Influence of Protease on Inhibitory and Stimulatory Effects of Interleukin 1β on β-Cell Function

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To elucidate the putative role of proteases in the action of interleukin 1β (IL-1β) on pancreatic β-cells, we studied the effects on islet function of different protease inhibitors when added together with recombinant IL-1β to isolated rat pancreatic islets. It was found that the trypsin inhibitor *Nα-p*-tosyl-L-lysine chloromethyl ketone (TLCK) counteracted the acute stimulatory effects of IL-1β on islet glucose oxidation, insulin release, and biosynthesis. TLCK also partially or completely counteracted the long-term inhibitory effects of IL-1β on islet glucose oxidation, insulin biosynthesis, content, and release. This protease inhibitor also counteracted IL-1β–induced β-cell cytotoxicity as assessed by DNA content measurements. Of the other group-specific protease inhibitors investigated, only *N*-tosyl-L-phenylalanine chloromethyl ketone, *Nα-p*-tosyl-L-arginine methyl ester, and chloromercuriphenylsulfonic acid were found to partially protect against IL-1β action. We concluded that protease activation, putatively a serine protease, may be an early and perhaps primary event in the action of IL-1β on β-cells. *Diabetes* 40:290–94, 1991

Interleukin 1 (IL-1) has been implicated in the pathogenesis of autoimmune insulin-dependent diabetes mellitus (1), because it has been shown that prolonged exposure in vitro to this cytokine strongly inhibits islet insulin secretion (1–4). We have found that the IL-1β–induced inhibition is paralleled by an inhibition of the islet mitochondrial metabolism of glucose (3,4). On the other hand, during acute exposure to IL-1β, it has been observed that the cytokine stimulates islet insulin secretion (5–7). Although it was recently demonstrated that insulin-secreting cells possess receptors for IL-1β (8), the intracellular mechanisms that convey the actions of recombinant (r) IL-1β in islets still remain unknown. There are experimental data suggesting that this is not achieved via the arachidonic metabolism (4,9), inositol trisphosphate formation via phospholipase C activation (7,10), or protein kinase C activation (7). Moreover, dimethyl urea (4,8), a hydroxyl radical scavenger, and nicotinamide, an inhibitor of poly(ADP-ribose) synthetase, failed to revert the suppression of insulin secretion induced by IL-1 (4).

It has been shown in other cells that IL-1β activates intracellular proteases (11). To test the hypothesis that IL-1β exerts its actions on the β-cell by activating a protease, we incubated pancreatic islets with different inhibitors of proteases and found that the trypsin inhibitor *Nα-p*-tosyl-L-lysine chloromethyl ketone (TLCK) was a potent inhibitor of the effect of IL-1β on β-cells.

RESEARCH DESIGN AND METHODS

We purchased RPMI-1640 and donor calf serum from Flow (Irvine, UK); bovine serum albumin (BSA) from Miles (Slough, UK); L-[4.5-^{3}H]leucine and D-[U-^{14}C]glucose from Amersham (Aylesbury, UK); leupeptin, epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64), chloromercuriphenylsulfonic acid (CMPSA), *Nα-p*-tosyl-L-arginine methyl ester (TAME), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), TLCK, pepstatin A, phenanthroline, benzamidine, iodoacetic acid, and HEPES buffer from Sigma (St. Louis, MO); and Trasylol from Bayer (Leverkusen, Germany).

Pancreatic islets were isolated by collagenase digestion from adult male Sprague-Dawley rats (Uppsala, Sweden). Groups of 150 islets were kept free floating in RPMI-1640 supplemented with 10% (vol/vol) of donor calf serum at 37°C in gas phase of 95% humidified air/5% CO_2. On day 5, islets were transferred to culture medium containing 25 U/ml of human rIL-1β and the different inhibitors of proteases, in combination or alone, and cultured for another 24 h. Then the islets were subjected to in vitro experiments as described later. In some experiments, the acute effects of rIL-1β and TLCK during 90- to 120-min incubations were studied. rIL-

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1β was provided by S. Gillis (Immunex, Seattle, WA). The biological activity of the rIL-1β was 5 U/ng (12).

