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Investigation into the Presence of Insulin-degrading Enzyme in Cultured Type II Alveolar Cells and the Effects of Enzyme Inhibitors on Pulmonary Bioavailability of Insulin in Rats

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Abstract

The purpose of this study was to investigate the role of insulin-degrading enzyme (IDE, EC 3.4.22.11) in insulin degradation in alveolar epithelium. The primary culture of isolated rat type-II pneumocytes was used for the in-vitro characterization of IDE. Insulin was then administered intratracheally with various inhibitors to assess the improvement in its pulmonary bioavailability.

In cultured type-II pneumocytes, the cytosolic insulin-degrading activity contributed 81% of total insulin degradation, reached a maximum at pH 7·5 and had an apparent Michaelis–Menten constant (K_m) of 135 nM. *N*-Ethylmaleimide, *p*-chloromercuribenzoic acid and 1,10-phenanthroline inhibited insulin-degrading activity almost completely in both crude homogenate and cytosol. An immunoprecipitation study showed that IDE contributed 74% of cytosolic insulin-degrading activity. Western blot analysis showing a single band of 110 kDa on reduced SDS (sodium dodecylsulphate) gels confirmed the presence of IDE in cultured type-II cells. When given intratracheally with insulin, inhibitors including *N*-ethylmaleimide, *p*-chloromercuribenzoic acid, and 1,10-phenanthroline significantly enhanced the absolute bioavailability of insulin and the compound's hypoglycaemic effects.

These results suggest that IDE is present in alveolar epithelium and might be involved in limiting insulin absorption in the lung.

Recently, pulmonary delivery of insulin has attracted much attention (O'Hagan & Illum 1990; Patton & Platz 1992). The lung provides a number of advantages for effective absorption, for example a very thin epithelial barrier and a large surface area well supplied by extensive blood circulation. Some success has been achieved in that absorption and pharmacological availability of insulin are higher than by other routes (Wigley et al 1971; Elliot et al 1987; Okumura et al 1992). However, insulin degradation was considerable when incubated with cultured rat alveolar epithelial cells (Yamahara et al 1993). Therefore, to improve pulmonary insulin absorption it is important to understand which enzyme is responsible for insulin degradation in the lung.

Insulin-degrading enzyme (IDE) (EC 3.4.22.11) has been implicated in intracellular degradation of insulin (Hamel et al 1987; Duckworth 1988). IDE accounts for most of the insulin-degrading activity in extracts of muscle, liver, kidney, fat cells, erythrocytes, fibroblasts, placenta and pancreas (Stentz et al 1985; Shii & Roth 1986; Duckworth 1990) with activity mostly in the cytoplasm (Duckworth et al 1972). IDE is inhibited by metal chelators (ethylenediaminetetraacetic acid, ethyleneglycol-bis-(β-aminoethyl ether), *N,N'*-tetraacetic acid and 1,10-phenanthroline) and sulphhydryl inhibitors (*N*-ethylmaleimide and *p*-chloromercuribenzoic acid) (Duckworth 1990). It has a pH optimum between 7·0 and 8·0 and when purified has a Michaelis-Menten constant (K_m) of 20 to 40 nM. The molecular weight of IDE is 110 000 on reducing sodium dodecylsulphate gel (Duckworth 1988). cDNA encoding IDE has been used to probe

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IDE transcripts in various tissues; the level in the lung was found to be low compared with that in other tissues (Kuo et al 1993). However, whether IDE is present in alveolar epithelium and involved in alveolar insulin degradation is unknown.

Alveolar epithelium, which consists of type-I and type-II pneumocytes with most of the surface area covered by type-I cells, separates air space from blood circulation. It is the first possible barrier drugs will encounter after pulmonary administration. Type-II pneumocytes proliferate and develop into type-I cells which cover over 90% of the alveolar surface (Weibel 1985). Type-II pneumocytes have type-I-like morphology after 5- to 7-day in-vitro culture (Cheek et al 1989), and have been used by others as a model to study pulmonary drug transport (Morimoto et al 1993; Yamahara et al 1994). The morphological change and the features of type-I cell phenotypes in cultured type-II pneumocytes have been demonstrated using type-I cell-specific monoclonal antibodies (Danto et al 1992). Therefore, cultured type-II cells serve as a suitable in-vitro model to study whether IDE is present in alveolar epithelium.

