Further evidence for the involvement of a membrane proteolytic step in insulin action

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The hypothesis that insulin action involves a membrane proteolytic step was further explored, by using isolated rat adipocytes and liver plasma membranes. (1) The maximal insulin stimulation of 2-deoxyglucose transport and lipogenesis in fat-cells was selectively inhibited (73–88%) by N^α -*p*-tosyl-L-lysine chloromethyl ketone (Tos-Lys-CH₂Cl; active-site inhibitor of trypsin; 30–125 μ M), *p*-nitrophenyl *p*'-guanidinobenzoate (active-site inhibitor of serine proteinases; 30–125 μ M) and *p*-tosyl-L-arginine methyl ester (arginine ester substrate analogue of proteinases; 1–2 mM), under conditions where neither the basal rate of each metabolic process nor insulin binding nor cellular ATP content were affected. In contrast, *N*-acetyl-L-alanyl-L-alanyl-L-alanine methyl ester (alanine ester substrate analogue of proteinases; 1–2 mM) was ineffective. (2) Endoproteinase Arg-C (0.25–40 μ g/ml) exerted dose-dependent insulin-like effects on both 2-deoxyglucose transport and lipogenesis in fat-cells, whereas endoproteinase Lys-C (5–100 μ g/ml) was ineffective. The maximal activation by endoproteinase Arg-C of both processes (200 and 177% of control values respectively) was shown to occur under conditions where membrane integrity (assessed by measurement of lactate dehydrogenase leakage and passive glucose diffusion) was preserved. This effect was inhibited by Tos-Lys-CH₂Cl (125 μ M) and was not additive with the maximal insulin effect. (3) Insulin (1–100 ng/ml) produced a dose-dependent increase in the trichloroacetic acid-soluble ¹²⁵I radioactivity released after a 30 min incubation at 37°C of ¹²⁵I-labelled liver plasma membranes, but was ineffective on ¹²⁵I-labelled bovine serum albumin. Insulin effects on both radio-labelled proteins were reproduced by wheat-germ agglutinin (20 μ g/ml), an insulin mimicker shown to act through the insulin receptor. These data provide further evidence for the hypothesis that insulin bioeffects involve the activation of a membrane serine proteinase with arginine specificity.

Insulin binds to specific plasma-membrane receptors in target cells and then initiates a wide spectrum of well-known biological responses (Czech, 1977; Kahn *et al.*, 1981). The mechanism(s), however, whereby insulin elicits these responses is poorly understood (Denton *et al.*,

Abbreviations used: Ac-Ala-Ala-Ala-OMe, *N*-acetyl-L-alanyl-L-alanyl-L-alanine methyl ester; KRP, Krebs-Ringer phosphate (129 mM-NaCl/5 mM-KCl/1.2 mM-KH₂PO₄ / 1.2 mM-MgSO₄ / 1.3 mM-CaCl₂ / 10 mM-Na₂HPO₄); NPGB, *p*-nitrophenyl *p*'-guanidinobenzoate; Tos-Arg-OMe, *p*-tosyl-L-arginine methyl ester; Tos-Lys-CH₂Cl, N^α -*p*-tosyl-L-lysine chloromethyl ketone; WGA, wheat-germ (*Triticum*) agglutinin.

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1981). Several observations support the hypothesis that insulin may be acting via a proteolytic mechanism implying a membrane serine proteinase with arginine specificity (Seals & Czech, 1980). (1) Low concentrations of trypsin produced pronounced insulinomimetic effects in fat-cells, including inhibition of adrenaline-induced lipolysis (Kono & Barham, 1971) and activation of 2-deoxyglucose transport (Clark *et al.*, 1980; Kikuchi *et al.*, 1981), glucose oxidation (Kono & Barham, 1971; Kikuchi *et al.*, 1981; Pilch *et al.*, 1981), glycogen synthase and pyruvate dehydrogenase (Kikuchi *et al.*, 1981). Furthermore, trypsin mimicked insulin's ability to generate mediator(s), whether adipocyte plasma membranes (Seals & Czech, 1980) or intact fat-cells (Kikuchi *et al.*, 1981) were used. (2) Anti-

proteolytic agents and arginine ester substrate analogues of proteinases blocked several insulin bioeffects in rat adipocytes (Larner *et al.*, 1981, 1982; Seals & Czech, 1982), as well as the insulin-dependent generation of mediator from fat-cell plasma membranes (Seals & Czech, 1980). (3) Finally, insulin stimulation of the insulin-receptor phosphorylation, a process believed to be an early event in the hormone's action (Kasuga *et al.*, 1982; Roth *et al.*, 1983), was mimicked by trypsin (Tamura *et al.*, 1983) and selectively inhibited by arginine ester substrate analogues of proteinases (Tamura *et al.*, 1984), in either partially or highly purified insulin-receptor fractions. However, Muchmore *et al.* (1982), using intact fat-cells to examine the antagonizing effects of a series of low-*M_r* inhibitors of proteinases on insulin bioactivity, obtained several data that cast doubt on the relevance of a trypsin-like proteinase to the mechanism of action of the hormone.

In an attempt to provide further experimental arguments for or against the proposed proteolytic mechanism of insulin action: (1) the ability of anti-proteolytic agents to antagonize selectively insulin bioeffects in fat-cells was re-evaluated by examining the concomitant effects of Tos-Lys-CH₂Cl (active-site inhibitor of trypsin), NPGB (active-site inhibitor of serine proteinases) and Tos-Arg-OMe (arginine ester substrate analogue of proteinases) on both basal and insulin-stimulated 2-deoxyglucose transport and lipogenesis as well as on fat-cell ATP content and insulin binding; (2) the ability of endoproteinase Arg-C and endoproteinase Lys-C, two serine proteinases that specifically cleave peptide bonds at the carboxyl end of arginine (Levy *et al.*, 1970; Schenkein *et al.*, 1977) or lysine (Jekel *et al.*, 1983) residues respectively, to mimic the effect of insulin on glucose transport and lipogenesis in rat adipocytes was studied; (3) the ability of insulin or WGA, an insulin mimicker shown to act through the insulin receptor (Cuatrecasas & Tell, 1973; Cherqui *et al.*, 1981, 1982, 1983; Shechter & Sela, 1981), to activate proteolysis was tested with either ¹²⁵I-labelled liver plasma membranes or ¹²⁵I-labelled bovine serum albumin as endogenous and exogenous substrates respectively.

