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Selective Inhibition of the Insulin-Stimulated Phosphorylation of the 95,000 Dalton Subunit of the Insulin Receptor by TAME or BAEE

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Added Nα-p-tosyl-l-arginine methyl ester or Nα-benzoyl-l-arginine ethyl ester inhibited the stimulation by insulin of phosphorylation of the 95,000 dalton subunit of the insulin receptor both in a partially purified insulin receptor fraction from rat adipocytes and in a highly purified insulin receptor preparation from human placenta. N-α-p-tosyl-l-lysine chloromethyl ketone, Nα-p-tosyl-l-lysine methyl ester, or N-acetyl-l-phenylalanine ethyl ester were much less potent, while N-benzoyl-l-alanine methyl ester was without effect. Inhibition of the phosphorylation by the arginine analogues did not require preincubation of the insulin receptor with inhibitors in the presence of insulin prior to phosphorylation. Inhibition by Nα-p-tosyl-l-arginine methyl ester was decreased by preincubation of the receptor fraction with cold ATP and $MnCl_2$. These results suggest that Nα-p-tosyl-l-arginine methyl ester inhibits an initial ATP and Mn^{2+} dependent reaction in insulin-stimulated phosphorylation process.

Following the initial demonstration of insulin mediator and the suggestion that mediator was a peptide or peptide-like molecule (1), Seals and Czech reported that treatment of adipocyte plasma membranes with insulin or trypsin generated a mediator which stimulated mitochondrial pyruvate dehydrogenase (2). They further reported that arginine ester substrate analogues which inhibited trypsin, also inhibited insulin-dependent generation of mediator and suggested that insulin activated a trypsin-like protease which generated mediator. Subsequently, we reported that treatment of intact adipocytes with trypsin generated insulin-like (but not insulin-identical) mediator which activated glycogen synthase phosphatase (3).

The abbreviations used are: TAME, Nα-p-tosyl-l-argine methyl ester; BAEE, Nα-benzoyl-l-argine ethyl ester; TLCK, Nα-p-tosyl-l-lysine chloromethyl ketone; PMSF, phenyl methyl sulfonylfluoride; HEPES, n-2-hydroxyethyl piperazine N-2-ethane sulfonic acid.

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Trypsin added to an insulin receptor fraction from adipocytes or to a highly purified insulin receptor from human placenta stimulated the phosphorylation of polypeptides derived from the α and β chains of the insulin receptor (4). Stimulation of insulin receptor phosphorylation by trypsin may thus be related to its metabolic and growth promoting actions now well documented in a number of cell types. We were therefore interested to determine whether an early proteolytic event is involved in the insulin dependent stimulation of phosphorylation of insulin receptor. To this end we had previously examined the effect of proteolytic inhibitors TLCK and bacitracin and found that they blocked insulin action in adipocytes (5). In the present communication we demonstrate that activation of insulin receptor phosphorylation by insulin is selectively inhibited by TAME or BAEE, arginine specific substrate analogue inhibitors of proteases. Inhibition by TAME is decreased by preincubation of the receptor with both cold ATP and Mn^{2+}. Two possible mechanisms are discussed.

Materials

Collagenase was obtained from Worthington. Crystalline porcine insulin was obtained from NOVO Laboratories (Copenhagen) and assayed at 25 units/mg. Wheat germ agglutinin coupled to agarose was from Miles. PMSF, Triton X-100, Hepes, N-acetylglucosamine, phosphorylase b, histone (f2b), TAME, BAEE, TLCK, Nα-p-tosyl-l-lysine methyl ester, N-benzoyl-l-alanine methyl ester, and N-acetyl-l-phenylalanine ethyl ester were from Sigma.

Pansorbin was from Calbiochem-Behring. All reagents for $NaDodSO_4$-polyacrylamide gel electrophoresis were from Bio-Rad. [γ-^{32}P]ATP was a gift from Dr. G. E. Vandenhoff (Diabetes Center, University of Virginia, Charlottesville, VA). Serum from a patient containing autoantibodies against the insulin receptor was a gift from Dr. C.R. Kahn (Joslin Research Laboratory, Boston, MA).

