), increased gene transcription (16), e.g., expression of the c-*fos* proto-oncogene (17), involvement of a serine protease (18), and increased nitric oxide formation (19) have all been suggested to occur.

At a time point when β-cell function is suppressed after IL-1β exposure, increased expression of heat shock protein 70 (hsp70) is observed in isolated islets (11,20–22). Hsp70 is the major stress protein, and it is induced by hyperthermia and also after other cellular assaults (23). It belongs to a family of proteins with various functions including protection against cellular damage, possibly by dissociating cellular aggregates (24) but also involving various stages of protein synthesis and intracellular transport (25–27). Induction of hsp70 in islet cells occurs as a consequence of exposure to specific stimuli such as IL-1 and streptozocin (21) but also in response to nonspecific thermal stress (28). In the cases of IL-1 (5,8) and thermal stress (29), but not after streptozocin (29,30), the β-cells may resume their function after a period of suppressed function. Thus, it is unclear whether hsp70 induction in IL-1β–incubated islets exerts a protective role in these cells against the cytokine. To address this issue, hsp70 was purified and introduced into islet cells by the liposome technique and subsequently exposed to IL-1β. The results suggest a protective role of hsp70 against IL-1β–induced impairment of β-cell function.

RESEARCH DESIGN AND METHODS

We purchased ATP agarose (type III) from Pharmacia (Uppsala, Sweden). [^{35}S]methionine, [^{3}H]sucrose, [^{32}P]dCTP, an oligonucleotide labeling kit, and Hyperfilm were from Amersham (Aylesbury, UK). Collagenase from *Clostridium histolyticum* was purchased from Boehringer Mannheim (Mannheim, Germany). Genescreen filters were from Du Pont-NEN (Boston, MA), and nitrocellulose filters were from Millipore (Worthington, MA). Culture medium RPMI-1640 and donor calf serum were obtained from Flow (Irvine, UK). Bovine serum albumin (BSA), ovalbumin, L-α-phosphatidylethanolamine (type III-E), cholesterol, and oleic acid were from Sigma (St. Louis, MO). Human recombinant IL-1β was kindly given by Dr. Klaus Bendtzen (Laboratory of Medical Immunology, Copenhagen Univ. Hospital).

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Received for publication 4 February 1991 and accepted in revised form 12 June 1991.

Hsp70 was purified from bovine muscle with ATP agarose as described (31). Approximately 10–25 mg/ml of purified hsp70 protein as determined according to Bradford (32) was used for liposome encapsulation.

Liposomes were prepared as follows. Cholesterol, phosphatidylethanolamine, and oleic acid (molar ratio 7:7:3) were suspended in diethyl ether (2 μmol/ml), and 0.5 ml of the suspension was transferred to a round-bottomed retort. The diethyl ether was evaporated under a stream of N_2, and 100 μl of a solution of 50 mM NaCl and 20 mM Tris (pH 8) was added. For synthesis of hsp70, BSA, and ovalbumin-containing liposomes, the aqueous solution contained purified protein at a concentration of 10–25 mg/ml. The lipid film was disrupted for 2 min in a sonication bath, and the resulting liposomes were concentrated by centrifugation for 5 min at 160,000 × *g*. The liposome pellet was suspended in phosphate-buffered saline (PBS) and added to the different islet cultures at 50 nmol lipid/ml.

Pancreatic islets were isolated from adult Sprague-Dawley rats belonging to a local colony (Biomedical Centre, Uppsala, Sweden) by a collagenase digestion procedure as described in detail elsewhere (6). Groups of 200 islets were incubated in 0.7 ml RPMI-1640 at 37°C in 95% O_2/5% CO_2 with or without addition of liposome solution. After 2 h, 4.5 ml RPMI-1640 + 0.5 ml calf serum, and in some groups also 25 U/ml IL-1β, was added, and the islets were kept under tissue culture conditions for 16 h as given above.

After the 2-h incubation of islets with liposomes, 25 islets were washed and then labeled with [^{35}S]methionine for 2 h at 37°C (95% O_2/5% CO_2) in 100 μl of Krebs-Ringer buffer (33) supplemented with 10 mM HEPES (KRBH), containing 16.7 mM glucose, 0.5 mg/ml BSA, and 150 μCi/ml [^{35}S]methionine. After the labeling period, the islets were washed once in KRBH without BSA and dissolved in 25 μl sodium dodecyl sulfate (SDS) sample buffer (34). The samples were electrophoresed on 10% acrylamide/0.3% bis-SDS gels (34), and proteins were then electrically transferred onto nitrocellulose filters. The blots were then incubated with antibodies against hsp70 (35) and stained with a horseradish peroxidase reaction (36). The filters were then autoradiographed with Hyperfilm to visualize the [^{35}S]methionine labeling of proteins.