Our data provide additional lines of evidence for the hypothesis that insulin bioeffects involve the activation of a membrane serine proteinase with arginine specificity.

Materials and methods

Materials

Crude collagenase (*Clostridium histolyticum*) (EC 3.4.24.3) type I (131 units/mg, where 1 unit liberates 1 μ mol of L-leucine equivalents from

collagen in 5 h at 37°C) was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. (lot CLS 42K290). Endoproteinase Arg-C from mouse submaxillary glands (250 units/mg of protein, where 1 unit hydrolyses 1 μ mol of Tos-Arg-OMe/min at 25°C and pH 8) (lot 11004505), endoproteinase Lys-C (*Lysobacter enzymogenes*) (12 units/mg of protein, where 1 unit hydrolyses 1 μ mol of Chromozym PL/min at 25°C and pH 7.7) (lot 1443308), and lactoperoxidase from cow milk (EC 1.11.1.7) [200 units/mg, where 1 unit catalyses the oxidation by H₂O₂ of 1 μ mol of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate) diammonium salt/min at 25°C] (lot 1392523) were from Boehringer-Mannheim. The lactate dehydrogenase test was obtained from Labo Express Service, Viry-Chatillon, France. Cytochalasin B, Tos-Arg-OMe, Tos-Lys-CH₂Cl, NPGB, Ac-Ala-Ala-OMe, bovine serum albumin (fraction V, essentially fatty acid-free) (lot 73F-9305) and the ATP-monitoring kit were from Sigma Chemical Co., St. Louis, MO, U.S.A.; dinonyl phthalate (density 0.98 kg/l) and Perhydrol (30%) were from Merck, Darmstadt, Germany; Bio-Gel P-30 was from Bio-Rad, Richmond, CA, U.S.A.; WGA was purchased from Reactifs IBF, Pharmindustrie, Villeneuve-la-Garenne, France; Aprotinin [200 000 peptidase-inhibitor units/ml, where 1 unit inhibits the activity of 1 S.T. (Schwert-Takenaka) unit of trypsin] was from Laboratoires Choay, Paris, France; 2-deoxy-D-[1-¹⁴C]glucose (sp. radioactivity 55 Ci/mol), [3-³H]glucose (sp. radioactivity 11.5 Ci/mmol) and ¹²⁵I-labelled insulin (sp. radioactivity 100 Ci/g) were purchased from New England Nuclear Corp., Boston, MA, U.S.A.; Na¹²⁵I (500–600 mCi/ml) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.; pig monocomponent insulin (25 units/mg, where 1 unit is equivalent to 0.04 mg) was from Novo Research Institute, Copenhagen, Denmark. Insulin was dissolved in 0.3 M-HCl at a concentration of 1 mg/ml and diluted in KRP buffer to the concentrations required in assays. All chemicals were of the highest grade available.

Preparation of isolated adipocytes and liver plasma membranes

Adipocytes were isolated from the epididymal fat-pads of male Wistar rats (150–175 g) by a modification of the method of Rodbell (1964) as previously described (Cherqui *et al.*, 1981). The pooled epididymal fat-cells were suspended in KRP buffer, pH 7.4, 37°C, containing 20 mg of defatted bovine serum albumin/ml. The adipocyte number was determined by counting portions of the cell suspension in a haemocytometer. Fat-cell viability was assessed by the Trypan Blue-exclusion test. Liver plasma membranes were prepared

by the method of Ray (1970). Microsomal contamination was evaluated by the ratio 5'-nucleotidase/glucose-6-phosphatase (>11). These enzyme activities were respectively measured by the methods of de Duve (1967) and Harper & Young (1959). Protein concentration was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. The insulin-binding activity of liver plasma membranes was measured as previously described (Caron *et al.*, 1978).

Endoproteinase digestion

The proteolytic activity of endoproteinase preparations was systematically tested by measuring the trichloroacetic acid-soluble ^{125}I radioactivity released from two appropriate substrates, ^{125}I -labelled insulin (60 nM; with Arg and Lys residues present respectively in positions A22 and A29) and ^{125}I -labelled bovine serum albumin (300 nM; 5.38 and 10.95% of Arg and Lys residues respectively). For routine assays, fat-cells (approx. 10^5 cells) were incubated for 15 min at 37°C in KRP buffer, pH 7.4, containing 5 mg of defatted bovine serum albumin/ml either in the presence or in the absence of graded concentrations of endoproteinases. The reaction was stopped by adding to the incubation medium an excess of aprotinin (200 peptidase-inhibitor units/ml). The leakage of lactate dehydrogenase activity from the cells during incubation with endoproteinases was used as an indicator of the structural integrity of the fat-cell membranes and was determined as the percentage of total lactate dehydrogenase activity recovered in sonicated cells. 2-Deoxyglucose transport and lipogenesis assays were then conducted in both control and endoproteinase-treated fat-cells as reported below.

Insulin-binding studies

Fat-cells (approx. 10^5 cells) were preincubated for 20 min at 37°C in KRP buffer, pH 7.6, containing 10 mg of bovine serum albumin/ml, either in the presence or in the absence of proteinase inhibitors (125 μM) or substrates (1 mM). The binding of ^{125}I -labelled insulin (75 pM) to fat-cells was then measured using the oil-centrifugation technique (Gliemann *et al.*, 1972) as previously described (Cherqui *et al.*, 1981). Non-specific binding (^{125}I -labelled insulin bound in the presence of 40 μg of unlabelled insulin/ml) was subtracted from the total binding to obtain the specifically bound ^{125}I -labelled insulin. Non-specific binding was never more than 15% of the total binding.