Methods

Adipocytes were prepared by standard methods from epididymal tissue of fed Sprague-Dawley rats weighing 140-180 g (6). Experiments were performed in plastic tubes using Krebs-Ringer phosphate buffer, pH 7.4, containing 3% bovine serum albumin (Fraction V, Sigma). Solubilized insulin receptor fraction was prepared from rat adipocytes as previously described (7), as was the highly purified insulin receptor from human placenta(8).

A 180 μl portion of solubilized insulin receptor fraction from rat adipocytes was incubated with or without insulin or other additions in the presence of 85 mM Hepes buffer, pH 7.6, containing 6.3 mM $MgCl_2$ and 0.004% bovine serum albumin in a final volume of 300 μl at 4° for 30 min. The phosphorylation reaction was begun by the addition of 75 μl of a solution containing 25 μM [γ-^{32}P]ATP (66.7 μCi/nmol) and 10 mM $MnCl_2$ and the reaction was continued for 10 min at 4°. Phosphorylation was stopped by adding 150 μl of a solution containing 40 mM Hepes buffer, pH 7.6, 0.4% Triton X-100, 20 mM EDTA, 200 mM KF, 100 mM ATP, 20 mM sodium pyrophosphate and 40 mM sodium phosphate. Each fraction was incubated with human serum containing anti-insulin receptor antibody (1:200 dilution) with 4 mM PMSF for 18 h at 4°. Immunoprecipitates were purified with protein A, solubilized and submitted to $NaDodSO_4$ polyacrylamide gel electrophoresis and autoradiography as described previously (4). Density of the 95,000 dalton bands was obtained by densitometric scan and used as a measure of relative degree of phosphorylation.

Glycogen synthase I was prepared from rabbit skeletal muscle as described previously (9,12). Catalytic subunit of cAMP dependent protein kinase was partially purified from the pH 6.1 supernatant of rabbit muscle by elution from DEAE cellulose by 5 mM cyclic AMP, followed by chromatography on hydroxyapatite. Glycogen synthase kinase-3 was prepared from rabbit muscle and assayed according to the method by Hemming et. al. (10). Phosphorylase kinase was prepared from rabbit muscle as reported by Cohen (11). Activity of the catalytic subunit of cAMP-dependent protein kinase was assayed using histone as substrate (12).

Results and Discussion

TAME and BAEE added to a solubilized insulin receptor fraction from rat adipocytes in the presence of insulin inhibited insulin stimulated phosphorylation of the 95,000 dalton subunit of the insulin receptor in a dose dependent manner (Fig. 1). Concentrations of 0.1, 1, or 5 mM inhibited by 22.1%, 31.3% and 87.8% respectively. BAEE, 0.1, 1, or 5 mM inhibited phosphorylation by 21.1%, 78.1% and 99.9% respectively. TLCK, Nα-p-tosyl-l-lysine methyl ester or N-acetyl-l-phenylalanine ethyl ester 5 mM were weakly inhibitory (26.0%, 27.1% or

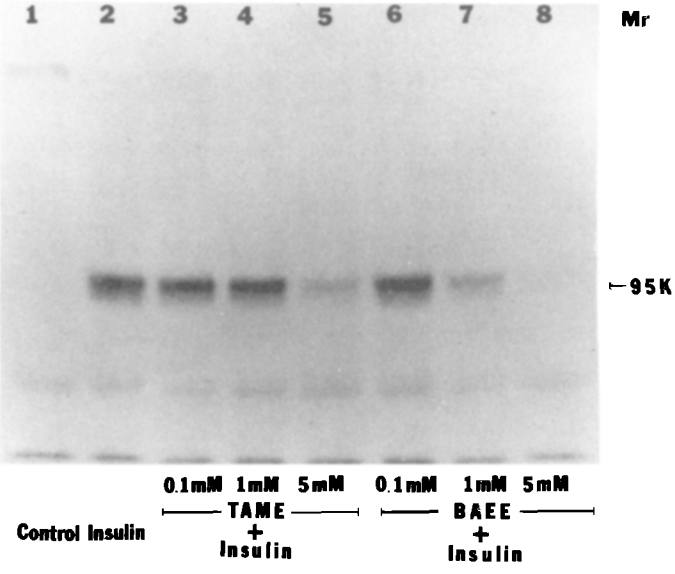

Figure 1. Dose response of TAME or BAEE effect on phosphorylation of insulin receptor.