Triplicate groups of 10 islets each were transferred to sealed glass vials containing 0.25 ml Krebs-Ringer bicarbonate buffer with 2 mg/ml BSA and 10 mM HEPES (KRBH). During the 1st h of incubation at 37°C (95% O_2/5% CO_2), the KRBH was supplemented with 1.7 mM glucose. The medium was then replaced by medium containing 16.7 mM glucose, and the incubation continued for a 2nd h. The insulin concentration of the incubation media was measured by radioimmunoassay. After the culture periods, samples were obtained for determination of the medium insulin accumulation per 24 h. After the incubations, islets cultured for 24 h with the test compounds were pooled and ultrasonically disrupted in 0.2 ml redistilled water. An aliquot of the aqueous homogenate was mixed with acid ethanol and the insulin extracted overnight at 4°C. DNA was measured by fluorophotometry in another fraction of the water homogenate (13).

Duplicate groups of 10 islets were incubated for 2 h at 37°C (95% air/5% CO_2) in 100 ml KRBH containing 16.7 mM glucose and 50 mCi/ml L-[4.5-^{3}H]leucine. After incubation, the islets were homogenized and sonicated in 0.2 ml redistilled water. Total protein biosynthesis was measured in trichloroacetic acid precipitates of the homogenates. Proinsulin (PI) biosynthesis was estimated by an immunoabsorption method (14).

Triplicate groups of 10 islets each were incubated for 90 min in glass vials containing KRBH without BSA but supplemented with D-[U-^{14}C]glucose and nonradioactive glucose to a final concentration of 16.7 mM. The islet glucose oxidation was measured as described previously (15).

Values were computed as means ± SE, and groups of data were compared via Student's paired *t* test.

RESULTS

When studying acute effects of rIL-1β, insulin secretion at 1.7 mM was slightly increased in islets incubated in the combination of the cytokine plus TLCK for 1 h only (Table 1). At a high glucose concentration, rIL-1β enhanced insulin release. TLCK markedly potentiated islet insulin secretion during the 2nd h at the high glucose concentration in control islets. No further elevation of insulin release was observed when rIL-1β was added to the incubation medium containing TLCK. Islet PI biosynthesis was acutely stimulated by rIL-1β (Table 1). This effect was abolished by the presence of TLCK, which otherwise did not affect either the PI or total protein biosynthesis rates.

The cytokine increased islet glucose oxidation rates at 16.7 mM glucose when studied in acute experiments (Table 2). On the other hand, after culture for 24 h, the rate of islet glucose oxidation was decreased by rIL-1β. TLCK alone failed to affect the islet glucose oxidation rates both acutely and after 24 h. Addition of TLCK prevented both the acute increase and the impaired islet glucose oxidation after 24 h after treatment with rIL-1β.

Exposure of islets to rIL-1β for 24 h reduced the medium insulin accumulation by ~75% and glucose-sensitive insulin release by 45% compared with control islets (Table 3). Addition of TLCK counteracted both the decrease in medium insulin accumulation and the decrease in glucose-stimulated insulin release induced by rIL-1β.

Culture for 24 h with rIL-1β or TLCK influenced neither the islet DNA nor islet insulin content (data not shown). However, culture for 48 h reduced the DNA content of islets exposed to rIL-1β (207 ± 13 vs. 239 ± 14 ng DNA/10 islets for rIL-1β and controls, respectively; $P < 0.05$, $n = 10$ for both). TLCK prevented this decrease in DNA (232 ± 10 vs. 247 ± 17 ng DNA/10 islets for rIL-1β + TLCK and TLCK alone, respectively; $n = 10$).

PI biosynthesis was inhibited when the islets were cultured for 24 h with rIL-1β but without TLCK, but there was no significant alteration of the total protein biosynthesis rates (Table 3). As a consequence, there was also a decrease of the fraction of newly formed PI to the total pool of newly synthesized proteins in these islets. TLCK counteracted the impairment of the islet PI biosynthesis induced by rIL-1β. TLCK itself did not influence either the PI or total protein biosynthesis rates of the islets.