2-Deoxyglucose transport

2-Deoxy-D-[1- ^{14}C]glucose transport in rat adipocytes (approx. 10^5 cells) was measured by using the oil-centrifugation technique (Gliemann *et al.*,

1972) as previously described (Cherqui *et al.*, 1982), except that the concentration of defatted bovine serum albumin in KRP buffer was lowered to 20 mg/ml. Non-specific diffusion and trapping in the extracellular space of 2-deoxy-D-[1- ^{14}C]glucose (0.25 mM) were estimated by measuring 2-deoxyglucose uptake in the presence of 50 μM -cytochalasin B, a concentration shown to inhibit completely facilitated glucose transport in fat-cells (Czech *et al.*, 1973). All data have been corrected for the amount of cell-associated radioactivity determined under this condition. In experiments in which the effects of insulin (100 ng/ml), proteinase inhibitors (30–250 μM) or substrates (1–2 mM) were studied, fat-cells were incubated with the agents at 37°C for 20 min either separately or together before 2-deoxyglucose-transport assays were initiated.

Lipogenesis

Lipogenesis was measured by the method of Moody *et al.* (1974) as the incorporation of [3- ^3H]glucose into toluene-extractable lipid. Assays were performed for 2 h at 37°C as previously described (Cherqui *et al.*, 1983), except that KRP buffer, pH 7.4, containing 20 mg of defatted bovine serum albumin/ml was used as incubation buffer. All data were corrected for non-metabolic ^3H labelling. This factor represented the amount of radioactivity that was counted in flasks in which fat-cell incubation was immediately stopped by addition of the toluene scintillation fluid (zero time). In experiments in which the effects of insulin (100 ng/ml), proteinase inhibitors (30–250 μM) or substrates (1–2 mM) were studied, fat-cells were incubated with the agents at 37°C for 20 min either separately or together before lipogenesis assays were initiated.

ATP determination

Fat-cells (approx. 2×10^6 cells) were incubated for 20 min at 37°C in the presence or in the absence of proteinase inhibitors (125 μM) or substrates (1 mM) in a final volume of 3 ml. At the end of incubation, the reaction was stopped by mixing with 1 ml of ice-cold 20% (w/v) trichloroacetic acid (final concn. 5%, w/v). Each sample was then sonicated and centrifuged at 30000g for 15 min at 4°C . After removal of trichloroacetic acid by diethyl ether extraction (2×7 vol.), ATP was determined in each sample with an ATP-monitoring kit. This assay is based on the determination of NAD generated in a phosphoglycerate kinase/glyceraldehyde phosphate dehydrogenase coupled reaction.

^{125}I -labelling of liver plasma membranes and bovine serum albumin

The ^{125}I -labelling of liver plasma membranes was performed by the method of Kull *et al.* (1983).

Liver plasma membranes (1 mg; approx. 3 pmol of insulin-binding activity) were suspended in 5 ml of 50 mM-sodium phosphate buffer, pH 7.5; 100 μ g of lactoperoxidase was added, followed by 2 mCi of Na¹²⁵I. A 20 μ l portion of 1 mM-H₂O₂ was added every 4 min for 12 min. The membranes were then washed three times with 50 mM-phosphate buffer by centrifugation at 50000g for 30 min. ¹²⁵I-labelled liver plasma membranes were kept frozen at -20°C and were used in the 3 days after their iodination. The specific radioactivity of ¹²⁵I-labelled liver plasma membranes, determined in three individual experiments, was $9 \pm 2 \mu$ Ci/mg of protein.

The ¹²⁵I-labelling of bovine serum albumin was conducted under similar conditions, except that the separation of ¹²⁵I-labelled bovine serum albumin from free Na¹²⁵I was achieved by gel filtration (Bio-Gel P-30). The specific radioactivity of ¹²⁵I-labelled bovine serum albumin was 15 μ Ci/mg.

Proteolysis assays

¹²⁵I-labelled liver plasma membranes (1.2×10^5 – 1.5×10^5 c.p.m.) or ¹²⁵I-labelled bovine serum albumin (2.3×10^5 c.p.m.) were incubated for 30 min at 37°C in 0.5 ml of 50 mM-sodium phosphate buffer, pH 7.5, either in the presence or in the absence of insulin (1–100 ng/ml) or WGA (20 μ g/ml). Incubations (performed in duplicate) were stopped by adding to the medium 30 μ l of 20% (w/v) bovine serum albumin solution, followed by 530 μ l of ice-cold 20% (w/v) trichloroacetic acid. After 15 min at 4°C and centrifugation at 5000 rev./min, the trichloroacetic acid-soluble ¹²⁵I radioactivity was counted in two 100 μ l samples of each supernatant. All results were corrected for the trichloroacetic acid-soluble ¹²⁵I radioactivity spontaneously released by control samples of ¹²⁵I-labelled liver plasma membranes or ¹²⁵I-labelled bovine serum albumin (zero time of incubation).

Statistical analysis

Results are given as means $\pm$ S.E.M. for the indicated numbers (*n*) of independently performed experiments. Differences between the mean values were evaluated by Student's *t* test.

Results

Effects of proteinase inhibitors or substrates on basal and insulin-stimulated 2-deoxyglucose transport and lipogenesis

To re-evaluate the ability of anti-proteolytic agents to antagonize the action of insulin in fat-cells (Larner *et al.*, 1982; Muchmore *et al.*, 1982; Seals & Czech, 1982), we first examined the effects of Tos-Lys-CH₂Cl (active-site inhibitor of trypsin),

NPGB (active-site inhibitor of serine proteinases), Tos-Arg-OMe and Ac-Ala-Ala-Ala-OMe (arginine and alanine methyl ester substrate analogues of proteinases) on basal and insulin-stimulated 2-deoxyglucose transport and lipogenesis.

When adipocytes were incubated with Tos-Lys-CH₂Cl or NPGB (30–250 μ M) in the absence of insulin, no alteration, as compared with controls, of either 2-deoxyglucose transport (Table 1) or lipogenesis (Table 2) was observed in the 30–125 μ M concentration range. Both metabolic processes were, however, significantly decreased by 250 μ M of these agents (Tables 1 and 2).