A 180 µl aliquot of the insulin receptor fraction was incubated in Hepes buffer (50 mM), pH 7.6, $MgCl_2$ (6.3 mM), an bovine serum albumin (0.004%) with the following additions in a final volume of 300 µl at 4° for 30 min, lane 1; no addition, lane 2; insulin (10 milliunits/ml), lane 3; insulin (10 milliunits/ml) and TAME (0.1 mM), lane 4; insulin (10 milliunits/ml) and TAME (1 mM), lane 5; insulin (10 milliunits/ml) and TAME (5 mM), lane 6; insulin (10 milliunits/ml) and BAEE (0.1 mM), lane 7; insulin (10 milliunits/ml) and BAEE (1 mM), lane 8; insulin (10 milliunits/ml) and BAEE (5 mM). The phosphorylation reaction was started by adding 75 µl of solution containing [γ-^{32}P]ATP (25 µM, 66.7 µCi/nmol) and $MnCl_2$ (10 mM) and incubated for 15 min at 4°. Other experimental conditions are described in the text. K represents 1000.

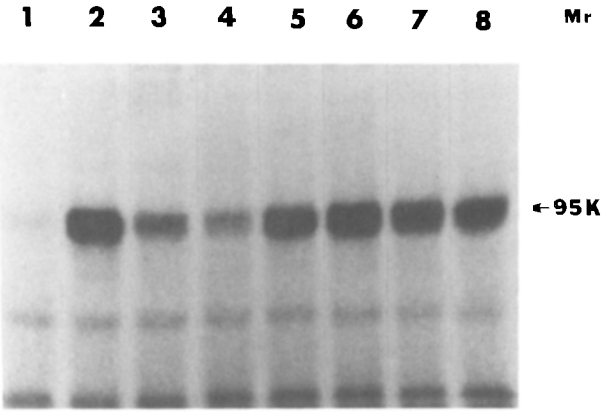

Figure 2. Specificity of amino acid analogue esters

A 180 µl aliquot of the insulin receptor fraction was incubated in Hepes buffer (50 mM), pH 7.6, $MgCl_2$ (6.3 mM), and bovine serum albumin (0.004%) with the following additions in a final volume of 300 µl at 4° for 30 min., lane 1; no addition, lane 2; insulin (10 milliunits/ml), lane 3; insulin (10 milliunits/ml) and TAME (5 mM), lane 4; insulin (10 milliunits/ml) and BAEE (5 mM), lane 5; insulin (10 milliunits/ml) and Nα-p-tosyl-l-lysine chloromethyl ketone (5 mM), lane 6; insulin (10 milliunits/ml) and N-benzoyl-l-alanine methyl ester (5 mM), lane 7; insulin (10 milliunits/ml) and Nα-p-tosyl-l-lysine methyl ester (5 mM) and lane 8; insulin (10 milliunits/ml) and N-acetyl-l-phenylalanine ethyl ester (5 mM). The phosphorylation reaction was started by adding 75 µl of solution containing [γ-^{32}P]ATP (25 µM, 66.7 µCi/nmol) and $MnCl_2$ (10 mM) and incubated for 15 min. at 4°. Other experimental conditions are described in the text. K represents 1000.

22.2% respectively) (Fig. 2). N-benzoyl-l-alanine methyl ester had no detectable effect. Thus, the insulin-stimulated phosphorylation of the 95,000 dalton receptor subunit is inhibited selectively by two arginine specific inhibitors with BAEE more potent than TAME (Fig. 2).

Added TAME or BAEE (5 mM) inhibited insulin-enhanced phosphorylation of the highly purified insulin receptor from human placenta by 72.6% or 87.6% respectively, suggesting that both TAME and BAEE act directly on the insulin receptor to inhibit insulin-enhanced phosphorylation (Fig. 3).

To study the mechanism of the action of TAME on phosphorylation, the action of TAME added initially together with insulin was compared with that of TAME added following insulin but with [γ-^{32}P]ATP and $MnCl_2$. As shown in Table 1, TAME added after insulin with [^{32}P]ATP and $MgCl_2$ inhibited receptor phosphorylation to the same extent as TAME added initially with insulin. Thus TAME most likely acts after the addition of [γ-^{32}P]ATP and $MnCl_2$ since these are common reagent additions in both conditions, whereas preincubation with insulin is not common.

