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TRIS(HYDROXMETHYL)AMINOMETHANE PERMITS THE EXPRESSION OF INSULIN-INDUCED RECEPTOR LOSS IN ISOLATED RAT ADIPOCYTES

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SUMMARY: The effect of Tris(hydroxymethyl)aminomethane on insulin-induced loss of cell-surface insulin receptors was studied in vitro using isolated rat adipocytes. In the presence of insulin, Tris(5-50mM) resulted in a dose-dependent loss of insulin receptors, whereas Tris alone was without effect. Loss of receptors after exposure to insulin and Tris was prevented by inhibiting endocytosis with 2,4,dinitrophenol or low temperature (16°), indicating Tris acts at an intracellular site distal to insulin binding. These results combined with our previous findings indicate that Tris mediates insulin-induced receptor loss by preventing insulin receptors from recycling.

INTRODUCTION: We have previously shown that incubation of isolated rat adipocytes with insulin leads to a rapid loss of cell-surface insulin receptors (1,2), and the first step in this process is most likely endocytotic uptake of receptor complexes after insulin binding (3-5). More recently, we have found that insulin induces a net loss of surface receptors only when cells are incubated in a simple buffer. In a more physiological "complete medium" down-regulation is not observed after insulin treatment, although these cells continue to internalize and degrade insulin by a receptor-mediated endocytotic process (5). Since it is likely that insulin and its receptor are internalized together (3-5), we explained these results by postulating that insulin-receptor complexes are internalized in complete medium; however, a net loss of cell-surface receptors does not occur because of concomitant insertion of insulin receptors into the plasma membrane. Since no large preformed pool of intracellular insulin receptors was found, and since inhibition of receptor synthesis by cycloheximide did not lead to the expression of insulin-

Abbreviations: Tris, Tris(hydroxymethyl)aminomethane; Tricine, Tris (hydroxymethyl)methyglycine; Hepes, 4-(2-hydroxymethyl)-1-piperazine-2-ethanosulfonic acid; BSA, bovine serum albumin; DNP, 2,4,dinitrophenol; MEM, minimal essential medium.

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induced receptor loss (5), these findings suggested that the absence of insulin-induced receptor loss in complete medium was due to recycling of insulin receptors.

The present investigation was undertaken to delineate either the constituents of the simple buffer which lead to a loss of receptors when cells are exposed to insulin, or the components of the complete medium which prevent insulin-induced receptor loss. These studies reveal that Tris(hydroxymethyl)aminomethane, a commonly used physiological buffering agent permits the expression of insulin-induced down-regulation, most probably by preventing the recycling of insulin receptors

MATERIALS AND METHODS: Isolated rat adipocytes were obtained from the epididymal fat pads of male Sprague-Dawley rats weighing 160 to 225 g as previously described (1).

Insulin Pretreatment and Dissociation Procedures: Adipocytes were suspended in pH 7.6 medium containing MEM, 10mM HEPES, 25mM Tricine and 1% BSA unless otherwise indicated, and incubated with 100 ng/ml insulin in polypropylene flasks. Cells were then gently agitated in a shaking water bath for the indicated times and temperatures. At the end of the incubation period, cells were transferred to 16 X 125mm polystyrene tubes, centrifuged at 200 rpm for 2 min, and the insulin containing buffer removed and replaced with insulin free medium at pH 7.0. Adipocytes were again centrifuged, resuspended in medium, transferred to 25 ml flasks and receptor-bound insulin allowed to dissociate at pH 7.0 for 1 h at 37°C. After the 1 h dissociation, cells were washed and resuspended in medium pH 7.6 for determination of specific ^{125}I-insulin binding. Measurements at the end of this dissociation period (at the lowered pH, 7.0) have shown that all receptor-bound insulin and any insulin subsequently internalized (including subsequently generated degradation products) are effectively dissociated or released by this procedure (1,2).

Binding Studies: Isolated adipocytes (2-3 X 10^5 cells) were incubated in a total volume of 1 ml (medium, pH 7.6) with ^{125}I-insulin (prepared as previously described) (1) in the absence or presence of 50 µg of unlabeled insulin. Incubations were performed in polypropylene tubes (17 X 100mm) and free ^{125}I-insulin separated from cell-bound radioactivity by removing aliquots (300µl) from the cell suspension and rapidly centrifuging the cells in plastic microtubes to which 100 µl of silicone oil had been added. The cells were then removed and the radioactivity was determined. Adipocyte-associated radioactivity was determined in triplicate from each incubation tube, and each experiment is a representative example of at least three similar experiments unless otherwise indicated. For all samples total and non-specific binding was determined, and the total binding was corrected to reflect specific insulin binding.