For insulin mRNA determination, groups of 30 islets were collected after the different exposures and extracted with SDS/phenol as described (37). The RNA was denatured with glyoxal (38) and dot-blotted onto Genescreen filters. pRI-7 (39) was labeled with an oligonucleotide labeling kit with [^{32}P]dCTP and hybridized to the dot blots as described (37). Insulin mRNA contents were determined by densitometry after autoradiography.

To measure insulin release, islets in triplicate groups of 30 were incubated in KRBH containing 2 mg/ml BSA and 1.7 mM glucose at 37°C (95% O_2/5% CO_2). After 60 min, the medium was gently removed and replaced with KRBH containing 16.7 mM glucose, and the islets were incubated for another 60 min. Insulin released to the media was measured by radioimmunoassay (40). After the incubations, the islets were pooled in groups of 30 and homogenized in water, and the DNA content was measured by fluorophotometry (41,42).

In previous studies, two experimental approaches were used to estimate intracellular islet uptake of liposomes. In one approach, the fluorescent compound 6-carboxyfluorescein (43) was used, and in the other, $^{14}CO_2$ output with [^{14}C]glucose-6-phosphate–containing liposomes (N.W., unpublished observations) was adopted. Both experimental techniques yielded rates of minimal intracellular liposomal release similar to 0.03% of islet vol/10 min. Extrapolated over a 2-h period, this corresponded to 0.4% of the islet volume. To assess the intracellular/extracellular hsp70 islet content (Fig. 1), liposomes containing [^{3}H]sucrose were synthesized and added to islets for 2 h, after which the islets were washed and incubated for 2 h in KRBH containing 16.7 mM glucose and 0.5 mg/ml BSA. The remaining ^{3}H counts in the islets in two separate experiments corresponded to a liposomal uptake of 4.5 and 5.5 pl/islet (i.e., 0.45 and 0.55% islet vol, assuming a mean islet vol of 1 nl). Thus, these figures are only slightly higher than the estimated minimal intracellular liposomal release referred to above.

Values are means ± SE, and groups of data were compared, with Student's paired *t* test. One observation represents islets isolated from one rat, and each observation was obtained on separate occasions with newly formed liposomes.

RESULTS

Islet content of hsp70 2 h after liposomal delivery, as assessed by immunoblot analysis, is shown in Fig 1. The content of hsp70 was greatly increased (Fig. 1*A*, *lane 2*). When the analysis was repeated 16 h later (Fig. 1*A*, *lane 4*), the islet content of hsp70 was nearly normal compared with islets exposed to liposomes containing no hsp70 (Fig. 1*A*, *lane 3*). The liposomal delivery of hsp70 did not affect protein synthesis (Fig. 1*B*).

Incubation of islets with control liposomes did not affect IL-1β–induced inhibition of insulin secretion (Table 1). Thus, a significant decrease in glucose (16.7-mM)-stimulated insulin release after IL-1β exposure was observed from the liposome-incubated islets. Neither liposomes nor IL-1β affected the insulin secretion at low glucose (1.7 mM). Because purification and liposome encapsulation of hsp70 is a major undertaking, a simplified protocol was designated to assess the effect of liposomes on the IL-1β effect. With this protocol, we found that the islets incubated with the hsp70-containing liposomes released a significantly larger amount of insulin in response to 16.7 mM glucose after IL-1β treatment than the islets pretreated with the control liposomes (Fig. 2). No differences in the islet DNA contents between the groups were detected (data not shown). Also, islet insulin content (ng insulin/10 islets) was unaffected by IL-1β or hsp70 (control 567 ± 153; control + IL-1β 460 ± 92; hsp70 584 ± 152; hsp70 + IL-1β 541 ± 119; n = 7 determinations in all groups).

To examine whether the effect of hsp70-containing liposomes against the suppression of insulin secretion was mainly due to nonspecific effects of protein delivery into the islet cells, the action of BSA and ovalbumin-containing liposomes was studied (Table 2). However, these liposome preparations and saline-containing liposomes (the same type of liposomes as in Table 1) failed to counteract the suppressive action of the cytokine on islet insulin secretion.