Materials and Methods

Materials

N-2-Hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), glucose, $CaCl_2$, ethylene glycol-bis(*β*-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), elastase, IgG, foetal bovine serum, DNase I, 1,10-phenanthroline, *N*-ethylmaleimide, ethylenediamine *N,N,N',N'*-tetraacetic acid (EDTA), *p*-chloromercuribenzoic acid, chymostatin, soybean trypsin inhibitor, leupeptin, aprotinin, diisopropylphosphofluorinate (DiPF), trichloroacetic acid, protein G, sepharose beads, Dulbecco's Modified Eagle's Medium (DMEM) and chemicals for Papanicolaou stain, including Harris's haematoxylin solution, lithium carbonate, ethanol, xylene and Mountant, were purchased from Sigma (St Louis, MO). Human recombinant insulin was kindly provided by Eli-Lilly (Indianapolis, IN). Human [^{125}I]insulin was purchased from Amersham (Arlington Heights, IL). Mouse monoclonal antibody to rat IDE was a gift from Dr Richard A. Roth (Stanford University Medical Center, Stanford, CA). Protein-assay kits and gel reagents were obtained from Bio-Rad (Richmond, CA). Coat-A-Count insulin radioimmunoassay kits were obtained from Diagnostics Products (Los Angeles, CA). Chemistrip bG and AccuChek II blood glucose monitor were purchased from Boehringer Mannheim Diagnostics (Indianapolis, IN).

Isolation and culture of type-II pneumocytes

Rat alveolar epithelial cells were isolated according to the methods of Kikkawa & Yoneda (1974) and Dobbs et al (1986) with some modification. Male Sprague–Dawley rats, 150–200 g, were anaesthetized by intraperitoneal injection of pentobarbital and heparin. The lung was then perfused via the pulmonary artery with solution I (140 mM NaCl, 5 nM KCl, 2·5 mM sodium phosphate buffer, 10 mM HEPES, and 6 mM glucose) until white in appearance. The trachea was cannulated and the lung was lavaged with 4–5 times the total lung capacity of iced EGTA solution to remove macrophages and then twice with solution II (containing the same components as solution I plus 10 mM $CaCl_2$ and 1·3 mM $MgSO_4$). Elastase solution (8·6 units mL^{-1}; 5 mL) was placed into the lung and distended by gravity for another 35 mL. After 20-min incubation at 37°C, the trachea and large airways were dissected and discarded. The lung was removed from the chest and minced to a final size of 1 mm^3. DNAase I solution (10 $\mu g\,mL^{-1}$, 4 mL) and foetal bovine serum (4·5 mL) were added and the tissue suspension was stirred at 37°C for 5 min. The resulting suspension was filtered sequentially through 4-layer cotton gauze and 150- and 15-μM Nitex mesh filters, and centrifuged for 10 min at 4°C. To obtain purified type-II cells macrophages and leukocytes were removed by panning the crude cell suspension on tissue-culture plates coated with immunoglobulin G (IgG) for 1 h at 37°C in an incubator with 5% CO_2. After incubation, the plates were tipped back and forth three times and non-adherent cells were dislodged to a tissue-culture dish for another 1 h adherence at room temperature to eliminate fibroblasts. Finally non-attached cells were pooled and centrifuged to obtain purified type-II cells. After IgG adherence, 1×10^7 cells per rat lung were obtained. Modified Papanicolaou stain which identifies type-II cell inclusion bodies was used to determine purity (Kikkawa & Yoneda 1974). The type-II cell purity was improved from 26·8 ± 6·0% to 86·5 ± 1·4% by IgG adherence.

After adherence, isolated type-II cell preparation was seeded with DMEM (10^6 cells mL^{-1}), containing 10% foetal bovine serum, penicillin (60 mg L^{-1}) and streptomycin (100 mg L^{-1}), plated at a density of 2×10^5 cells cm^{-2} and maintained at 37°C in a humidified incubator with 5% CO_2. Cell cultures were routinely monitored by phase-contrast microscopy. Over 1–7 days in culture, type-II cells underwent extensive morphological changes. After 24 h most of the attached cells were flattened and dispersed in a monolayer. By 2–3 days the cells were further flattened and confluent. After 5–7

days, cells became large and irregular. These cultured type-II cells were then detached and homogenized for in-vitro studies.

