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Insulin-like Activity of Proteases. VIII.[1] Stimulation of Lipogenesis and Pyruvate Dehydrogenase and Suppression of Epinephrine-Sensitive Lipolysis in Rat Epididymal Adipose Tissue by an *N*-Succinyl-L-trialanine *p*-Nitroanilide-Hydrolyzing Protease from Pronase

HIROSHI UEKI,*[,a] YUKO MATSUNAMI,[b] AIICHIRO MOTOSHIMA,[a] TAKAYUKI FUNAKOSHI,[b] SHOZO SHOJI,[b] and YUKIHO KUBOTA[b]

Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University,[a] 985 Higashi-muramachi, Fukuyama, Hiroshima 729–02, Japan and Faculty of Pharmaceutical Sciences, Kumamoto University,[b] 5–1 Ohe-honcho, Kumamoto 862, Japan

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Insulin-like effects of *N*-succinyl-L-trialanine *p*-nitroanilide-hydrolyzing protease (STA-protease) purified from Pronase were investigated in rat epididymal adipose tissue. The enzyme stimulated the conversion of [3-^{3}H] glucose into lipid and suppressed the epinephrine-stimulated lipolysis in the fat cells, like trypsin and insulin. When the fat pads were incubated with STA-protease in the presence of glucose, pyruvate dehydrogenase activity in the homogenate of the incubated fat pads was stimulated markedly. In the absence of glucose, STA-protease did not stimulate pyruvate dehydrogenase activity though trypsin and insulin showed a slight but significant stimulation. Further, the stimulatory effect of STA-protease in the presence of glucose was inhibited by the addition of 3-*O*-methylglucose or phlorizin to the incubation medium of the fat pads. Trypsin and insulin still showed a significant stimulation under similar conditions. When the homogenate of intact fat pads was incubated with STA-protease, no stimulation was observed throughout the range of effective concentrations. These results suggest that STA-protease also reacts with the cell surface and consequently mimics the actions of insulin, though a slight difference between STA-protease and trypsin may exist as regards the mechanism of stimulation of pyruvate dehydrogenase.

Keywords——insulin-like activity; protease; fat pad; isolated fat cell; lipogenesis; antilipolysis; pyruvate dehydrogenase

Insulin shows such cellular effects as the stimulation of transport of glucose and amino acids, the promotion of glycogen, lipid, and protein syntheses, and the suppression of the hormone-dependent lipolysis in rat fat cells.[2] Proteases, such as Pronase, subtilisin, trypsin, and α-chymotrypsin, can to various extents mimic the actions of insulin on rat and mouse diaphragms, rat fat cells, and frog sartorius muscles.[3–7] We reported previously that an acid-stable *N*-succinyl-L-trialanine *p*-nitroanilide-hydrolyzing protease (STA-protease) purified from Pronase E was a type of serine-protease, hydrolyzing *N*-succinyl-L-trialanine *p*-nitroanilide at a high rate and glutaryl-L-phenylalanine *p*-nitroanilide at a lower rate, but showing no hydrolytic activity towards benzoyl-L-arginine *p*-nitroanilide.[8] Therefore, the enzyme appeared to be an elastase-like enzyme but not a trypsin-like one.

The purpose of the present work was to clarify whether or not STA-protease shows insulin-like effects on rat epididymal adipose tissue, like trypsin.

Materials and Methods

Animals——Male Wistar rats weighing 100—150 g were fed a pellet diet (CE-2, Clea Japan Co., Tokyo) for at

least three days before use.

Chemicals——Pronase (from *Streptomyces griseus*, type E, 70 PUK/mg) was a gift from Kaken Chemical Co., Tokyo. Elastase (from porcine pancreas, 326 EL.U/mg) and α-chymotrypsin (from porcine pancreas, 40 U/mg) were gifts from Eisai Co., Tokyo. Subtilisin BPN′ (*Bacillus subtilis* alkaline protease) was purchased from Nagase Sangyo Co., Osaka. Trypsin (from porcine pancreas, 4500 U/mg) was obtained from Miles Research Products Co., Elkhart, and purified by an affinity chromatography on soybean trypsin inhibitor-Sepharose 4B after treatment with L-(1-tosylamide-2-phenyl)ethyl chloromethyl ketone.[9] Alkaline protease inhibitor (API-2c, from *Streptomyces griseoincarnatus*) was donated by Professor M. Shibata and Dr. M. Uyeda of Kumamoto University. Insulin (from bovine pancreas, 24 IU/mg) and phlorizin were obtained from Sigma Chemical Co., St. Louis. [3-^{3}H]Glucose (17.54 Ci/mmol), sodium [1-^{14}C]pyruvate (8 mCi/mmol), and Omnifluor were purchased from New England Nuclear, Boston. All other chemicals used were of analytical grade.