In contrast, when fat-cell incubations with concentrations of Tos-Lys-CH₂Cl or NPGB shown not to alter basal 2-deoxyglucose transport or lipogenesis (30–125 μ M) were carried out in the presence of a supramaximal concentration of insulin (100 ng/ml), a dose-dependent decrease in the insulin stimulation of glucose metabolism was observed (Tables 1 and 2). Maximal inhibitions of 73 or 74% in 2-deoxyglucose transport (Table 1) and 83 or 84% in lipogenesis (Table 2) were observed at 125 μ M-Tos-Lys-CH₂Cl or -NPGB respectively.

The effects of Tos-Arg-OMe and Ac-Ala-Ala-Ala-OMe on basal and insulin-stimulated 2-deoxyglucose transport or lipogenesis are shown in Tables 1 and 2 respectively. In the absence of insulin, neither Tos-Arg-OMe nor Ac-Ala-Ala-Ala-OMe (1–2 mM) appreciably modified glucose metabolism in fat-cells (Tables 1 and 2). However, when fat-cells were incubated in the presence of insulin (100 ng/ml), Tos-Arg-OMe and Ac-Ala-Ala-Ala-OMe (1–2 mM) exerted different effects, whatever the metabolic process considered. The former (1–2 mM) inhibited 88 or 73% of the maximal insulin stimulation of 2-deoxyglucose transport (Table 1) and lipogenesis (Table 2) respectively, whereas the latter remained ineffective (Tables 1 and 2).

Lack of effects of Tos-Lys-CH₂Cl, NPGB and Tos-Arg-OMe on fat-cell ATP content

Since insulin stimulation of glucose metabolism is an energy-dependent process (Chandramouli *et al.*, 1977; Kono *et al.*, 1977; Siegel & Olefsky, 1980; Haring *et al.*, 1981), the possibility was envisaged that the Tos-Lys-CH₂Cl-, NPGB- and Tos-Arg-OMe-induced decreases in the hormonal action might result from a side effect of these agents on fat-cell ATP content (Muchmore *et al.*, 1982) rather than from proteinase inhibition. Thus comparisons were made of ATP concentrations in fat-cells incubated for 20 min at 37°C in the absence or in the presence of the anti-proteolytic agents. Table 3 shows that the treatment of fat-cells with Tos-Lys-CH₂Cl, NPGB or Tos-Arg-

Table 1. *Effect of proteinase inhibitors or substrates on basal and insulin-stimulated 2-deoxyglucose transport in rat adipocytes*

Fat-cells (approx. 10^5 cells) were incubated for 20 min at 37°C without or with 100 ng of insulin/ml either in the absence or in the presence of graded concentrations of proteinase inhibitors or substrates. 2-Deoxy-D-[1- 14 C]glucose was then added (final concn. 0.25 mM) and the uptake was measured at the end of a 2 min incubation period as described in the Materials and methods section. The control values of 2-deoxy-D-[1- 14 C]glucose transported in fat-cells were 82.7 ± 7.4 and 163.0 ± 13.1 pmol/min per 10^5 cells for non-treated and insulin-treated fat-cells respectively. All data were corrected for 2-deoxy-D-[1- 14 C]glucose trapping in the extracellular space and for non-specific diffusion, both determined in the presence of 50 μ M-cytochalasin B. This non-specific uptake was never more than 13% of the total uptake. Values are means $\pm$ S.E.M. for quadruplicate determinations from the indicated numbers of separate experiments (numbers in parentheses). Values significantly different from the control value with no hormone are indicated: ** $P < 0.02$; *** $P < 0.01$. Values significantly different from the control value with insulin are indicated: † $P < 0.05$; †† $P < 0.02$; ††† $P < 0.01$; †††† $P < 0.001$. Abbreviation: n.d., not determined. Maximal insulin stimulation was calculated as follows: $100 \times (163.0 - 82.7)/82.7 = 97\%$ increase above the basal rate. The percentage of the maximal stimulation represents the ratio: (% increase above the basal rate in cells treated simultaneously with insulin and anti-proteolytic agents/% increase above the basal rate in cells treated with insulin alone) $\times 100$.

Additions	2-Deoxy-D-[1- 14 C]glucose transport	
	No hormone (% of control)	Insulin (100 ng/ml) (% of maximal stimulation)
None	100 (28)	100 (13)
Tos-Lys-CH ₂ Cl (μ M)		
30	101 ± 7 (3)	65 ± 15 (3)
60	93 ± 8 (3)	38 ± 10 † (3)
125	91 ± 14 (3)	27 ± 8 ††† (5)
250	43 ± 8 *** (4)	n.d.
NPGb (μ M)		
30	92 ± 5 (3)	66 ± 12 (3)
60	89 ± 7 (3)	31 ± 19 † (4)
125	83 ± 11 (3)	26 ± 14 †† (4)
250	58 ± 5 ** (3)	n.d.
Tos-Arg-OMe (mM)		
1	115 ± 9 (3)	12 ± 6 †††† (5)
2	100 ± 11 (3)	13 ± 7 ††† (4)
Ac-Ala-Ala-Ala-OMe (mM)		
1	95 ± 8 (3)	94 ± 6 (3)
2	97 ± 9 (3)	90 ± 13 (3)

OMe, at concentrations shown to decrease maximally the insulin stimulation of glucose metabolism (125 μ M or 1 mM respectively), did not appreciably modify cellular ATP content, making the hypothesis of ATP depletion in anti-proteinase-treated fat-cells highly unlikely.

Lack of effects of Tos-Lys-CH₂Cl, NPGb and Tos-Arg-OMe on insulin binding to fat-cells

To rule out the possibility that these three agents modified the insulin-receptor interaction, their effects on insulin binding were examined. In these experiments, fat-cells were preincubated under the same conditions (20 min at 37°C) as those described for studies of 2-deoxyglucose transport and lipogenesis. Binding assays were then conducted at 22°C as indicated in the Materials and methods section. It was observed that, after fat-cells were treated with concentrations of Tos-Lys-CH₂Cl (125 μ M), NPGb (125 μ M) or Tos-Arg-OMe (1 mM) shown to decrease maximally insulin action

(Tables 1 and 2), the binding of 125 I-labelled insulin (75 pM) was not significantly altered [88.7 ± 11.9 ($n = 8$), 100.0 ± 3.0 ($n = 3$), and 89.0 ± 15.1 ($n = 6$) % of the control value respectively].