Studies of insulin-degrading activity
Cultured type-II cells were homogenized manually with a glass-Teflon homogenizer. Centrifugation was performed at 100 000 *g* and 4°C for 1 h to obtain the cytosol. Insulin-degrading activity was determined by the trichloroacetic acid method (Bai & Hsu 1995). [^{125}I] [A14]-insulin was incubated with homogenates or cell cytosol at 37°C in the presence or absence of the agents listed in Table 1. In studies of the pH-dependence of insulin-degrading activity acetate buffer (pH 4·5, 5·5) phosphate buffer (pH 5·5, 6·5, 7·5) and Tris buffer (pH 7·5, 8·5) were used. Each sample was assayed in triplicate and tissue protein concentrations were appropriately diluted to maintain the amount of insulin degradation below 10–15%. Incubation was maintained at 37°C for different times (depending on the insulin-degrading velocity), triplicate samples were taken and mixed with trichloroacetic acid solution (final concentration 10%), and then centrifuged at 10 000 *g*. The appearance of radioactivity soluble in trichloroacetic acid solution was taken as a measure of initial insulin degradation and was expressed as pmol min^{-1} (g protein)$^{-1}$. The kinetics of insulin degradation were studied by adding a trace amount of [^{125}I] [A14]-insulin to insulin at various concentrations (1, 25, 100, 300, 500, 1000 nM). The Michaelis–Menten constant (K_m) was calculated with the Sigma plot program using non-linear fitting to the Michaelis–Menten equation.

Immunoprecipitation and Western blot analysis
Immunoprecipitation and Western blot analysis were performed as described elsewhere (Bai & Hsu 1995). For immunoprecipitation the ratio of the amounts of cytosolic protein to antibody was 1 : 1. Non-specific mouse immunoglobulin served as a negative control.

In-vivo intratracheal administration of insulin
Male Sprague–Dawley rats, 250–350 g, were fasted for 16–20 h before the experiment. The animals were anaesthetized and, after exposing the trachea through a longitudinal incision along the ventral line of the neck, the trachea was cut transversely, halfway through, between the fourth and fifth tracheal rings. Body temperature was maintained by an overhead light. Before intratracheal instillation, the rat was placed in a head-up position at an angle of 60°. Insulin solution (0·1 mL approx.) with or without inhibitors was administered into the lung through PE-50 tubing inserted through the trachea incision to a depth of 2 cm. At this distance, the tip of the tubing was located 1–2 mm above the bifurcation of the trachea (Schanker & Less 1977). The solution was discharged over a period of 1–2 s and the tubing was then withdrawn completely. Blood samples (0·23 mL) were withdrawn through the cannulated femoral vein at predetermined times and then replaced with equal volume of warm (37°C) 0·9% saline solution. Blood glucose level was determined immediately using Chemistrip bG and an AccuChek II blood-glucose monitor. Plasma insulin levels were quantitated using Coat-A-Count insulin radioimmunoassay kits with an assay sensitivity of 1 μunit mL^{-1}.

Data analysis
The endogenous insulin level in blood was determined before insulin administration. The area under the plot of plasma insulin concentration against time (AUC) was calculated by the trapezoidal method, after taking into account the endogenous insulin level. The absolute bioavailability (F) and clearance (CL) were calculated according to non-compartmental analysis (Gibaldi & Perrier 1982). The area above the blood glucose curve and under the baseline at time 0 (AAC) was calculated using the trapezoidal method. The pharmacological availability (f) was calculated by equation 1.

$$f = (AAC_{i.t.}/AAC_{i.v.})/(Dose_{i.v.}/dose_{i.t.}) \quad (1)$$

The paired, two-tailed Student's *t*-test was used to determine statistical significance.

Results and Discussion

Characteristics of insulin-degrading activity in cultured type-II cells
Akiyama et al (1990) reported that insulin-degrading activity in the liver, kidney, brain and muscle was 870, 760, 350, and 110 (pmol min^{-1} (g protein)$^{-1}$), respectively. Insulin-degrading activity in the crude homogenate of cultured type-II cells was 34·7 pmol min^{-1} (g protein)$^{-1}$, approximately one order of magnitude lower than the values reported above. Considering the specific activity per unit amount of protein and the total amount of protein in each subcellular fraction, the activity in cytosol contributed 80·9% of total degradation. This result is in accord with the observations that the majority of cellular IDE is cytoplasmic (Duckworth 1988). Figure 1 shows the pH-dependence of cytosolic insulin-degrading activity with a maximum activity at 7·5, in agreement with reports that IDE is a neutral protease (Duckworth 1988). Besides IDE, proteasome, a