Preparation of STA-Protease from Pronase——STA-protease was purified from Pronase E by affinity chromatography on arginine peptides-Sepharose and soybean trypsin inhibitor-Sepharose, as described in a previous paper.[8]

Preparation of Fat Pads and Cells——Rat epididymal adipose tissue was cut into pieces weighing 80—100 mg each. The fat pads obtained were preincubated gently in 5 ml of Krebs–Ringer bicarbonate buffer, pH 7.4, containing 1% bovine serum albumin (KRBA) and glucose at a desired concentration for 30 min and then incubated in the fresh medium for assays.[10] The fat cells were isolated by the collagenase digestion method.[11,12] The adipose tissue (about 700 mg) was digested with collagenase (2.5 mg) in 2 ml of the KRBA (but with 4% bovine serum albumin) containing 3 mM glucose for 1 h. The cells were then washed twice with the KRBA and used for assays. All incubations were carried out at 37 °C in a plastic vial containing the KRBA under an atmosphere of 95% O_2–5% CO_2.

Assays——The rate of conversion of [3-^{3}H]glucose into lipid in the fat cells was determined by the method of Moody *et al.*[13] The epinephrine-dependent lipolysis was assayed by determining the amount of free fatty acids accumulated in the incubation medium by the colorimetric method.[14,15] Pyruvate dehydrogenase activity was assayed by determining the rate of formation of $^{14}CO_2$ from [1-^{14}C]pyruvate with the use of the homogenate of the incubated fat pads, essentially according to the method of Sakamoto and Kuzuya.[10] Radioactivity was measured with an Aloka (LSC-700) liquid scintillation spectrometer. The results are expressed as the mean value ±S.E. of four or five observations.

Results and Discussion

Figure 1 shows the rate of conversion of [3-^{3}H]glucose into lipid at various times of incubation with the fat cells and STA-protease or insulin. The conversion in both cases increased almost linearly up to 180 min.

When the fat cells were incubated with STA-protease at concentrations of 1—50 μg/ml, a

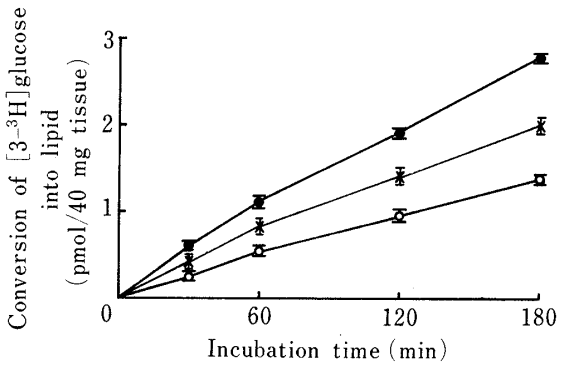

Fig. 1. Time Course of Lipogenesis-Stimulating Effects of STA-Protease and Insulin

The fat cells (40 mg) were incubated with STA-protease or insulin in 1 ml of the KRBA containing 0.55 mM glucose and 6 nM [3-^{3}H]glucose for 0—180 min. The reaction was stopped by the addition of 15 ml of toluene-based scintillant and the radioactivity was measured.

●, STA-protease (3 μg/ml); ×, insulin (1 mU/ml); ○, control (20 μl of physiological saline).

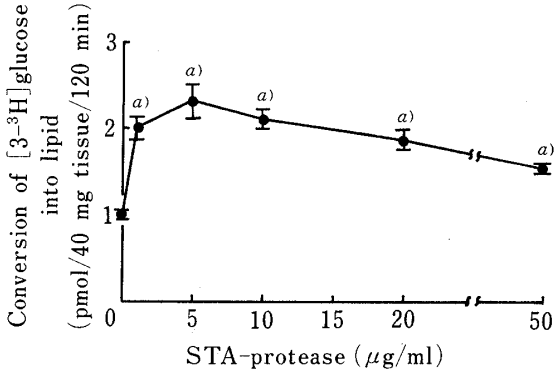

Fig. 2. Dose-Response Relation for the Lipogenesis-Stimulating Effect of STA-Protease

The fat cells were incubated with STA-protease (1—50 μg/ml) for 120 min in the incubation medium as described in the legend to Fig. 1.

a) Significantly different from the control (without STA-protease) at $p<0.01$.