RESULTS AND DISCUSSION: Adipocytes preincubated for 2 h at 37° in simple buffer containing 100 ng/ml of insulin bound 49% less ^{125}I-insulin than cells similarly treated but not exposed to insulin (Table 1). Scatchard analysis of insulin binding data revealed that the reduced binding capacity was due to a decrease in insulin receptor number, rather than a change in receptor affinity (not shown). In complete medium specific insulin binding was unaffected by insulin treatment (Table 1).

TABLE 1: Insulin-Induced Receptor Loss in a Simple Buffer and a Complete Medium.

Treatment	Specific ^{125}I-Insulin Binding (%)	Loss of Insulin Receptors
Simple buffer	4.68 ± .21	
Simple buffer + 100 ng/ml Insulin	2.39 ± .13	49%
Complete medium	4.76 ± .29	
Complete medium + 100 ng/ml Insulin	4.61 ± .34	3%

Adipocytes suspended in a simple buffer (120mM NaCl, 10mM dextrose, 35mM Tris, 1.2mM $MgSO_4$, 2mM Ca_2Cl, 2.5mM KCl and 1% BSA) or a complete medium (MEM, 10mM Hepes, 25mM Tricine, 1% BSA and 15% fetal calf serum) were incubated in the presence or absence 100 ng/ml insulin for 2 h at 37°. Specific ^{125}I-insulin binding was then determined at 16° after extracellular insulin was removed and receptor-bound insulin dissociated. Insulin binding represents the mean ± SEM of duplicate incubations sampled in triplicate, and is expressed as a percentage of the total ^{125}I-insulin added.

To more clearly define the incubation conditions that result in insulin-induced receptor loss in buffer but not in medium, we evaluated several components of these two incubation systems. Cells were preincubated with insulin (100 ng/ml) for 2 h at 37° under the conditions depicted in Fig. 1, washed to remove extracellular and

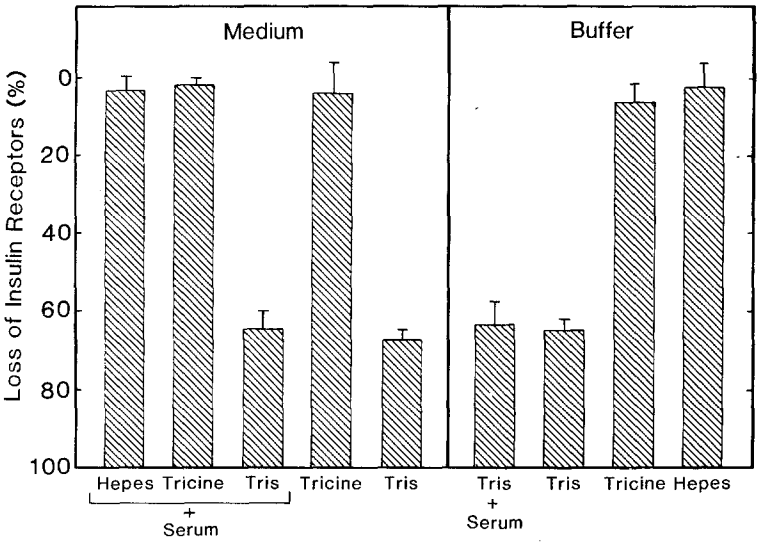

FIGURE 1: Effect of several buffering agents and 15% fetal calf serum on insulin-induced receptor loss. Cells were preincubated with 100 ng/ml insulin for 2 h at 37° in either complete medium or simple buffer containing 35mM of the indicated buffer agents. Receptor-bound insulin was then dissociated and specific ^{125}I-insulin binding determined at 16° after 3 h. The results are expressed as a percentage decrease in specific binding compared to control cells similarly treated but not exposed to insulin. Values represent the mean ± SEM 3 separate experiments.

TABLE 2: Effect of 2,4,Dinitrophenol and Low Temperature (16°) on Tris-Mediated Insulin-Induced Receptor Loss.