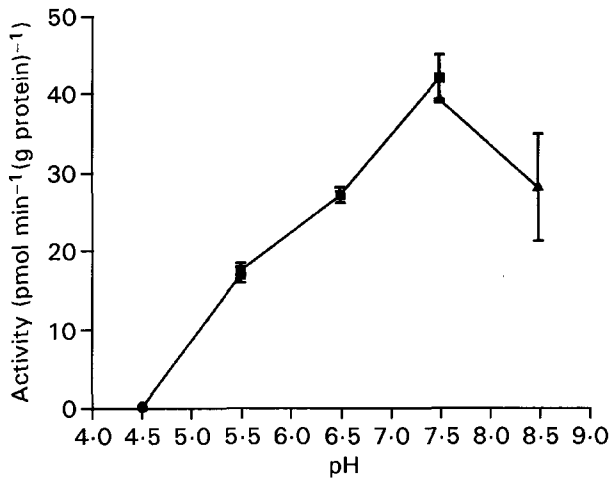

Figure 1. The pH-dependence of insulin-degrading activity in the cytosolic fraction of cultured type-II cells. Results are mean ± s.e.m., n = 3; ● acetate buffer; ■ phosphate buffer; ▲ Tris buffer.

high molecular-weight non-lysosomal protease, has recently been reported to have trypsin-, chymotrypsin- and cucumsin-like activity and it is therefore possible that it is involved in insulin metabolism (Rivett 1989; Orlowski 1990). Therefore, inhibitors of these two enzymes were tested. Table 1 summarizes the effects of various inhibitors on insulin-degrading activity in crude homogenate and cytosol of cultured type-II cells. *N*-Ethylmaleimide, *p*-chloromercuribenzoic acid and 1,10-phenanthroline inhibited insulin-degrading activity almost completely in both crude homogenate and cytosol whereas EDTA inhibited 52% of cytosolic insulin-degrading activity. These data agree with the reported characteristics of IDE as being those of a thiol metalloprotease (Duckworth 1990). In contrast, aprotinin and soybean trypsin inhibitor, which inhibit trypsin-like activity, resulted in little inhibition. Leupeptin and chymostatin which block chymotrypsin-like activity had no effect on insulin degradation. Because proteasome was not inhibited by metal chelators but was inhibited by leupeptin and chymostatin (Rivett 1989), the results suggest that involvement of proteasome is unlikely.

The apparent Michaelis–Menten constant (K_m) for alveolar epithelial cell cytosol was 135 nM (Figure 2), larger than the 20–40 nM for purified IDE but comparable with the value for non-purified IDE (Duckworth 1990). The K_m value reported for crude tissues was 290 mM for mouse pancreatic acini (Goldfine et al 1984), 140 mM for cultured rat hepatoma cells (Harada et al 1993) and 150 mM for rat islet-derived tunour-cell lines (Bhathena et al 1985). On the other hand, the K_m value of glutathione insulin transhydrogenase, which splits insulin into two chains by disulphide bond reduction, is 5–60 μM (Duckworth 1988), much higher than that of IDE. Proteasome has an even larger K_m, approximately 0·1–2 mM (Brange & Langkjar 1993). Therefore, on the basis of the kinetic study IDE seems to be the enzyme dominating intracellular insulin degradation. The immunoprecipitation study showed that 26% insulin-degrading activity remained in the cytosol after treatment with the mouse monoclonal anti-IDE antibody, compared with the control treated with non-specific mouse immunoglobulin. This result demonstrates that IDE is responsible for most insulin degradation in alveolar epithelial cell cytosol, even though other enzymes might play a small part. Western blot analysis, showing a single band of 110 kDa on reducing SDS (sodium dodecylsulphate) gels (Figure 3), confirmed that insulin protease in rat type-II pneumocytes is IDE.

Effect of inhibitors on intratracheal administration of insulin

Though the mechanism and pathway of insulin absorption across the alveolar membrane is not well characterized, studies have found that IDE is also

Table 1. Effects of different inhibitors on insulin-degrading activity in cultured type-II cells.