The IL-1β–treated islets that had been incubated with hsp70 liposomes contained more insulin mRNA than those

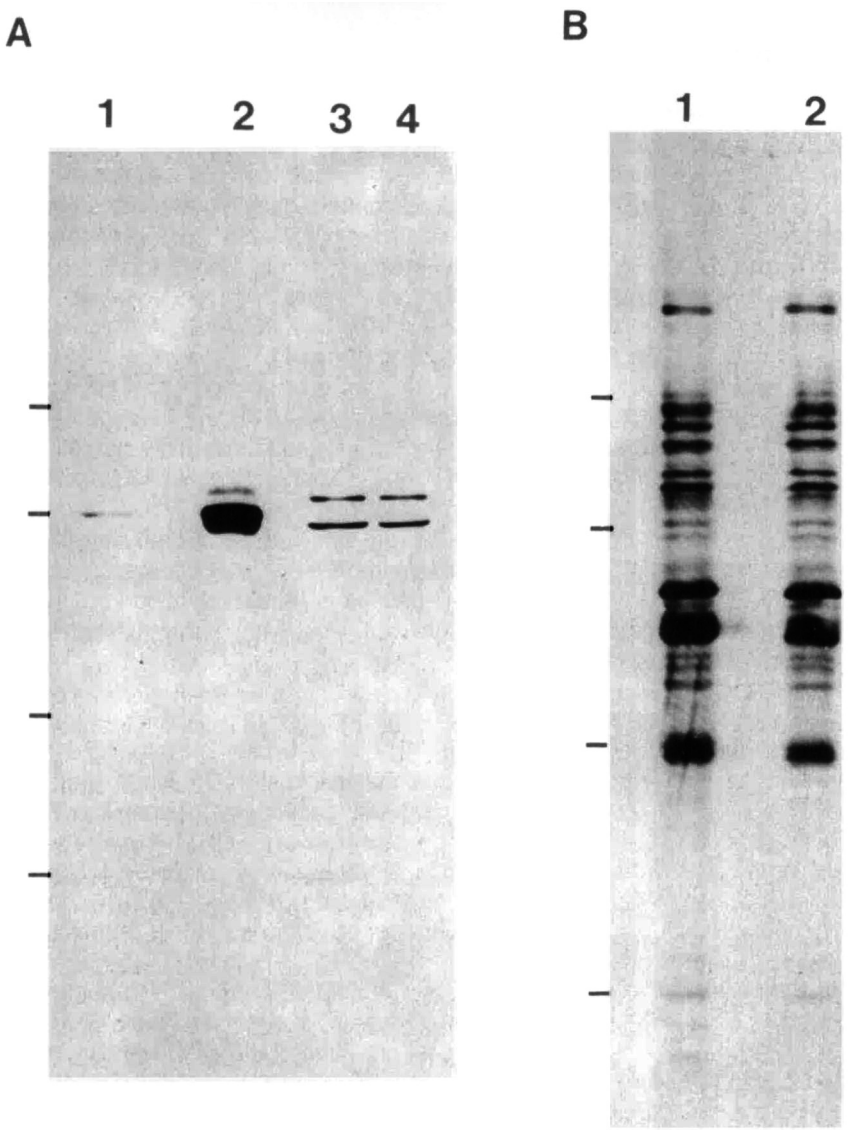

FIG. 1. Islet content of heat shock protein 70 (hsp70; *A*) and protein synthesis (*B*) after incubation with hsp70-containing liposomes. After incubation of groups of 200 islets for 2 h with control liposomes (*lanes 1* and *3*) or hsp70-containing liposomes (*lanes 2* and *4*), groups of 25 islets were labeled for 2 h with [^{35}S]methionine (*lanes 1* and *2*) and then subjected to sodium dodecyl sulfate gel electrophoresis and immunoblotting. *A* shows horseradish peroxidase staining for hsp70, whereas *B* shows pattern of protein biosynthesis after autoradiography. In *lanes 3* and *4*, islets were maintained in culture for 16 h before electrophoresis. *Bars*, molecular-weight markers for (from *bottom* to *top*) 31,000, 46,000, 69,000, and 97,000 M_r.

incubated with the control liposomes (Fig. 3). The insulin mRNA content of islets incubated with control (empty) liposomes was 106 ± 7% of the corresponding value for islets not incubated with liposomes but otherwise treated identically in three experiments.

DISCUSSION

To assess the putative role of increased hsp70 expression after IL-1β exposure of pancreatic islets (11,20–22), hsp70 was purified and artificially introduced into islet cells. The method used for hsp70 delivery was liposome encapsulation. This technique has previously been shown to be efficient for introducing impermeable compounds into islet cells, and it was estimated that ~0.03% of the islet cell volume was of liposomal origin after a 10-min incubation (43). Starting out with an intraliposomal concentration of 25 mg/ml hsp70, the intraislet hsp70 uptake would then be ~50 pg/islet 1 h after liposome addition. Although difficult to assess precisely, the immunoblot results indicate an ~10-fold increase in the hsp70 content, which is compatible with the estimated liposome delivery. Furthermore, experiments with [^{3}H]sucrose-containing liposomes also suggest that most of the hsp70, observed by immunoblot analysis, is of intracellular origin. It was considered of special importance to investigate whether the lipids constituting the liposomes affected IL-1β action in the islet cells. However, the liposomes themselves did not interfere with the IL-1β–induced suppression of insulin release (Table 1).