Treatment			Specific ^{125}I-Insulin Binding (%)	Loss of Insulin Receptors (% of Control)
Exp. 1	Control	37°	3.86 ± .07	
	Insulin			
	100 ng/ml	37°	1.75 ± 0.2	54.6
	100 ng/ml	16°	3.85 ± .14	0
Exp. 2	Control	37°	4.46 .16	
	Insulin			
	100 ng/ml	37°	2.48 .04	44.4
	100 ng/ml + DNP	37°	4.10 .06	8.1

Experiment 1: Cells were suspended in medium and exposed to insulin and Tris (35mM) for 2 h at either 16° or 37°, while control cells were exposed to Tris alone. After receptor-bound insulin was dissociated, specific ^{125}I-insulin binding was measured at 16°. Experiment 2: Cells were incubated for 2 h at 37° with Tris alone (controls) or Tris plus insulin in the absence or presence of 0.5mM DNP. Insulin binding represents the mean ±SEM of duplicate incubations sampled in triplicate, and is expressed as a percentage of the total ^{125}I-insulin added.

receptor-bound insulin, and specific ^{125}I-insulin binding then measured at 16°. These results show that insulin-treatment did not produce a loss of insulin receptors when cells were suspended in buffer or medium that contained Hepes, Tricine or fetal calf serum. However, when Tris was included in either the medium or buffer along with insulin, a marked insulin-induced receptor loss was seen. For each treatment group in Fig. 1 a control group was included that was similarly treated but not exposed to insulin, and no differences in binding were noted among these groups (data not shown). Thus, the Tris-mediated loss of receptors occurs only in the presence of insulin, and indicates that Tris neither inhibits insulin binding nor independently accelerates receptor loss.

To ascertain whether Tris mediates insulin-induced receptor loss at an intracellular site, we inhibited endocytosis by using low temperature (16°) or a metabolic energy depleter (DNP), and then measured receptor loss after insulin and Tris treatment (Table 2). We (6,7) and others (8-11) have previously demonstrated that these conditions prevent receptor-mediated internalization of ^{125}I-insulin, and our current results show that these same conditions prevent insulin receptor loss when cells are exposed to both insulin and Tris. Therefore, we infer

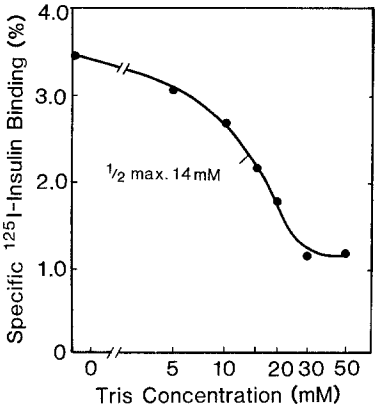

FIGURE 2: Insulin-induced receptor loss in the presence of several concentrations of Tris. Adipocytes were preincubated in medium with 100 ng/ml and the indicated concentrations of Tris for 3 h at 37°. Specific ^{125}I-insulin binding was subsequently determined at 16°, and is expressed as a percent of the total ^{125}I-insulin added. Each point represents the mean of triplicate determinations.

that Tris acts distal to insulin binding and uptake (at an intracellular site) to mediate insulin-induced down-regulation.

Inhibition of receptor recycling appears to be the mechanism whereby Tris mediate insulin-mediated down-regulation, and we base this conclusion on several lines of evidence. First, we have previously presented data indicating that adipocytes exposed to insulin in "complete medium" internalize insulin receptors then recycle these receptors back to the cell-surface so there is nominal loss of cell-surface receptors, whereas in simple buffer recycling is impaired resulting in a rapid loss of receptors during exposure to insulin (5). The present studies show that Tris (hydroxymethyl)aminomethane mediates insulin-induced down-regulation at an intracellular site. Thus, the absence of down-regulation in "complete medium" is not due to the more "physiological incubation conditions", but rather to the omission of Tris. Other possibilities also exist to explain the ability of Tris to mediate insulin-induced receptor loss. For example, Tris could interact directly with insulin receptors to inhibit binding, it could accelerate receptor endocytosis independent of insulin, or Tris could decrease the rate at which newly synthesized receptors are inserted into the plasma membrane. However, since Tris in the absence of insulin did not affect subsequently measured specific ^{125}I-insulin binding, these possibilities seem less likely. Therefore, when all the observations