Inhibitor	Activity remaining (%)	
	Cytosol	Crude homogenate
None	100	100
N-Ethylmaleimide (2 mM)	2·6 ± 1·4	3·0 ± 1·5
p-Chloromercuribenzoic acid (0·2 mM)	0	2·3 ± 0·5
Ethylenediamine tetraacetic acid (5 mM)	48·3 ± 5·4	42·3 ± 5·1
1,10-Phenanthroline (2 mM)	7·5 ± 1·3	5·1 ± 1·1
Aprotinin (0·5 mg mL^{-1})	71·7 ± 3·5	NT
Trypsin (0·1 mg mL^{-1})	81·3 ± 5·5	NT
Chymostatin (0·07 mg mL^{-1})	95·2 ± 5·4	NT
Diisopropylphosphofluoridate (1 mM)	83·1 ± 1·5	NT
Leupeptin (0·1 mg mL^{-1})	93·5 ± 13·4	NT

Data are means ± s.e.m., n = 3. NT = not tested..

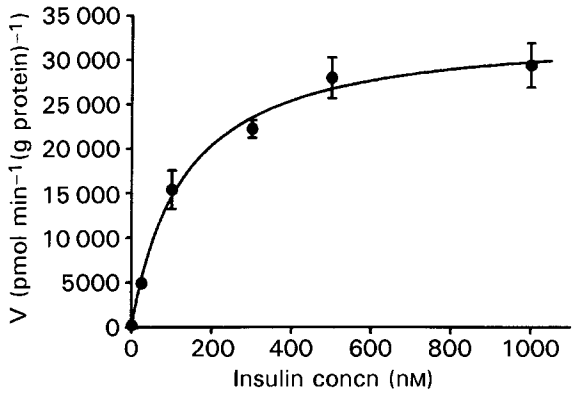

Figure 2. Determination of the Michaelis–Menten constant (K_m) for enzymatic degradation of insulin in the cytosol of cultured type-II cells. Results are mean ± s.e.m., n = 3.

present on the cell membrane (Duckworth 1988; Chang & Bai 1996). Therefore, irrespective of the absorption pathway insulin molecules have chances of encountering IDE either on the cell membrane or in the cytosol during the course of absorption toward the alveolar membrane and across the membrane. Because of this in-vitro evidence the effects of inhibitors on the pulmonary bioavailability of insulin were further tested.

Plasma insulin concentration–time profiles after intravenous administration (0·2 units kg^{-1}) and intratracheal instillation (3 units kg^{-1}) of *N*-ethylmaleimide are shown in Figure 4. *N*-Ethylmaleimide at 0·5 and 1 mM enhanced insulin absorption dramatically within 15 min (inset Figure 4). The insulin concentration reached a maximum (C_{max}) after 0·5 min (T_{max}) and remained high, about 100 μunits mL^{-1} above the normal level, for 90 min in the presence of 1 mM *N*-ethylmaleimide, whereas 0·5 mM *N*-ethylmaleimide had a smaller effect with a maximum concentration after 1·5 min. Because there is no information about the absorption and distribution of *N*-ethylmaleimide in the body, the effect of *N*-ethylmaleimide alone after intratracheal administration was examined; no effects were observed on either plasma insulin concentrations or blood glucose levels (data not shown). The effect of *N*-ethylmaleimide on improved insulin absorption is, therefore, not systemic but local. Table 2 summarizes the effects of enzyme inhibitors on the absolute bioavailability of insulin after intratracheal administration. Absolute bioavailability increased more than fourfold and the C_{max} increased twofold when the *N*-ethylmaleimide concentration was doubled (from 0·5 to 1 mM); 1,10-phenanthroline and *p*-chloromercuribenzoic acid resulted in weaker but significant enhancement of insulin absorption. Figure 5 shows the hypoglycaemic effects after intratracheal instillation of

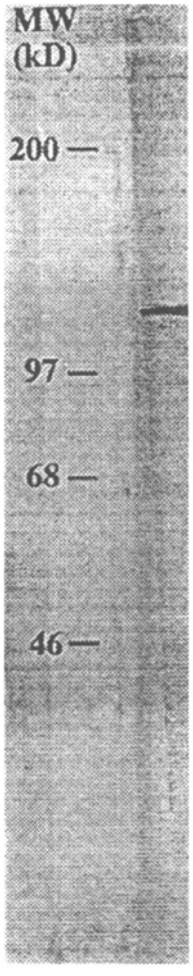

Figure 3. Western blot analysis using type-II cell cytosol and monoclonal anti-IDE antibody. Molecular weight markers are ovalbumin (46 kD), bovine albumin (68 kD), phosphorylase (97 kD) and myosin (200 kD).