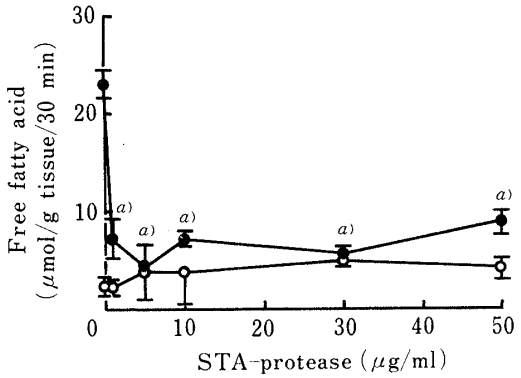

Fig. 3. Suppressive Effect of STA-Protease on Lipolysis Stimulated by Epinephrine

The fat cells (20 mg) were incubated with STA-protease (1—50 μg/ml) in 1 ml of the KRBA in the presence or absence of epinephrine for 30 min. The amount of free fatty acids accumulated in the incubation medium was determined as described in Materials and Methods.

●, 0.55 μM epinephrine; ○, without epinephrine.

a) Significantly different from the control (without STA-protease) at $p<0.01$.

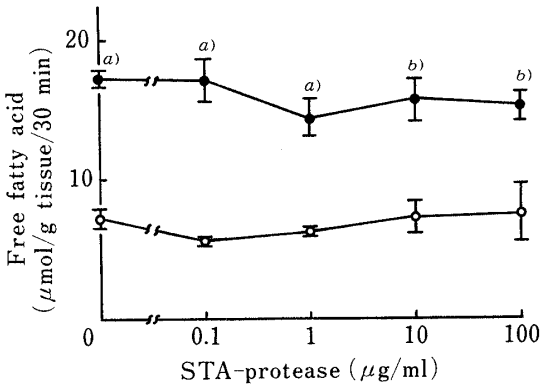

Fig. 4. Stimulatory Effect of Epinephrine on Lipolysis in the Fat Cells pretreated with STA-Protease

Epinephrine was added to the incubation medium containing fat cells which had been pretreated with STA-protease for 30 min, and incubation was carried out for an additional 30 min. In order to stop the reaction, one equivalent of API-2c with respect to the enzyme was added. Other details were as described in Materials and Methods.

●, 0.55 μM epinephrine; ○, without epinephrine.

a, b) Significantly different from the control (without epinephrine) at $p<0.01$ and $p<0.05$, respectively.

TABLE I. Stimulation of Lipogenesis and Suppression of Lipolysis

	Lipogenic activity[a]		Lipolytic activity[b]	
	Concentration of protease		Concentration of protease	
	0[c]	10 (μg/ml)	0[c]	10 (μg/ml)
STA-protease	1.01±0.04	2.10±0.10[e]	23.0±1.4	7.2±0.8[e]
Pronase E	1.08±0.03	1.89±0.05[e]	19.7±1.1	12.6±1.4[f]
α-Chymotrypsin	1.08±0.03	1.75±0.03[e]	19.7±1.1	11.6±1.3[f]
Subtilisin BPN′	1.08±0.03	1.74±0.01[e]	21.1±1.3	16.1±1.0[f]
Trypsin	1.08±0.03	1.72±0.08[e]	21.1±1.3	11.3±0.6[e]
Elastase	1.08±0.03	1.95±0.09[e]	20.9±1.2	20.3±1.0
Insulin[d]	1.08±0.03	1.80±0.04[e]	23.0±1.4	11.4±1.1[e]

a) The rate of conversion of [3-^{3}H]glucose into lipid (pmol/40 mg tissue/120 min).

b) The amount of free fatty acids accumulated in the incubation medium, in the presence of 0.55 μM epinephrine (μmol/g tissue/30 min).

c) 20 μl of physiological saline.

d) 3 mU/ml.

e, f) Significantly different from the control (without protease) at $p<0.01$ and $p<0.05$, respectively.

significant increase from the control (without the enzyme) was observed at all concentrations tested (Fig. 2). The maximum value was obtained at a concentration of 5 μg/ml, but decreased with further increase in concentration. This is presumably due to unselective digestion of the cell membrane by the proteolytic action of the enzyme at the higher concentrations.