The possibility that TLCK would directly interfere with rIL-1β was checked by preincubating concentrated rIL-1β (6250 U/ml) with 100 μM TLCK for 24 h at 37°C and subsequently adding the rIL-1β solution (25 U/ml final concn) to islets in culture for 24 h. However, this did not alter the degree of rIL-1β–induced impairment of islet glucose oxidation at 16.7 mM glucose (342 ± 56.4 vs. 698 ± 104

TABLE 1
Effects of acute exposure to human recombinant interleukin 1β (rIL-1β) and *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) on glucose-stimulated insulin release and proinsulin (PI) and total protein (TOT) biosynthesis

rIL-1β (U/ml)	TLCK (μM)	Islet insulin release (ng · 10 islets^{-1} · 60 min^{-1}) 1.7 mM glucose	16.7 mM glucose	PI (dpm × 10^3 · 10 islets^{-1} · 2 h^{-1})	TOT (dpm × 10^3 · 10 islets^{-1} · 2 h^{-1})	PI/TOT × 100 (%)
0	0	4.2 ± 0.7	51.2 ± 8.0	6.4 ± 0.9	21.4 ± 3.1	30.5 ± 0.7
25	0	4.4 ± 0.4	69.7 ± 11*	7.6 ± 0.5*	21.5 ± 1.9	35.9 ± 1.6†
0	100	5.5 ± 0.9	128 ± 13‡	5.1 ± 0.7	20.0 ± 2.3	27.6 ± 1.9
25	100	6.1 ± 0.6†	118 ± 7.9‡	6.0 ± 0.9	19.5 ± 2.8	30.8 ± 1.3

Values are means ± SE for 7 experiments. Islets were incubated in 0.25 ml Krebs-Ringer bicarbonate buffer, 2 mg/ml bovine serum albumin, and 10 mM HEPES (KRBH) medium with rIL-1β and TLCK at concentrations noted. During 1st h of insulin release experiment, glucose concentration was 1.7 mM, and during 2nd h, it was 16.7 mM. For biosynthesis experiments, islets were incubated acutely for 2 h at 37°C with rIL-1β and TLCK as noted in 100 μl KRBH containing 50 μCi/ml of L-[4.5-^{3}H]leucine and 16.7 mM glucose. After incubations, islets were washed and homogenized in water. TOT and PI biosynthesis were measured as described in METHODS. PI/TOT × 100 denotes percentage contribution of labeled PI to total pool of labeled islet proteins.
*$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$, vs. islets incubated in absence of rIL-1β and TLCK via Student's paired *t* test.

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TABLE 2
Effects of acute and 24-h exposure to human recombinant interleukin 1β (rIL-1β) and Nα-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) on glucose oxidation of rat pancreatic islets

rIL-1β (U/ml)	TLCK (μM)	Glucose oxidation ($pmol \cdot 10\ islets^{-1} \cdot 90\ min^{-1}$)	
		Acute	24 h
0	0	740 ± 51.5	731 ± 54.9
25	0	903 ± 65.2*	576 ± 32.5*
0	100	781 ± 38.9	738 ± 71.2
25	100	779 ± 48.8	680 ± 38.3

Values are means ± SE for 8 (acute) and 10 (24-h) experiments. Islets were studied either with rIL-1β and TLCK present during the glucose oxidation incubations (acute) or after 24-h culture similar to experiments in Table 1. In the latter experiments, TLCK and rIL-1β were not present during islet incubation. Islets were incubated in 0.25 ml Krebs-Ringer bicarbonate buffer, 2 mg/ml bovine serum albumin, and 10 mM HEPES at 16.7 mM glucose in presence of D-[U-^{14}C]glucose for 90 min at 37°C (95% O_2/5% CO_2).
*$P < 0.05$ vs. corresponding group with no rIL-1β and no TLCK via Student's paired *t* test.

$pmol \cdot 10\ islets^{-1} \cdot 90\ min^{-1}$; $P < 0.01$ via paired *t* test; $n = 4$ in each group).

In a separate series of experiments, the effects of several protease inhibitors with different specificities were studied in conjunction with rIL-1β–induced impairment of islet glucose oxidation after 24-h exposure to the cytokine (Table 4). Only TAME (at 1 mM but not at 100 μM), TPCK, and CMPSA partially counteracted the inhibitory effect exerted by rIL-1β. Two of the inhibitors (TPCK and iodoacetic acid) significantly decreased islet glucose oxidation compared with control islets.