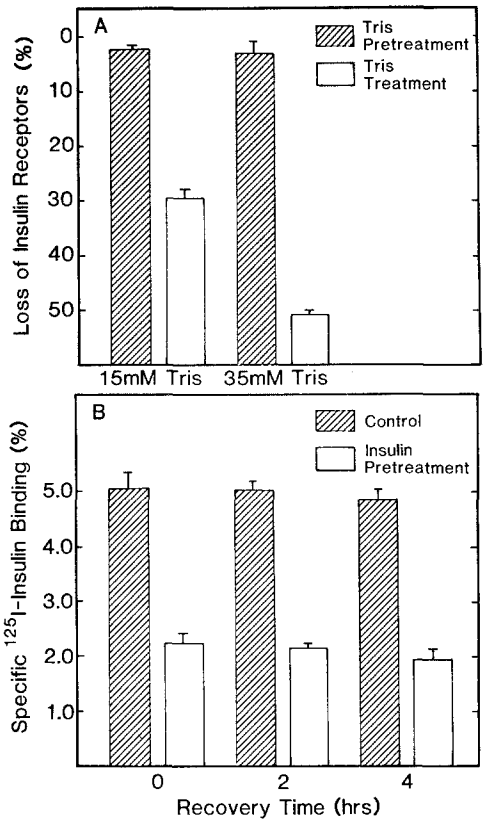

FIGURE 3: (A) Effect of Tris pretreatment on subsequent insulin-induced receptor loss. Cells were incubated with Tris at 37° for 2 h, then incubated in Tris-free medium for an additional hour (hatched bars). Insulin (100 ng/ml) was then added and the cells were further incubated for 2 h before the extent of receptor loss was assessed by measuring specific ^{125}I-insulin binding at 16°. The white bars represent cells in which Tris was added only during the final 2 h incubation with insulin. Results are expressed as a percentage decrease in specific insulin binding compared to control cells similarly treated but not exposed to insulin. Bars represent the mean ± SEM of 3 separate experiments.

(B) Specific ^{125}I-insulin binding over time in down-regulated cells incubated in Tris-free medium. Insulin receptors were down-regulated by exposing cells to insulin (100 ng/ml) in the presence of Tris (35mM) for 2 h at 37°. Cells were then washed to remove both Tris and insulin, and receptor-bound insulin was dissociated. Adipocytes were then incubated in Tris-free medium at 37° for the indicated times before specific ^{125}I-insulin binding was measured at 16° (white bars). Control cells (hatched bars) were similarly treated but not exposed to insulin. Binding is expressed as a percent of the total ^{125}I-insulin added and represents the mean ± SEM of 3 separate experiments.

are considered together, it appears that Tris mediates insulin-induced receptor loss by preventing internalized receptors from recycling.

The relationship between the concentration of Tris and the extent of insulin-induced receptor loss is depicted in Fig. 2. Cells preincubated for 3 h at 37°

with a maximally effective insulin concentration (100 ng/ml) and several concentrations of Tris (5-50mM) showed a dose-dependent decrease in ^{125}I-insulin binding, with a 1/2 maximally effective Tris concentration of 14mM, a maximal effect at 30 mM, and a plateau of receptor loss at 30-50mM Tris.

To determine if pretreatment of adipocytes with Tris results in receptor loss when cells are subsequently exposed to insulin alone, we pretreated cells with either 15 or 35mM Tris for 2 h at 37°, incubated adipocytes for another hour at 37° in Tris-free medium, then exposed cells to 100 ng/ml insulin for 2 h at 37°. Control cells were similarly incubated, except Tris was included in the final 2 h incubation with insulin. The results of this experiment (Fig. 3A) show that both Tris and insulin must be present together in order for cells to undergo receptor down-regulation. The loss of insulin receptors under these conditions appears irreversible since reappearance of insulin receptors is not observed when down-regulated cells are incubated in Tris-free medium for up to 4 h (Fig. 3B). This suggests that receptors which are internalized by cells treated with Tris plus insulin are either degraded intracellularly, or are sequestered within cells such that they are no longer able to recycle.

The finding that a commonly used physiological buffering agent, Tris, can permit the expression of insulin-induced receptor loss should have important implications in the interpretation of receptor studies involving down-regulation and recycling, and may provide a valuable research tool for future studies investigating these cellular phenomenon.

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