Figure 3 shows the effect of STA-protease on the lipolysis stimulated by epinephrine. When the fat cells were incubated with epinephrine at a concentration of 0.55 μM, the amount of free fatty acids that accumulated in the incubation medium was increased from 2.4±1.0 to 23.0±1.4 μmol/g tissue/30 min. The stimulation of lipolysis was markedly suppressed by

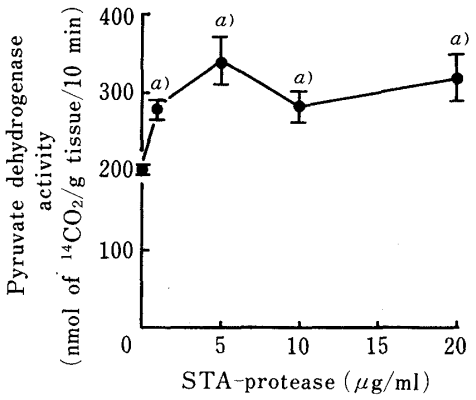

Fig. 5. Dose-Response Relation for the Pyruvate Dehydrogenase-Stimulating Effect of STA-Protease

The fat pads (100 mg) were incubated with STA-protease (1—20 μg/ml) in 5 ml of the KRBA containing 11 mM glucose for 30 min. The protease and insulin were washed out with the incubation medium, then the washed fat pads were frozen on dry ice, homogenized in 1 ml of 30 mM phosphate buffer, pH 7.0, containing 30 mM NaCl and 0.5 mM dithiothreitol, and centrifuged at $700 \times g$ for 10 min. The rate of formation of $^{14}CO_2$ from [1-^{14}C]pyruvate was determined with the intermediate fraction.[10] The incubation was carried out in a plastic vial covered with a rubber seal, and containing a small plastic cup holding a piece of Whatman glass-fiber paper and 0.2 ml of β-phenethylamine. The fraction (0.4 ml) was added through the seal with a syringe to the vial, which held 0.5 ml of the buffer containing 50 mM NaCl, 1 mM NAD and cocarboxylase, 0.5 mM dithiothreitol, 0.27 mM Co A, 0.25 mM pyruvate, and 0.5 μM [1-^{14}C]pyruvate. After incubation for 5 min, the reaction was stopped by cooling the vial in an ice-cold bath followed by injection of 0.6 ml of 2.5 M sulfuric acid through the seal. The vial was then gently shaken at 24 °C for 30 min. The glass-fiber paper was transferred to a plastic minivial containing 5 ml of toluene–ethanol-Omnifluor scintillant, and the radioactivity was measured.

a) Significantly different from the control (without STA-protease) at $p < 0.05$.

TABLE II. Stimulatory Effects of Proteases and Insulin on Pyruvate Dehydrogenase Activity in the Presence or Absence of Glucose, 3-*O*-Methylglucose, or Phlorizin

	Pyruvate dehydrogenase activity[a)]				
	Glucose (mM)			2 mM glucose + 20 mM 3-*O*-methylglucose	2 mM glucose + 4 mM phlorizin
	0	2	11		
Control[b)]	72±2	76±11	224±12	71±11	55± 2
STA-protease	75±8	161±17[c)]	383±25[c)]	89± 5	49±12
Elastase	70±4	155±16[c)]	382±30[c)]	80±10	61±10
Trypsin	89±7[d)]	260±10[c)]	403±45[c)]	260±15[c)]	120±18[c)]
Insulin	110±6[c)]	482±30[c)]	498±56[c)]	477±54[c)]	179±36[d)]

The concentrations of proteases and insulin were 5 μg/ml and 1 mU/ml, respectively. Pyruvate dehydrogenase activity was assayed as described in the legend to Fig. 5.

a) nmol of $^{14}CO_2$/g tissue/10 min.

b) 0.1 ml of physiological saline.

c, *d*) Significantly different from the control at $p < 0.01$ and $p < 0.05$, respectively.

STA-protease over the concentration range of 1—50 μg/ml. The maximum antilipolytic effect was found at the concentration of 5 μg/ml. In the absence of epinephrine, no antilipolytic effect was observed at any concentration tested.

When the fat cells pretreated with STA-protease were incubated with 0.55 μM epinephrine, a significant stimulation of lipolysis was observed over the concentration range of 0.1—100 μg/ml (Fig. 4). These results suggest that incubation with STA-protease does not damage the epinephrine receptor on the cell surface. Therefore, the possibility that STA-

protease directly attacks the epinephrine receptor to cause the antilipolytic effect may be excluded.

