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STUDIES ON THE EFFECTS OF PROTEASE SUBSTRATE ANALOGUES ON SOME OF THE ACTIONS OF INSULIN

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SUMMARY. Added TAME ($N\alpha$-p-tosyl-l-anginine methyl ester) or BAME (benzoyl-anginine methyl ester) inhibited insulin induced activation of glucose oxidation and fat cell PDH activation without affecting spermine action on PDH activation and glucose oxidation in fat cells. BAME inhibited insulin-induced generation of both PDH stimulator and PDH inhibitor from liver particulate fraction. In contrast, insulin-induced internalization of insulin receptors and negative cooperativity of insulin receptors were not affected by protease substrate inhibitors. These results suggest that certain actions of insulin (glucose oxidation, generation of PDH regulators) are mediated by proteolytic events, while insulin-induced down regulation and negative cooperativity of insulin receptors are not mediated by activation of endogenous proteases.

Insulin's interaction with its plasma membrane receptors results in the generation of low molecular weight substances which regulate a number of intracellular enzymes (1-5). Recent studies have also suggested that insulin activates an intrinsic plasma membrane protease in generating the chemical mediators (3,6). Two lines of evidence have been presented to suggest that the factor(s) may be produced by tryptic proteolysis of a membrane bound precursor protein: 1)low concentrations of trypsin mimicked the ability of insulin to stimulate the production of PDH activator by isolated adipocyte plasma membranes (3) or intact adipocytes (6); 2)protease inhibitors and exogenous protease substrate analogues prevented the appearance of the factor in insulin stimulated plasma membrane supernatant fractions (3). More recent studies from this laboratory have shown that insulin activation of fat cell PDH was abolished in the presence of protease substrate analogues while no inhibition by these substrates was observed on the spermine-induced

Abbreviations: PDH, pyruvate dehydrogenase; KRB, Krebs-Ringer bicarbonate; NS, not significant

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activation of fat cell PDH (7). Endoproteases have also been implicated in insulin stimulation of glucose transport in rat fat cells (8). The present studies were undertaken to investigate whether protease activation is involved in insulin stimulation of 1)glucose oxidation, 2)internalization of insulin receptors, 3)negative cooperativity of the receptors and 4)production of PDH inhibitor. Therefore, we have studied the effects of TAME and BAME on some of the actions of insulin in intact adipocytes and in isolated liver particulate fractions.

MATERIALS AND METHODS

Porcine insulin was a gift from Dr. Mary Root (Eli Lilly), [1-^{14}C]pyruvate, U-^{14}C glucose, ^{125}I-sodium iodide were purchased from Amersham Searle. TAME, BAME, Spermine, pyruvic acid and the cofactors for PDH assay were obtained from Sigma. Collagenase was from Worthington. Bovine serum albumin (BSA) was a product of Reheis.

Animals. Young male Sprague-Dawley rats (120-130 g) were used in all experiments. All rats had access to food and water ad libitum.

Studies on Fat Cells. Adipocytes were prepared by collagenase digestion of epididymal fat pads (9), washed 4 times in KRB buffer and were used for the following studies.

Glucose Oxidation. Fat cells (0.025 ml) were added to vials containing 2 ml of 0.01% glucose-KRB buffer with 2% BSA and traces of U-^{14}C glucose in the presence and absence of insulin (100 μU/ml) or Spermine (1 mM) with and without TAME (5 mM), or BAME (5 mM). The vials were sealed, gassed and incubated for 1 hr at 37°C. The CO_2 was absorbed on NCS and radio activity counted.

Insulin Pretreatment, Dissociation and Binding Studies. Adipocytes (2 ml) suspended in Tris-albumin buffer (pH 7.6) were added to vials containing insulin (100 ng/ml) in the presence and absence of BAME (5 mM). The vials were incubated for 2-3 hr at 37°C (10), the bound insulin was dissociated in Tris buffer (pH 7.0). Aliquots of the washed cells (0.1 ml) were used for ^{125}I-insulin binding studies (11) performed at 16°C for 3 hr using 0.5 ng/ml ^{125}I-insulin. ^{125}I-insulin was prepared by the method of Freychet et al (12). Non-specific binding was subtracted from the total binding as described (11).

Studies on Negative Cooperativity. Adipocytes were allowed to associate with ^{125}I-insulin (0.5 ng/ml) for 3 hr at 16°C in a final volume of 5 ml. The cells were centrifuged to remove the buffer and resuspended in insulin-free ice cold buffer (pH 7.6). Aliquots (0.1 ml) were distributed into tubes containing buffer or buffer + insulin (100 ng/ml) in the presence and absence of BAME (5 mM). The tubes were incubated at 16°C for the indicated times. The cells in these tubes were centrifuged through 0.5 ml silicone oil and the radioactivity of the cells counted (13).