DISCUSSION

The results of this study show that TLCK could partially or completely counteract both the acute and long-term effects of rIL-1β on pancreatic islet function. TLCK is an active site–directed reagent. Its tosyl-lysine group enables it to form a substratelike complex with enzymes that recognize basic and hydrophobic amino acid residues, and its chloromethyl ketone group subsequently alkylates a catalytically active histidine, leading to an irreversible inhibition of the enzyme (16). TLCK is an inhibitor of trypsin and has also been shown to inhibit the activity of other proteases, e.g., plasmin, papain, and thrombin. Because IL-1β was itself not affected by TLCK, and because the protease inhibitors TAME and TPCK also protected against rIL-1β–induced alterations of β-cell functions, it appears that a serine protease is involved in the rIL-1β action. TAME, a synthetic substrate analogue, inhibits enzymes with trypsinlike specificity by binding noncovalently to the active site of the protease. The observed similarities in action between TAME and TLCK are not surprising, because both arginine and lysine have long and basic side chains. TPCK, the chloromethyl ketone derivative of tosyl-phenylalanine, acts like TLCK but inhibits chymotrypsin rather than trypsin (17). Therefore, the protective effect of TPCK was somewhat unexpected, especially because TPCK itself impaired β-cell function. The other trypsin inhibitors, Trasylol and benzamidine, did not counteract rIL-1β action. However, Trasylol is a macromolecule that is thought to act exclusively at the external surface of the cells. This is in contrast to TLCK, which is known to act intracellularly (18). The lack of effect of benzamidine may be explained by the modest concentration used (100 μM). Unfortunately, the use of higher concentrations was not possible due to adverse effects of benzamidine on β-cell function.

Group-specific inhibitors of cysteine (thiol) proteases are leupeptin, E-64, iodoacetic acid, and CMPSA. The specific inhibitors leupeptin and E-64 and the nonspecific thiol reagent iodoacetic acid did not protect against the effects of rIL-1β, whereas CMPSA exerted a weak protection. However, because CMPSA is a nonspecific thiol reagent, its effect is not necessarily mediated by inhibition of cysteine proteases. Finally, phenanthroline, a metalloproteinase inhibitor, and pepstatin A, an aspartic protease inhibitor, did not counteract the rIL-1β action. Taken together, these results implicate the involvement of a serine protease, and not cysteine, metalloprotein, or aspartic proteases, in mediating the rIL-1β–induced action on β-cells.

However, some caution should be taken in interpreting the results obtained with the chloromethyl ketones. Their activities are not confined to proteases only, because TLCK-induced inhibition of the cAMP-dependent protein kinase (19) and a tyrosine kinase (20) has been demonstrated. Fur-

TABLE 3
Effects of 24-h exposure to human recombinant interleukin 1β (rIL-1β) and Nα-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) on medium insulin secretion, glucose-stimulated insulin release, and proinsulin (PI) and total protein (TOT) biosynthesis

rIL-1β (U/ml)	TLCK (μM)	Medium insulin accumulation ($ng \cdot 75\ islets^{-1}\ 24\ h^{-1}$)	Islet insulin release ($ng \cdot 10\ islets^{-1} \cdot 60\ min^{-1}$)		PI (dpm × 10^3/10 islets)	TOT (dpm × 10^3/10 islets)	PI/TOT × 100 (%)
			1.7 mM glucose	16.7 mM glucose			
0	0	4070 ± 707	4.6 ± 0.4	33.6 ± 3.9	15.3 ± 3.7	49.7 ± 15.5	33.4 ± 3.0
25	0	984 ± 260*	2.5 ± 0.3†	19.3 ± 2.1†	7.9 ± 2.8‡	38.0 ± 11.0	17.3 ± 2.5†
0	100	5400 ± 909	5.4 ± 0.4	59.6 ± 12.8	14.1 ± 3.4	46.9 ± 11.8	30.4 ± 2.6
25	100	3140 ± 511‡	3.9 ± 0.6†	46.5 ± 8.2	12.5 ± 3.1	53.0 ± 14.6	25.1 ± 2.7

Values are means ± SE for 7 experiments. Rat pancreatic islets in groups of 75 were cultured for 24 h in 5 ml of RPMI-1640 + 10% calf serum with or without addition of rIL-1β or TLCK as noted, and a sample was subsequently taken for measurements of medium insulin accumulation. Islet insulin release to glucose was determined in batch-type incubations for the 1st h in presence of 1.7 mM glucose and during the 2nd h in presence of 16.7 mM glucose. Biosynthesis experiments were performed as described in Table 1. During these incubations, rIL-1β and TLCK were not present.
*$P < 0.001$, †$P < 0.01$, ‡$P < 0.05$, vs. islets not cultured with rIL-1β via Student's paired *t* test.