As reported by Kuo *et al.*,[3,4] proteases, such as Pronase E, α-chymotrypsin, subtilisin BPN′, and trypsin, stimulated lipogenesis and suppressed the epinephrine-stimulated lipolysis in the fat cells. Elastase did not show the antilipolytic effect though it stimulated the lipogenesis.

When the fat pads were incubated with STA-protease in the presence of 11 mM glucose, a significant stimulation of pyruvate dehydrogenase in the homogenate of the incubated fat pads was observed over the concentration range of 1—20 μg/ml (Fig. 5). The maximum value was obtained at 5 μg/ml. When the homogenate of intact fat pads was incubated with STA-protease, no stimulation was observed over the concentration range of 0.05—5 μg/ml.

Table II shows the changes in the stimulation of pyruvate dehydrogenase by proteases and insulin when the fat pads were incubated in the presence or absence of glucose, 3-*O*-methylglucose, or phlorizin. In the absence of glucose, no stimulation was observed with STA-protease and elastase, whereas trypsin and insulin showed slight but significant stimulation. For the stimulation of pyruvate dehydrogenase by STA-protease, the presence of glucose was necessary in the incubation medium of the fat pads. Further, in the presence of 2 mM glucose, the stimulatory effects of STA-protease and elastase were inhibited by the addition of 20 mM 3-*O*-methylglucose or 4 mM phlorizin to the incubation medium. Trypsin and insulin still showed a significant stimulation under similar conditions. It is well known that 3-*O*-methylglucose and phlorizin are inhibitors of glucose transport into cells. Weiss *et al.*[16] reported that when glucose was replaced by fructose or mannose in the incubation medium, the stimulation of pyruvate dehydrogenase by insulin was still observed distinctly, whereas xylose was essentially ineffective. Sugars which are metabolized by the fat cells appeared to be effective. Hughes *et al.*[17] reported that the stimulation of pyruvate dehydrogenase by insulin was associated with a decrease in the overall steady state incorporation of ^{32}P into the α-subunits of the enzyme, whereas the relative phosphorylation of all three serine residues appeared to be similar in ^{32}P-labelled α-subunits in both control and insulin-treated fat cells. One possible explanation for our results is that STA-protease and elastase facilitate glucose transport to change the intracellular concentrations of glucose and its metabolites, and/or the ratio of ATP to ADP, and consequently pyruvate dehydrogenase will be stimulated. As regards the mechanism of action of STA-protease, the facilitation of glucose transport may play an important role in the stimulation of pyruvate dehydrogenase, though the antilipolytic effect of STA-protease may be produced by a mechanism similar to that of insulin.

Kono and Barham[6] reported that trypsin showed lipogenesis-stimulating and antilipolytic effects similar to those of insulin on rat fat cells. Kikuchi *et al.*[18] reported that trypsin activated glycogen synthetase and pyruvate dehydrogenase in the fat cells and the activations were mediated by peptide-like substances which were generated from cell membrane isolated from fat cells pretreated with trypsin as well as insulin.[19-21] More recently, Tamura *et al.*[22] reported that trypsin stimulated the phosphorylation of tyrosine residues alone in polypeptides of a partially purified insulin receptor preparation from rat fat cells and that trypsin treatment of the insulin receptor fraction also stimulated the phosphorylation of an exogenous substrate of tyrosine kinase, as did insulin treatment. Thus, trypsin almost wholly mimics the actions of insulin on rat fat cells. It is considered that the first step in the insulin-like effects of proteases is a partial digestion of the cell surface, probably at the insulin receptor or in its vicinity, by proteolytic action. Massague *et al.*[23] described a unique proteolytic cleavage of the insulin receptor isolated from rat fat cells: the β-subunit (Mr=90000) in the receptor complex $(\alpha\beta)_2$ (Mr=350000) was converted to β_1-subunit (Mr=49000) by incubation with elastase but not by incubation with trypsin, α-

chymotrypsin, or *Staphylococcus aureus* protease. The proteolytic effects on the cell surface may differ among trypsin, STA-protease, and elastase due to the different substrate specificities (*e.g.*, STA-protease and trypsin have quite low activities against elastin-congo red, which is hydrolyzed by elastase at a high rate.)[24] Therefore, these proteases may mimic the actions of insulin by somewhat different mechanisms.

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