The counteraction by the hsp70 liposomes of the IL-1β suppression of islet insulin release and insulin mRNA content can be explained by a protective effect of this stress protein against the noxious action of the cytokine. Hsp70 is thought to disassemble harmful intracellular aggregates of denatured protein after heat shock (22), which may also be the case in this situation. Thus, if IL-1β activates a protease or a protease substrate (18), induces formation of free oxygen radicals (11,12) or nitric oxide (19), or leads to expression of new genes (16), the end result may be toxic or damaging aggregates of denatured proteins, which the delivered hsp70 may help dissolve. However, other experiments indicated a striking difference in the behavior of islet hsp70 after heat shock compared with IL-1β treatment (35). By use of an antibody effective in binding hsp70, binding was blocked after heat shock, probably by masking of the epitope

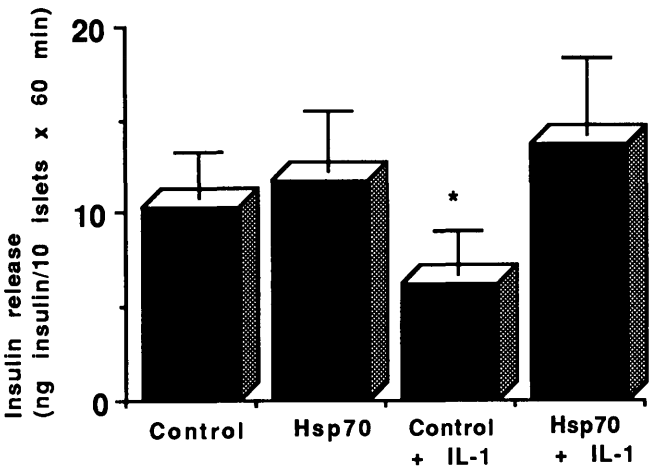

FIG. 2. Effects of liposomes containing purified heat shock protein 70 (hsp70) on insulin release. Freshly isolated rat pancreatic islets in groups of 200 were either incubated with 50 nmol/ml control or hsp70-containing liposomes in volume of 0.7 ml RPMI-1640 for 2 h at 37°C in 95% O_2/5% CO_2. Islets were then transferred to dishes containing RPMI-1640 + 5% calf serum with or without 25 U/ml interleukin 1β (IL-1β) and maintained in culture for 16 h. For insulin release determinations, islets were preincubated for 1 h at 37°C in 95% O_2/5% CO_2 in HEPES-supplemented Krebs-Ringer buffer containing 1.7 mM glucose and 2 mg/ml bovine serum albumin. Rates of insulin secretion were then determined during 2nd h under identical conditions but at glucose concentration of 16.7 mM. Values are means ± SE for 7–8 experiments. *$P < 0.05$ vs. IL-1β–exposed islets pretreated with hsp70-containing liposomes by paired *t* test.

on hsp70 recognized by the antibody by other substrates. Such a masking effect was not seen after IL-1β treatment, suggesting that IL-1β does not cause massive denaturation of proteins but rather a more specific destructive event. Moreover, our results do not exclude the possibility that liposomal delivery of hsp70 fortuitously interferes with a process that indirectly protects the β-cells against the IL-1β action, e.g., by affecting factors involved in gene or protease activation. However, the experiments with BSA- or ovalbumin-containing liposomes suggest that the protective effect was specific for the hsp70 liposomes.

So far, most studies dealing with the pathogenesis of insulin-dependent diabetes mellitus (IDDM) have focused on the process causing destruction of β-cells. However, it could also be rewarding to investigate the repair mechanisms triggered in the β-cells after injury. In this context, it is conceivable that not only genetic differences in the immune response but also genetic differences in β-cell repair capacity can be relevant when studying suceptibility to IDDM. Indeed, it was recently reported that the frequency of hsp70 alleles differed in IDDM patients and nondiabetic control subjects (44). Regardless, if the protective effects of liposomal hsp70 against IL-1β are direct or indirect, the results of this study point toward new possibilities in saving β-cells after a harmful assault.