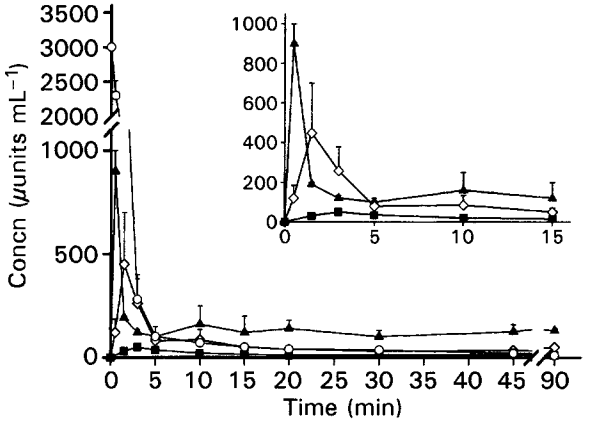

Figure 4. Plasma insulin concentration profiles after intravenous and intratracheal administration of insulin with and without *N*-ethylmaleimide. ○ Intravenous insulin (0·2 units kg^{-1}); ■ intratracheal insulin (3 units kg^{-1}); ▲ intratracheal insulin (3 units kg^{-1}) + *N*-ethylmaleimide (1 mM); ◇ intratracheal insulin (3 units kg^{-1}) + *N*-ethylmaleimide (0·5 mM). Inset: profiles during the first 15 min after intratracheal administration. Results are means ± s.e.m., n = 3.

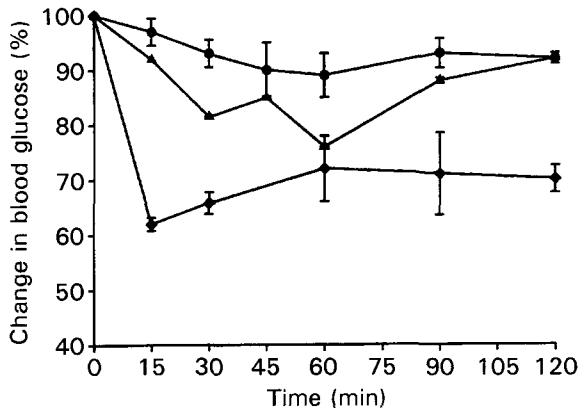

Figure 5. Effect of *N*-ethylmaleimide on blood glucose levels after administration of insulin: ◆ intravenous insulin (0·2 units kg^{-1}); ● intratracheal insulin (3 units kg^{-1}); ▲ intratracheal insulin (3 units kg^{-1}) + 0·5 mM *N*-ethylmaleimide). Results are means ± s.e.m., n = 3.

insulin with *N*-ethylmaleimide. Table 3 summarizes the effects of various inhibitors on the hypoglycaemic response of insulin. The pharmacological availability was also increased significantly by *N*-ethylmaleimide, 1,10-phenanthroline and *p*-chloromercuribenzoic acid. Although these inhibitors are not specific IDE inhibitors, the results seem consistent with in-vitro enzyme characteristics.

Insulin exists as a monomer at concentrations lower than 0·1 μM but forms dimers and large aggregates at higher concentrations (Brange & Langkjar 1993). Therefore, the effect of *N*-ethylmaleimide at an insulin concentration of 0·1 μM was also tested, the same dose of insulin (0·0045 units kg^{-1}) being used for intravenous and intratracheal administration (Table 4). In the control intratracheal administration of *N*-ethylmaleimide was followed immediately by intravenous administration of insulin; *N*-ethylmaleimide had no significant effect on intravenous absorption of insulin. Importantly, at 0·0045 units kg^{-1} with 1 mM *N*-ethylmaleimide the absolute bioavailability was > 80%. Because the clearance of insulin did not change significantly within the range 0·0045–0·2 units kg^{-1} (40 ± 15 compared with 22 ± 1 mL min^{-1}), the increase in the bioavailability of insulin reflects a significant possibility that *N*-ethylmaleimide enhanced insulin absorption in the lung but not systemically. The bioavailability increased more than threefold as the insulin dose was reduced from 3 to 0·1 units kg^{-1} (Table 2). As reported in a previous extensive study (Bai & Chang 1996), *N*-ethylmaleimide had no detectable damaging effect on the integrity of the intestinal epithelium. Moreover, during the course of

Table 2. The absolute bioavailability of insulin after intratracheal administration under various conditions.