Effect of endoproteinase Arg-C and endoproteinase Lys-C on 2-deoxyglucose transport and lipogenesis

To characterize further the amino acid specificity involved in the proteinase-like effect of insulin, the ability of endoproteinase Arg-C and endoproteinase Lys-C to mimic the effect of insulin (Fig. 1a) on 2-deoxyglucose transport and lipogenesis was examined. Endoproteinase Arg-C and endoproteinase Lys-C are highly specific serine proteinases which hydrolyse peptide bonds exclusively at the carboxyl end of arginine (Levy *et al.*, 1970; Schenkein *et al.*, 1977) or lysine (Jekel *et al.*, 1983) residues respectively.

Fig. 1(b) shows that digestion of fat-cells for 15 min at 37°C with graded concentrations of endo-

Table 2. *Effect of proteinase inhibitors or substrates on basal and insulin-stimulated lipogenesis in rat adipocytes*
 Fat-cells (approx. 10^5 cells) were incubated for 20 min at 37°C without or with 100 ng of insulin/ml either in the absence or in the presence of graded concentrations of proteinase inhibitors or substrates. Assays for lipogenesis from [$3\text{-}^3\text{H}$]glucose (0.25 μCi ; final concn. 0.5 mM) were then conducted at 37°C for 2 h. Total extractable lipids were determined as described in the Materials and methods section. The control values of [$3\text{-}^3\text{H}$]glucose converted into total ^3H -labelled lipids were 5.9 ± 0.6 and 15.1 ± 1.3 nmol/2 h per 10^5 cells for non-treated and insulin-treated fat-cells respectively. Values are means $\pm$ S.E.M. for duplicate determinations from the indicated numbers of experiments (numbers in parentheses). Values significantly different from the control value with no hormone are indicated: $^{**}P < 0.02$; $^{***}P < 0.01$. Values significantly different from the control value with insulin are indicated: $^{\dagger}P < 0.05$; $^{\dagger\dagger\dagger}P < 0.01$; $^{\dagger\dagger\dagger\dagger}P < 0.001$. Abbreviation: n.d., not determined. Maximal insulin stimulation was calculated as follows: $100 \times (15.1 - 5.9)/5.9 = 156\%$ increase above the basal rate; the percentage of maximal stimulation was obtained as indicated in the legend to Table 1.

Additions	[$3\text{-}^3\text{H}$]Glucose converted into ^3H -labelled lipids	
	No hormone (% of control)	Insulin (100 ng/ml) (% of maximal stimulation)
None	100 (40)	100 (30)
Tos-Lys-CH ₂ Cl (μM)		
30	110 ± 14 (3)	71 ± 7 (3)
60	109 ± 9 (5)	$45 \pm 5^{\dagger\dagger\dagger}$ (4)
125	100 ± 8 (8)	$17 \pm 7^{\dagger\dagger\dagger\dagger}$ (8)
250	$64 \pm 5^{***}$ (4)	n.d.
NPGb (μM)		
30	94 ± 5 (3)	81 ± 7 (3)
60	95 ± 6 (3)	$58 \pm 13^{\dagger}$ (4)
125	92 ± 5 (3)	$16 \pm 3^{\dagger\dagger\dagger\dagger}$ (4)
250	$49 \pm 10^{**}$ (4)	n.d.
Tos-Arg-OMe (mM)		
1	104 ± 11 (3)	$27 \pm 6^{\dagger\dagger\dagger}$ (3)
2	110 ± 10 (3)	$28 \pm 15^{\dagger}$ (3)
Ac-Ala-Ala-Ala-OMe (mM)		
1	109 ± 6 (3)	97 ± 3 (4)
2	111 ± 6 (3)	100 ± 10 (3)

Table 3. *Effect of proteinase inhibitors or substrates on fat-cell ATP content*

Fat-cells (approx. 2×10^6 cells) were incubated for 20 min at 37°C in the absence or in the presence of the indicated concentrations of Tos-Lys-CH₂Cl, NPGb or Tos-Arg-OMe (final volume 3 ml). At the end of incubation, 1 ml of ice-cold 20% (w/v) trichloroacetic acid (final concn. 5%, w/v) was added and fat-cells were sonicated. After centrifugation (30000 g, 15 min), trichloroacetic acid was removed by diethyl ether extraction (2×7 vol.). Assays for ATP determination were then carried out as described in the Materials and methods section. Values are means $\pm$ S.E.M. for duplicate determinations from the indicated numbers of separate experiments (numbers in parentheses).

Additions	ATP (nmol/ 10^6 cells)
None	25 ± 7 (9)
Tos-Lys-CH ₂ Cl (125 μM)	24 ± 8 (5)
NPGb (125 μM)	25 ± 7 (5)
Tos-Arg-OMe (1 mM)	30 ± 8 (3)

proteinase Arg-C produced a dose-dependent increase in both 2-deoxyglucose transport and lipogenesis. The maximal effects of endoproteinase

Arg-C (200.0 ± 23.0 and $177.0 \pm 18.5\%$ of the control value for 2-deoxyglucose transport and lipogenesis respectively) were observed at an enzyme concentration of 40 $\mu\text{g}/\text{ml}$ (Fig. 1b). These effects were 105 or 67% of the effects elicited by a supramaximal concentration of insulin (100 ng/ml) on both processes (189.9 ± 13.9 and $264.0 \pm 22.9\%$ of the control value for 2-deoxyglucose transport and lipogenesis respectively) (Fig. 1a). Maximal enzyme and insulin effects were not additive: fat-cell digestion with a maximally effective concentration of endoproteinase Arg-C (40 $\mu\text{g}/\text{ml}$) did not enhance basal 2-deoxyglucose transport or lipogenesis beyond the maximal effect obtained with insulin alone (Fig. 1b). When fat-cells were treated with endoproteinase Arg-C (40 $\mu\text{g}/\text{ml}$) in the presence of Tos-Lys-CH₂Cl (125 μM), stimulation of both 2-deoxyglucose transport and lipogenesis was almost completely abolished (Fig. 1b).