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TABLE 4
Effects of 24-h exposure to human recombinant interleukin 1β (rIL-1β) in absence (no addition) or presence of different enzyme inhibitors on glucose oxidation rates (pmol · 10 islets^{-1} · 90 min^{-1}) in rat pancreatic islets

Inhibitor	No rIL-1β (% of no addition)	n	25 U/ml rIL-1β (% of corresponding control)	n
No addition	100		32.0 ± 2.5	24
Nα-p-tosyl-L-arginine methyl ester				
100 μM	114 ± 18.4	7	32.9 ± 8.2	7
1 mM	85.9 ± 13.5	5	59.2 ± 11.3	5
N-tosyl-L-phenylalanine chloromethyl ketone (100 μM)	76.2 ± 9.6*	7	61.7 ± 7.0	7
Trasylol (35 μg/ml)	97.9 ± 9.6	6	45.2 ± 7.0	6
Benzamidine (100 μM)	75.0 ± 18.1	5	39.6 ± 10.9	5
Leupeptin (50 μM)	91.5 ± 16.4	5	34.4 ± 5.3	5
Epoxysuccinyl-L-leucylamido-(4-guanidino) butane (50 μM)	85.8 ± 16.8	6	48.3 ± 9.1	6
Iodoacetic acid (20 μM)	56.7 ± 11.7*	5	31.5 ± 3.3	5
Chloromercuriphenylsulfonic acid (10 μM)	88.7 ± 7.7	5	44.5 ± 4.2	5
Phenanthroline (10 μM)	72.3 ± 13.0	4	20.8 ± 2.4	4
Pepstatin A (50 μM)	118 ± 8.7	3	41.7 ± 20.3	3

Values are means ± SE for *n* experiments. Islet glucose oxidation was measured at 16.7 mM glucose as described in Table 2 after 24-h exposure to rIL-1β in absence or presence of different inhibitors. The no rIL-1β column shows effect of inhibitors themselves (without rIL-1β) expressed as percentage of glucose oxidation rate with no addition of inhibitor. In absence of inhibitors and cytokine, islet glucose oxidation rate was 797.0 ± 55.6 pmol · 10 islets^{-1} · 90 min^{-1} ($n = 24$). The 25 U/ml rIL-1β column gives oxidation rates with rIL-1β + inhibitor as percentage of corresponding inhibitor only.
*$P < 0.05$ vs. no addition of inhibitor via Student's paired *t* test.

thermore, the chloromethyl ketone groups of TLCK and TPCK are known to react nonspecifically with thiol groups (16).

The findings that TLCK could prevent both the early stimulatory and the late inhibitory effects of rIL-1β suggest that both of these are regulated via the same pathway and that TLCK interferes at an early stage in the cytokine-induced chain of events. Interestingly, Hughes et al. (21) recently reported that, 17 h after a 1-h exposure of islets to rIL-1β, inhibition of β-cell function could still be detected. This was interpreted to indicate that the main mode of action of rIL-1β is by altering β-cell gene expression. If this is the case, a TLCK-sensitive protease may function as the second messenger linking IL-1–receptor activation to an altered gene expression (8). Alternatively, an altered gene expression may lead to de novo transcription of a protease-encoding gene, leading to the rIL-1β–induced stimulatory and inhibitory effects. Finally, it cannot be excluded that the protease preexists and that rIL-1β induces some other protein on which the TLCK-sensitive protease acts.

The reason for the strong potentiation of the glucose-stimulated insulin secretion exerted by TLCK is unclear. However, TLCK could react nonspecifically with thiol groups in the plasma membrane and thereby stimulate insulin release, an effect similar to that induced by low concentrations of iodoacetic acid (22). Nevertheless, we did not observe any inhibitory or cytotoxic effects of TLCK on isolated islets when studied during 24- to 48-h incubations.

In conclusion, this study demonstrates that the active site–directed reagent TLCK markedly counteracts both the stimulatory and inhibitory effects of rIL-1β in pancreatic islets, indicating the involvement of a serine protease. This finding may prove valuable in the elucidation of the precise mechanisms by which rIL-1β acts in β-cells.

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