Generation of Insulin Mediators. Liver particulate fractions were prepared by the method of Saltiel et al (4) from 5 g of liver tissue. Aliquots of particulate fractions were treated with and without BAME (2 mM) at 4°C for 30 min followed by incubation with insulin (100 μU/ml) for 10 min at 37°C. The mediators released into the supernatant were acidified, extracted and lyophilized. The inhibitory material was separated by alcohol extraction as described (14). Aliquots of alcohol soluble and insoluble fractions were tested on liver mitochondria for their ability to inhibit or stimulate PDH activity. Liver mitochondria were prepared by the method of Parsons et al (15). PDH was assayed by monitoring the conversion of 1-^{14}C pyruvate to $^{14}CO_2$ (16) by rat liver mitochondria.

RESULTS AND DISCUSSION

In confirmation of the hypothesis suggested by Seals and Czech (3), we also found that the presence of arginine specific protease substrate analogues TAME and BAME, during the insulin exposure of fat cells, resulted in dose dependent inhibition of insulin's effect on glucose oxidation without affecting the basal state (Table 1). In contrast to this, spermine activation of glucose oxidation was not affected by either BAME or TAME (Table 2). Earlier studies from this laboratory have also shown that protease substrate analogues failed to inhibit the spermine's effect on fat cell PDH (7) suggesting that the mechanism of action of spermine on glucose oxidation in the present studies as well as on PDH activation reported earlier was different from that of insulin. The lack of effect of protease inhibitors on spermine stimulation of glucose oxidation further supports the view that its insulin-like effects are limited and independent of early events which mediate certain metabolic effects of insulin. The inhibition of insulin's effect by BAME was not due to interference of the substrate with the binding of insulin to its receptors (Table 3). TAME inhibited the insulin binding by about 30% while BAME was without effect. Hence in the rest of the experiments, only BAME was used to study the effects on insulin

Table 1: Effects of protease inhibitors on insulin-stimulated glucose oxidation by fat cells

Additions	Glucose oxidation (nmoles $^{14}CO_2$ produced/assay/hr)			
	No insulin	+ Insulin	Δ	% Stimulation
1)None	8.5±1.50	48.1±5.46	39.6±4.04	466±40.1
2)TAME (5mM)	9.8±1.88	27.8±2.32	18.0±5.96	184±27.7
3)TAME (7.5mM)	10.3±2.43	20.5±1.59	10.2±0.90	99±36.3
4)BAME (5mM)	10.9±2.79	33.6±4.03	22.7±1.65	208± 6.0
P Value				
1 vs 2 or 3	NS	< 0.001	< 0.001	< 0.001
1 VS 4	NS	NS	< 0.010	< 0.001

Fat cells (0.025 ml) were incubated with and without insulin (100 μU/ml) in the presence and absence of TAME or BAME in 0.01% glucose-KRB buffer containing 2% BSA and traces of U-^{14}C glucose for 1 hr at 37°C. The $^{14}CO_2$ released was counted. Results are the mean ± SE of 5 individual experiments performed in triplicate.

Table 2: Effects of protease inhibitors on spermine-induced activation of glucose oxidation

Additions	Glucose oxidation (nmoles $^{14}CO_2$ produced/assay/hr)			
	No spermine	+ Spermine	Δ	% Stimulation
None	13.7±2.88	38.6±4.89	24.9±4.05	182±51.6
TAME	12.9±2.55	34.3±5.16	21.4±4.38	166±44.5
BAME	16.3±3.90	42.1±5.86	25.8±3.38	158±49.5
P value				
None vs TAME	NS	NS	NS	NS
None vs BAME	NS	NS	NS	NS

Fat cells were incubated with and without spermine (1 mM) in the presence and absence of TAME or BAME (5 mM) as described in Table I. Results are the mean ± SE of five individual experiments performed in triplicate.

induced internalization, negative cooperativity of insulin receptors and generation of insulin's second messenger from liver particulate fractions.

Studies on insulin-induced down regulation of insulin receptors showed that in contrast to the inhibitory effects of BAME on insulin-induced glucose oxidation, insulin-induced internalization of insulin receptor was not affected by BAME (Table 4). Experiments on insulin-induced enhancement of dissociation of hormone from receptors (Fig. 1) also showed that proteolytic inhibitors do not interfere with insulin's effect on negative cooperativity. Therefore, it appears that proteolytic events do not appear to be required for receptor internalization nor the postulated affinity change responsible for the "DeMeyts" effect. A recent report by Simpson and Hedo (17) suggests that receptor phosphorylation is not required for certain insulin effects.

Table 3: Effects of protease inhibitors on cell surface ^{125}I-insulin binding to adipocytes

Additions	Insulin Bound (pg/10^5 cells)
None	9.1±1.48
TAME	6.0±1.00
BAME	9.3±1.26
P value	
None vs TAME	< 0.05
None vs BAME	NS

Aliquots (0.1 ml) of fat cells were added to tubes containing 0.9 ml of Tris-albumin buffer with 0.5 ng ^{125}I-insulin with and without BAME or TAME (5 mM). The tubes were incubated for 3 hr at 16°C and the radioactivity in cells was counted. Non-specific binding was determined in the presence of excess of unlabeled insulin (100 μg/tube). Results are the mean ± SE of 4 individual experiments performed in triplicate.