The possibility that the endoproteinase-Arg-C-induced activation of glucose metabolism might reflect an alteration of fat-cell membranes and a consequent increase in passive glucose diffusion was ruled out by the following results: (1) the lactate dehydrogenase activity recovered in the cell-free medium after a 15 min incubation of fat-

Fig. 1. Comparative effects of insulin and endoproteinase Arg-C or endoproteinase Lys-C on 2-deoxyglucose transport and lipogenesis in rat adipocytes

Fat-cells (approx. 10^5 cells) were preincubated for 15 min at 37°C without (■) or with (□) the indicated concentrations of endoproteinases. In some experiments, Tos-Lys-CH₂Cl ($125\ \mu\text{M}$) was added at the same time as endoproteinase Arg-C ($40\ \mu\text{g/ml}$) (▨). Digestion reactions were stopped by the addition of aprotinin (200 peptidase-inhibitor units/ml) to the incubation medium. After a further 20 min incubation period at 37°C in the absence or in the presence of $100\ \text{ng}$ of insulin/ml (▤), assays for 2-deoxyglucose transport and lipogenesis were conducted as described in the Materials and methods section. The control values of 2-deoxy-D-[1- ^{14}C]glucose transported and [3- ^3H]glucose converted into total ^3H -labelled lipids were respectively $79.8 \pm 8.4\ \text{pmol/min}$ per 10^5 cells and $5.5 \pm 0.8\ \text{nmol/2h}$ per 10^5 cells. Values are means $\pm$ S.E.M. for quadruplicate (2-deoxyglucose transport) or duplicate (lipogenesis) determinations from the indicated numbers of experiments (numbers in parentheses). Values significantly different from the control values are indicated: * $P < 0.05$; *** $P < 0.01$; **** $P < 0.001$.

cells at 37°C either in the presence or in the absence of endoproteinase Arg-C ($40\ \mu\text{g/ml}$) was 8.1 ± 0.2 and 8.0 ± 0.5 munits/ 10^5 cells ($n = 3$) respectively and represented about 9–10% of the total lactate dehydrogenase activity measured in sonicated cells; (2) in the presence of $50\ \mu\text{M}$ -cytochalasin B, a concentration that completely inhibits facilitated glucose transport in fat-cells (Czech *et al.*, 1973), 2-deoxyglucose uptake was 23.0 ± 3.0 and $23.4 \pm 0.9\ \text{pmol/10}^5$ cells after a 2 min-transport assay with control or endoproteinase-Arg-C-treated fat-cells respectively ($n = 3$).

In contrast, fat-cell digestion with graded concentrations of endoproteinase Lys-C (5 – $100\ \mu\text{g/ml}$) did not result in any activation of either 2-deoxyglucose transport or lipogenesis (Fig. 1c). The difference in the specific activities of both enzyme preparations (12 units/mg of protein for endoproteinase Lys-C, 250 units/mg of protein for

endoproteinase Arg-C) could not account for this result, since: (1) at a concentration of $100\ \mu\text{g/ml}$ (i.e. 1.2 units/ml), endoproteinase Lys-C was still ineffective in stimulating 2-deoxyglucose transport or lipogenesis (99.2 ± 8.0 or $100.8 \pm 8.0\%$ of the control value respectively) (Fig. 1c), whereas at a concentration as low as $2\ \mu\text{g/ml}$ (i.e. 0.5 unit/ml) endoproteinase Arg-C produced a significant increase in both metabolic processes (153.0 ± 9.0 and $137.6 \pm 5.8\%$ of the control value for 2-deoxyglucose transport and lipogenesis respectively); (2) preliminary assays showed that, at the same concentration (0.5 unit/ml), endoproteinase Lys-C was as efficient as, or even more efficient than, endoproteinase Arg-C in hydrolysing both ^{125}I -labelled insulin ($60\ \text{nM}$) and ^{125}I -labelled bovine serum albumin ($300\ \text{nM}$) (1359 ± 20 and 6093 ± 172 c.p.m. released as trichloroacetic acid-soluble ^{125}I radioactivity for endoproteinase Lys-C, and

1459 ± 88 and 2873 ± 134 c.p.m. for endoproteinase Arg-C).

Insulin- and WGA-induced increases in membrane proteolysis

To provide direct evidence for the ability of insulin to activate a membrane proteolytic step, the effect of the hormone on the release of trichloroacetic acid-soluble ^{125}I radioactivity from ^{125}I -labelled liver plasma membranes was tested. For these studies, the presence of insulin receptors in liver plasma-membrane preparations was systematically verified (29 ± 2 fmol of ^{125}I -labelled insulin specifically bound/mg of protein at 28 pM hormone) before ^{125}I -labelling experiments were carried out. Table 4 shows that insulin, at concentrations currently used for studying its metabolic effects (1–100 ng/ml), induced a dose-

dependent increase in the release of trichloroacetic acid-soluble ^{125}I radioactivity from ^{125}I -labelled liver plasma membranes.

To gain insight into a possible relationship between insulin's ability to activate membrane proteolysis and insulin action, we examined the effect of WGA, a lectin shown to interfere with insulin binding (Cuatrecasas & Tell, 1973; Cherqui *et al.*, 1981, 1982) and to mimic insulin bioeffects on glucose metabolism in fat-cells (Cuatrecasas & Tell, 1973; Shechter & Sela, 1981; Cherqui *et al.*, 1982, 1983) on membrane proteolysis. Table 4 shows that, at 20 $\mu\text{g}/\text{ml}$, a concentration shown to be maximally effective in stimulating lipogenesis in fat-cells (Cherqui *et al.*, 1983), WGA produced a significant increase in the release of trichloroacetic acid-soluble ^{125}I radioactivity from ^{125}I -labelled liver plasma membranes.