TABLE 1
Effects of control liposomes and recombinant interleukin 1β (rIL-1β) on glucose-induced insulin release of rat pancreatic islets

Liposome addition (nmol/ml)	IL-1β addition (U/ml)	Islet insulin release (ng · 10 islets^{-1} · 60 min^{-1}) 1.7 mM glucose	16.7 mM glucose
0	0	13.3 ± 2.3	28.3 ± 3.6
0	25	11.3 ± 2.4	12.7 ± 2.5*
50	0	13.6 ± 1.8	26.8 ± 6.3
50	25	9.9 ± 1.4	15.4 ± 3.6†

Values are means ± SE for 14 experiments/group. Freshly isolated rat islets were incubated in 0.7 ml of medium RPMI-1640 under tissue culture conditions with or without liposome addition. After 2 h, 4.5 ml RPMI-1640 + 0.5 ml calf serum and IL-1β (IL-1β addition) were added, and islets were maintained in culture for 16 h. Subsequently, islet glucose–stimulated insulin release was studied by incubating islets during a 1st h in HEPES-supplemented Krebs-Ringer buffer containing 1.7 mM glucose and during a 2nd h with 16.7 mM glucose at 37°C (95% O_2/5% CO_2). Insulin released during the incubations was measured by radioimmunoassay.
*$P < 0.001$, †$P < 0.05$, vs. corresponding islets not exposed to IL-1β.

TABLE 2
Effects of different protein liposomes and interleukin 1β (IL-1β) on insulin release of rat pancreatic islets

Liposome addition (50 nmol/ml)	IL-1β addition (U/ml)	Islet insulin release (ng · 10 islets^{-1} · 60 min^{-1}) at 16.7 mM glucose
None	0	57.4 ± 14.4
	25	19.1 ± 2.0*
Control	0	55.0 ± 0.5
	25	18.8 ± 10.0*
Bovine serum albumin	0	67.9 ± 13.6
	25	20.0 ± 7.2*
Ovalbumin	0	49.7 ± 14.3
	25	16.5 ± 4.0*

Values are means ± SE for 3 experiments. Islets were isolated and treated as described in Table 1, and islet insulin release at 16.7 mM glucose is given. The liposome concentration added was 50 nmol/ml.
*$P < 0.05$ vs. corresponding islets not exposed to IL-1β.

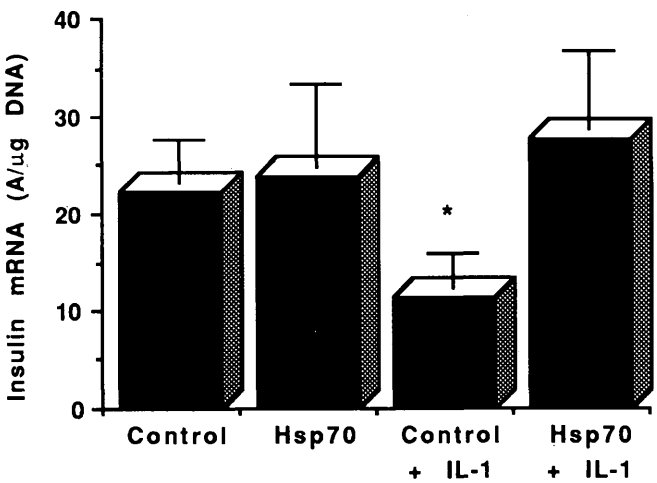

FIG. 3. Islets were treated as described in Fig. 2. Insulin mRNA contents were then determined by dot-blot analysis and autoradiography. Values are given as densitometric absorbance (A) per islet DNA in parallel determinations and are means ± SE for 5–6 observations. *$P < 0.05$ vs. interleukin 1β (IL-1β)–exposed islets pretreated with heat shock protein 70 (hsp70)-containing liposomes by paired *t* test.

ACKNOWLEDGMENTS

This work was supported by Grants 12X-109, 12X-8273, and 12X-9237 (connected to the β-Cell Transplant Concerted European Action for the Treatment of Diabetes) from the Swedish Medical Research Council; the Swedish Diabetes Association; the Juvenile Diabetes Foundation International; the Swedish Society for Medicine; the Nordic Insulin Fund; the Hoechst Diabetes Foundation; the Ragnar and Torsten Söderberg Foundation; the Ernfors Fund; and Magnus Bergvalls Stiftelse. D.L.E. and M.W. are recipients of a Fellowship and a Career Development Award, respectively, from the Juvenile Diabetes Foundation International.

We are grateful to Eva Forsbeck, Ing-Britt Hallgren, Astrid Nordin, and Sigran Svanholm for excellent technical assistance and Agneta Snellman for careful typing of the manuscript.

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