Table 4: Effect of protease inhibitor on insulin-induced internalization of insulin receptors

Additions	Cell surface insulin binding (pg/10^5 cells)		
	No insulin	+ Insulin	% Decrease in cell surface binding
None	10.6±1.93	3.9±0.67	63±3.8
BAME	10.7±1.88	5.0±1.05	54±6.2
P value			
None vs BAME	NS	NS	NS

Aliquots (2 ml) of fat cells were pretreated with and without insulin (100 ng/ml) in the presence and absence of BAME (5 mM) for 2 hr at 37°C, allowed to dissociate for 1 hr at 37°C, washed and used for binding studies as described in Table III. Results are the mean ± SE of 5 individual experiments performed in triplicate.

The results reported in this paper support that conclusion for hormone internalization and the DeMeyts effect which are not inhibited under conditions reported to decrease insulin induced receptor phosphorylation (18).

Tables 5 and 6 indicate that the protease inhibitor BAME inhibits the generation of both the inhibitor and stimulator of PDH by insulin exposed liver particulate fractions. Thus it may be suggested that proteolytic events are required for second messenger generation by insulin in both

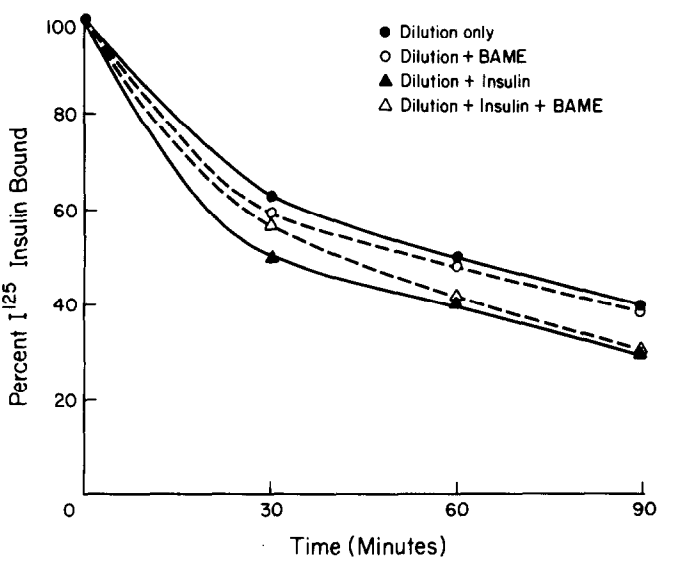

Figure 1. Dissociation of previously bound ^{125}I-insulin at 16°C into insulin free buffer and buffer containing unlabeled insulin (100 ng/ml) in the presence and absence of BAME. Studies were performed with isolated adipocytes.

Table 5: Effect of protease inhibitor on insulin-induced generation of PDH stimulator from liver particulate fractions

Additions	Mitochondrial PDH (nmoles $^{14}CO_2$ produced/mg protein/10 min) Basal activity	+ control mediator	+ Insulin mediator	Δ	% Stimulation
None	2.6±0.49	3.6±0.68	5.4±0.76	1.8±0.16	50± 9.7
BAME		3.5±0.42	3.0±0.64	(-)0.5±0.36	(-)14±10.7
P value					
None vs BAME		NS	< 0.05	< 0.005	< 0.005

Liver particulate fractions were treated with and without BAME (2 mM) at 4°C for 30 min, then incubated with and without insulin (100 μU/ml) for 10 min at 37°C. The mediators released into the supernatant were extracted and lyophilized. The alcohol insoluble residue was resuspended in 1 mM formic acid and 10 μl aliquots were tested for their ability to activate mitochondrial PDH. Results are the mean ± SE of 4 individual experiments performed in triplicate.

adipocyte plasma membranes (3) as well as liver particulate fractions and for stimulation of glucose oxidation. Studies by Tamura et al (18) also showed that insulin-induced phosphorylation of insulin receptor involves a proteolytic event and this was supported by the observations that trypsin could mimick insulin's effect on receptor phosphorylation.

In summary, we have shown that certain actions of insulin, namely glucose oxidation and second messenger generation are mediated by protease activation, while insulin-induced internalization and negative cooperativity of the receptors do not appear to involve proteolytic events.

Table 6: Effect of protease inhibitor on insulin-induced generation of PDH inhibitor by liver particulate fractions.

Additions	Mitochondrial PDH (nmoles CO_2 produced/mg protein/10 min) Basal activity	+ Control inhibitor	+ Insulin inhibitor	Δ	% Inhibition
None	4.7±0.25	3.1±0.21	2.0±0.20	1.1±0.10	36±3.0
BAME		3.5±0.04	3.2±0.24	0.3±0.25	9±7.0
P value					
None vs BAME		NS	< 0.010	< 0.025	< 0.025

Insulin mediators were prepared from BAME (2 mM) exposed liver particulate fractions as described in Table V. The inhibitery material was separated by alcohol extraction. The alcohol soluble material was evaporated to dryness, resuspended in 1 mM formic acid and aliquotes ((10μl) were tested for their ability to inhibit liver mitochondrial PDH in the absence of ATP. Results are the mean ± SE of 4 individual experiments performed in triplicate.

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