To address the possibility that insulin and WGA might exhibit proteolytic activity on other protein substrates, we tested the effects of the hormone and the lectin on ^{125}I -labelled bovine serum albumin. Table 4 shows that, at concentrations shown to be effective with ^{125}I -labelled liver plasma membranes, neither insulin nor WGA was able to increase the trichloroacetic acid-soluble ^{125}I radioactivity released from ^{125}I -labelled bovine serum albumin.

Table 4. Effect of insulin and WGA on the trichloroacetic acid-soluble ^{125}I radioactivity released from ^{125}I -labelled liver plasma membranes or ^{125}I -labelled bovine serum albumin

^{125}I -labelled liver plasma membranes (1.2×10^5 – 1.5×10^5 c.p.m.) or ^{125}I -labelled bovine serum albumin (2.3×10^5 c.p.m.) were incubated for 30 min at 37°C in 50 mM-sodium phosphate buffer (0.5 ml), pH 7.5, in the absence or in the presence of the indicated concentrations of insulin or WGA. At the end of incubation, 30 μl of 20% (w/v) bovine serum albumin solution was added, followed by 530 μl of ice-cold 20% (w/v) trichloroacetic acid. Samples (duplicate) were centrifuged for 5 min at 5000 rev./min, and two 100 μl portions of each supernatant were counted for evaluation of the trichloroacetic acid-soluble ^{125}I radioactivity. The results, expressed as a percentage of the total radioactivity added to the medium, were corrected for the trichloroacetic acid-soluble ^{125}I radioactivity released at zero time of incubation. Values are means $\pm$ S.E.M. for quadruplicate determinations from the indicated numbers of experiments (numbers in parentheses). Values significantly different from the control values are indicated: * $P < 0.05$; *** $P < 0.01$.

Additions	Trichloroacetic acid-soluble ^{125}I radioactivity (% of total c.p.m. added) released from:	
	^{125}I -labelled liver plasma membranes	^{125}I -labelled bovine serum albumin
None	3.8 $\pm$ 0.5 (11)	2.0 $\pm$ 0.5 (3)
Insulin (ng/ml)		
1	4.6 $\pm$ 0.8 (8)	1.7 $\pm$ 0.4 (3)
10	5.7 $\pm$ 0.3*** (11)	1.5 $\pm$ 0.3 (3)
100	5.8 $\pm$ 0.9* (5)	1.8 $\pm$ 0.4 (3)
WGA ($\mu\text{g}/\text{ml}$)		
20	5.4 $\pm$ 0.2* (5)	1.8 $\pm$ 0.1 (3)

Discussion

Both indirect and direct methodological approaches were successively developed in order to explore further the proposed (Seals & Czech, 1980) proteolytic mechanism of action of insulin.

The technical problems related to the procedures used for isolation of fat-cells, measurement of insulin binding, glucose transport and lipogenesis have been described in detail previously (Cherqui *et al.*, 1981, 1982, 1983). The present discussion only deals with the specificity of endoproteinases and the choice of liver plasma membranes for studying membrane proteolysis. (1) The high specificity of endoproteinase Arg-C and endoproteinase Lys-C, which are now regarded as valuable tools in the sequencing of proteins, has been assessed by previous studies using either synthetic or natural proteins of known sequence (Schenkein *et al.*, 1977; Jekel *et al.*, 1983). (2) Liver plasma membranes were judged to be more appropriate than adipocyte plasma membranes for testing the effect of insulin on membrane proteolysis. This choice allowed us to obviate a problem inherent in the preparation of adipocyte plasma membranes, that is the achievement of fat-cell disruption along with subsequent centrifugation of the resulting homogenate at a temperature of 20°C (Avruch & Wallach, 1971; Cherqui *et al.*, 1979).

These conditions were suspected to preserve imperfectly the integrity of plasma-membrane proteins and insulin receptors, inasmuch as these latter were shown to be highly sensitive to the proteolytic activity of a lysosome-containing cellular fraction (Massague *et al.*, 1981).

Tos-Lys-CH₂Cl, NPGB and Tos-Arg-OMe exerted an antagonizing effect that was selective on insulin action, as indicated by the following observations. (1) All three compounds exhibited non-parallel effects on basal and insulin-stimulated glucose transport or lipogenesis. Thus, at 125 μ M-Tos-Lys-CH₂Cl or -NPGB or at 1 mM-Tos-Arg-OMe, concentrations that did not impair basal rates of both metabolic processes, the effect of insulin was found to be almost completely abolished, indicating an exclusive action of the proteinase inhibitors on insulin bioeffects. Similar concentrations of either NPGB or Tos-Arg-OMe have previously been shown to inhibit potently the insulin stimulation of glycogen synthase in fat-cells (Seals & Czech, 1982). For Tos-Lys-CH₂Cl, however, it is worth noting that the range of effective concentrations observed in the present study (30–125 μ M) is far below that reported elsewhere (1–2 mM) (Larner *et al.*, 1982). The reason for this discrepancy is not clearly understood. One possible explanation may be that only freshly prepared solutions of Tos-Lys-CH₂Cl were used in our experiments, since a substantial loss of activity was noted in solutions subjected to successive freezings and thawings. (2) At concentrations shown to be maximally inhibitory on insulin action, Tos-Lys-CH₂Cl, NPGB and Tos-Arg-OMe failed to impair the insulin-receptor interaction. This finding, showing that these agents exclusively affected a post-binding step, agrees with the result reported for Tos-Lys-CH₂Cl alone (Larner *et al.*, 1981). (3) Tos-Lys-CH₂Cl, NPGB and Tos-Arg-OMe did not impair the ATP content of fat-cells, even though these agents were tested at concentrations shown to be maximally effective in inhibiting insulin action on glucose metabolism. This finding, which agrees with that reported by Cheng *et al.* (1984) for Tos-Lys-CH₂Cl alone, allowed us to rule out the possibility that fat-cell ATP depletion rather than an actual blocking of a proteolytic step is responsible for the inhibitory effects of the three agents on the insulin stimulation of 2-deoxyglucose transport and lipogenesis. ATP-depletion-induced effects have actually been demonstrated elsewhere (Muchmore *et al.*, 1982) but with different low-*M_r* proteinase inhibitors such as *p*-amino-benzamidine, phenylguanidine or di-isopropyl fluorophosphate, used at concentrations of 0.3–10 mM.