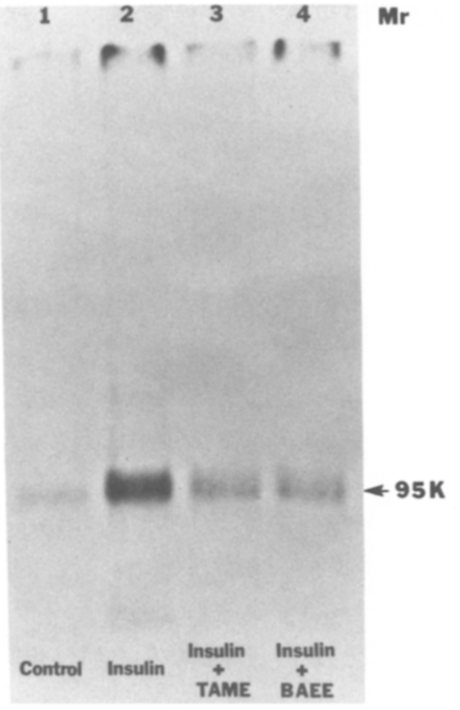

Figure 3. Effect of TAME or BAEE on phosphorylation of highly purified insulin receptor.

Purified insulin receptor from human placenta was incubated in buffer containing Tris-HCl (50 mM), pH 7.5, Triton X-100 (0.1%) without (lane 1) or with (lane 2 to 4) insulin (10 milliunits/ml) in a final volume of 40 µl at 4° for 18 h. Samples were then incubated with [γ-^{32}P]ATP (5 M, 32 µCi/nmol) and $MnCl_2$ (2 mM) without (lane 1 and 2) or with TAME (5 mM, lane 3) or BAEE (5 mM, lane 4) in a final volume of 50 µl at 25° for 1 h. Phosphorylation was stopped and the insulin receptor precipitated by adding anti-insulin receptor antibody containing serum. Other experimental conditions are described in the text. K represents 1000.

TABLE I

	Relative Phosphorylation
	%
1 control	6.5
2 insulin	100
3 insulin and TAME	32.2
4 insulin, then TAME	33.5

A 180 µl aliquot of the insulin receptor fraction was incubated in Hepes buffer (50 mM), pH 7.6, $MgCl_2$ (6.3 mM), and bovine serum albumin (0.004%) with (Experiment 3) or without (Experiment 1,2 or 4) TAME (5 mM) in the presence (Experiments 2,3 or 4) or absence (Experiment 1) of insulin (10 milliunits/ml) in a final volume of 300 µl.

The sample was then incubated with [γ-^{32}P]ATP (5 µM, 55.7 µCi/nmol) and $MnCl_2$ (2 mM) with (Experiment 4) or without (Experiment 1,2 or 3) addition of TAME (final concentration: 5 mM) in a final volume of 375 µl for 15 min at 4°. Other experimental conditions are described in the text.

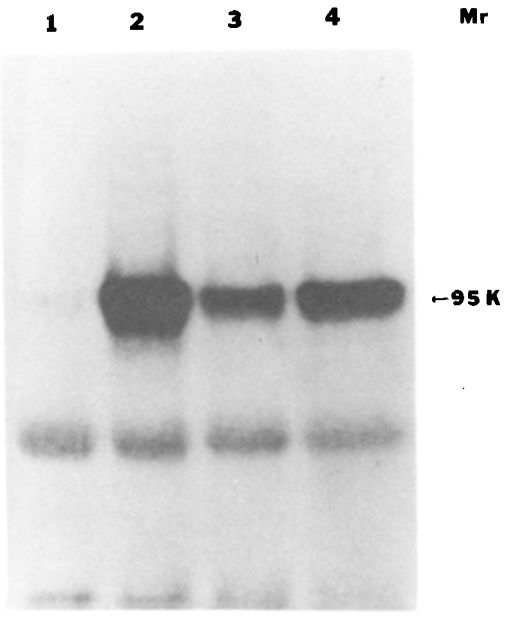

Figure 4 Effect of pretreatment with ATP and $MnCl_2$.
Insulin receptor fraction was incubated without (lane 1) or with (lane 2 to 4) insulin (10 milliunits/ml) at 4° for 30 min and was then incubated with ATP (4 μM) and $MnCl_2$ (2 mM) for 7 min at 4° in the absence (lane 1,2 and 4) or presence of TAME (5 mM). Samples were then added with [γ-^{32}P]ATP (final concentration 5 M, 66.7 μCi/nmol) together without (lane 1 to 3) or with (lane 4) the addition of TAME (final concentration; 5 mM) for 15 min at 4°. Other experimental conditions are described in the text. K represents 1000.