Conditions	Maximum concentration (μunits mL^{-1})	Time of maximum concentration (min)	AUC (min μunits mL^{-1})	Absolute bioavailability (%)
Insulin (0·2 units kg^{-1}, i.v.)			9180 ± 170	100
Insulin (3 units kg^{-1}, i.t.)	55 ± 8	3	590 ± 160	0·4 ± 0·1
+*N*-ethylmaleimide (1 nM)	902 ± 80	0·5	13600 ± 1800	9·9 ± 1·3*
+*N*-ethylmaleimide (0·5 mM)	456 ± 204	1·5	3060 ± 700	2·2 ± 0·5*
+chymostatin (70 μg mL^{-1})	53 ± 0	3	230 ± 70	0·2 ± 0·1**
+1,10-phenanthroline (2 mM)	83 ± 20	3	2610 ± 420	1·9 ± 0·3*
+*p*-chloromercuribenzoic acid (0·2 mM)	82 ± 13	1·5	3190 ± 390	2·3 ± 0·3*
Insulin (0·1 units kg^{-1} i.t.) + *N*-ethylmaleimide (1 nM)	78 ± 9	1·5	1580 ± 330	34·4 ± 7·1*

AUC = area under the plasma insulin concentration–time curve. Data are means ± s.e.m. (n = 3). * $P < 0·05$ significantly different from result for insulin (3 units kg^{-1}, i.t.). ** $P > 0·1$.

Table 3. Effects of various inhibitors on the hypoglycaemic response of insulin.

Conditions	AAC (min μunits mL^{-1})	Pharmacological availability (%)
Insulin (0·2 units kg^{-1}, i.v.)	3500 ± 670	100
Insulin (3 units kg^{-1}, i.t.)	850 ± 250	1·6 ± 0·5
+*N*-ethylmaleimide (0·5 mM)	1650 ± 190	3·1 ± 0·4*
+1,10-phenanthroline (2 mM)	2250 ± 710	4·3 ± 1·3*
+*p*-chloromercuribenzoic acid (0·2 mM)	2850 ± 1060	5·4 ± 2·0*

AAC = area above the blood glucose curve and under the baseline at time 0. Data are means ± s.e.m., n = 3. * $P < 0·05$ significantly different from result for insulin (3 units kg^{-1}, i.t.).

Table 4. Effects of *N*-ethylmaleimide on insulin absorption after intravenous and intratracheal administration.

Conditions	AUC (min μunits mL^{-1})	Absolute bioavailability (%)
Insulin (0·0045 units kg^{-1}, i.v.)	185 ± 98	-
Insulin (0·0045 units kg^{-1}, i.v.) + *N*-ethylmaleimide (1 mM, i.t.)	170 ± 45*	92 ± 24
Insulin (0·0045 units kg^{-1}, i.t.)	0	0
Insulin (0·0045 units kg^{-1}, i.t.) + *N*-ethylmaleimide (1 mM, i.t.)	151 ± 24	82 ± 12

AUC = area under the plasma insulin concentration–time curve. Data are means ± s.e.m., n = 3. * $P > 0.05$ significantly different from result for insulin (0·0045 units kg^{-1}, i.v.).

experiments rats in control and experimental groups showed no difference in breathing. It is therefore concluded that *N*-ethylmaleimide enhances insulin absorption by inhibiting enzyme activity. Furthermore, at high insulin concentrations pulmonary absorption is reduced, as reported previously (Liu et al 1993).

In conclusion, the biochemical characteristics of insulin-degrading activity in cultured type-II pneumocytes, including subcellular distribution, optimum pH and proteolytic kinetics, resembled those of IDE. Importantly, immuno-precipitation and Western blot analysis confirmed that IDE is present in cultured type-II pneumocytes and contributes the majority of total insulin-degrading activity. With the mutual support of in-vivo and in-vitro results this study provides evidence suggesting that IDE might be involved in reducing pulmonary insulin absorption.

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