Endoproteinase Arg-C, but not endoproteinase Lys-C, was shown to activate glucose metabolism

in fat-cells. The specificity of the endoproteinase-Arg-C-induced stimulation of 2-deoxyglucose transport and lipogenesis is indicated by the fact that the enzyme's insulin-like effects on both metabolic processes were almost completely abolished by Tos-Lys-CH₂Cl, a trypsin-blocking agent shown also to inhibit endoproteinase Arg-C activity (Levy *et al.*, 1970; Boesman *et al.*, 1976). In addition, since fat-cell digestion with a maximally effective concentration of endoproteinase Arg-C failed to increase either lactate dehydrogenase leakage or passive glucose diffusion, it can be reasonably envisaged that, under the conditions of our study, the enzyme treatment resulted in a limited proteolysis of fat-cell plasma membranes. In support of this hypothesis, endoproteinase Arg-C (40 μ g/ml) was found to hydrolyse about 2.5% of ¹²⁵I-labelled liver plasma-membrane proteins after 30 min incubation at 37°C (results not shown).

The insulin-like effect of endoproteinase Arg-C, which, to our knowledge, has not been described previously, agrees with other results reporting the ability of trypsin to mimic several insulin bio-effects on glucose metabolism, including activation of glucose transport (Clark *et al.*, 1980; Kikuchi *et al.*, 1981) and oxidation (Kono & Barham, 1971; Kikuchi *et al.*, 1981; Pilch *et al.*, 1981) as well as stimulation of glycogen synthase and pyruvate dehydrogenase (Kikuchi *et al.*, 1981). In this regard, the finding that endoproteinase Arg-C, at a maximally effective concentration was unable to enhance further the stimulation of 2-deoxyglucose transport or lipogenesis elicited by a supramaximal concentration of insulin, is similar to the lack of additivity of maximal effects of insulin and trypsin on glucose oxidation (Kikuchi *et al.*, 1981). This observation moreover suggests a common site of action for both endoproteinase Arg-C and insulin. A further similarity to trypsin, which was found to be as effective as insulin in stimulating glucose oxidation, but less effective than the hormone in activating glycogen synthase (Kikuchi *et al.*, 1981), lies in the fact that endoproteinase Arg-C fully mimicked the insulin effect on 2-deoxyglucose transport, but was not as potent as the hormone in stimulating lipogenesis. This finding is not clearly understood at present.

The concomitant observations that endoproteinase Arg-C mimicked insulin bioactivity, whereas endoproteinase Lys-C failed to do so, extends the findings obtained by others with trypsin alone. Thus our study clearly demonstrates that the cleavage of peptide bonds involving arginine residues is essential for the insulin-receptor activation. Although the molecular basis of the insulinomimetic effect of endoproteinase Arg-C remains unknown, it is tempting to speculate that this cleavage may release steric con-

straints in the nearby environment of the receptor and/or on the receptor structure itself. This process would activate the receptor, which would then in turn transduce the observed effects on glucose metabolism. Such an arginine-specific cleavage is most probably also involved in the bioactivity of insulin itself, since the hormone effects in fat-cells were found to be selectively antagonized by Tos-Arg-OMe, which is in agreement with previous results reported by others (Seals & Czech, 1980, 1982; Tamura *et al.*, 1984).

Insulin, at concentrations currently used for studying its metabolic effects (1–100 ng/ml), was shown to increase selectively the trichloroacetic acid-soluble ^{125}I radioactivity released from ^{125}I -labelled liver plasma membranes. Although two different studies reported that the insulin molecule itself might possess proteolytic activity (Rieser & Rieser, 1964; Huang & Cuatrecasas, 1975), this property most probably does not account for our finding, as indicated by the following observations. (1) In the range of concentrations shown to be effective in stimulating membrane proteolysis, insulin failed to exhibit any proteolytic activity when ^{125}I -labelled bovine serum albumin was used as an exogenous substrate. In fact, the proteolytic activity of insulin was initially demonstrated at very high concentrations (1.67 or 13 μM), which do not have any biological significance. (2) WGA, a well-known insulin mimicker acting through the insulin receptor (Cuatrecasas & Tell, 1973; Cherqui *et al.*, 1981, 1982, 1983; Shechter & Sela, 1981), reproduced the insulin activation of membrane proteolysis, but, like the hormone, was ineffective on ^{125}I -labelled bovine serum albumin. This finding suggested that the interaction of either insulin or WGA with the insulin receptor is a prerequisite for the expression of the proteinase-like activities of both ligands. Taken together, these data strongly suggest that an endogenous insulin-sensitive proteinase is responsible for the hormone-induced activation of liver plasma-membrane proteolysis. Whether or not the insulin receptor itself may serve as a substrate for the proteinase could not be determined with our experimentation. In any case, if that were true, it would not be the sole substrate, since the amount of membrane proteins that are hydrolysed in the presence of insulin (Table 4) is far higher (about 20-fold) than the concentration of insulin receptors in liver plasma membranes, as estimated from Scatchard analysis of insulin-binding data (Pottick *et al.*, 1981; Caron *et al.*, 1983).

In conclusion, our investigation yields several experimental results which provide additional evidence for the hypothesis that insulin acts via a proteolytic mechanism implying a membrane serine proteinase with arginine specificity. Such a

pathway for mediation of the regulatory effects of insulin on cellular metabolism raises theoretical problems which are related to proteinase substrate regeneration. Anyhow, in order to confirm the hypothesis of a proteolytic mechanism of insulin action, several questions need to be addressed. (1) What is the molecular mechanism of the transduction of the initial insulin-binding event into the proteinase activation? (2) Which protein species in the plasma membrane serves as a substrate for the insulin-sensitive proteinase? (3) How is the insulin activation of the membrane proteinase mechanistically related to the hormone stimulation of the insulin-receptor kinase?

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