We therefore next examined the ATP and Mn^{2+} requirement of inhibition of phosphorylation by TAME. To accomplish this, we employed 2 incubations with ATP, the first with cold, then a second with labeled [γ-^{32}P]ATP. For these experiments, the concentration of ATP was divided such that the initial cold ATP concentration was 4 μM followed by a concentration of [γ-^{32}P]-ATP of 1 μM in place of the usual final concentration of [γ-^{32}P]-ATP of 5 μM. After incubating insulin receptor with insulin and then with cold ATP and $MnCl_2$, we compared the effect of adding TAME with cold ATP and $MnCl_2$ with the effect of adding TAME with the subsequent [γ-^{32}P]-ATP and $MnCl_2$ following the preincubation with cold ATP (Fig. 4). The inhibition of insulin-stimulated phosphorylation of the insulin receptor by TAME added together with cold ATP and $MnCl_2$ (early addition) was 81.2% (Fig. 4, lane 2 vs lane 3). TAME added together with [γ-^{32}P]ATP after prior incubation of the insulin receptor with cold ATP and $MnCl_2$ without TAME (late addition) inhibited phosphorylation of the insulin receptor by 49.5% (lane 2 vs lane 4). Thus adding TAME early together with

cold ATP and $MnCl_2$ resulted in increased inhibition compared to that observed when TAME was added later with [^{32}P]ATP. This experiment suggests that inhibition by TAME added early is dependent on the presence of ATP and $MnCl_2$. When the insulin receptor fraction was incubated in the same format with either cold ATP or $MnCl_2$ separately, adding TAME late did not decrease the inhibition by TAME of insulin-enhanced receptor phosphorylation (data not shown). This demonstrates that both cold ATP and Mn^{2+} are required to decrease the inhibition by late addition of TAME.

We next examined the time course of incubating insulin receptor with cold ATP and $MnCl_2$ prior to late addition of TAME. Inhibition by TAME of phosphorylation of insulin receptor was a time dependent reaction suggesting that it is an enzymatic reaction (data not shown).

The following two possibilities should be considered for explaining the mechanism of inhibition by TAME of insulin-enhanced receptor phosphorylation. (I) TAME inhibits an ATP and Mn^{2+} stimulated arginine specific protease whose activation controls insulin-enhanced receptor phosphorylaton. (2) TAME directly inhibits an insulin stimulated protein kinase.

If possibility (1) is the case, ATP and Mn^{2+} are requirements for the protease for the following two reasons.

a) TAME added together with [γ-^{32}P]ATP and $MnCl_2$ after incubation of the insulin receptor fraction with insulin, inhibited insulin enhanced phosphorylation to the same extent as TAME added at the same time as insulin.

b) Incubation of the insulin receptor with insulin and cold ATP and $MnCl_2$ in the presence of TAME before the addition of [γ-^{32}P]ATP increased the inhibition by TAME of the phosphorylation of the insulin receptor.

Thus (1) binding of insulin to its surface receptor may stimulate a protease in the insulin receptor which requires ATP and Mn^{2+}. Stimulation of the protease subsequently triggers activation of protein kinase by proteolysis. Such proteolytic activation is well known with several protein kinases (13,14). Possibility (2), however, cannot be ruled out. When we tested the effect of TAME on 3 protein kinases, TAME (10 mM) inhibited the C-subunit of cAMP-dependent protein kinase,

phosphorylase b kinase and glycogen synthase kinase 3 by 47%, 38%, or 30% respectively. TAME however at a concentration lower than 5 mM or BAEE at concentrations up to and including 10 mM were without effect (data not shown). These results demonstrate that at least TAME can act as a non-specific inhibitor of protein kinases.

If possibility (2) is the case, the present data indicate that there are at least 2 separate phosphorylation reactions. The first kinase reaction is extremely sensitive to TAME, while the second is much less sensitive.

Regardless of the mechanism, the present experiments demonstrate that arginine esters specifically inhibit insulin-stimulated receptor phosphorylation and are therefore suggested as potentially valuable reagents to probe insulin receptor phosphorylation chemistry and function.

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