STIMULATION BY INSULIN OF CYCLIC AMP PHOSPHODIESTERASE. ROLE OF GLUTATHIONE-INSULIN TRANSHYDROGENASE.*

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SUMMARY: In agreement with other workers, exposure of isolated rat fat cells to insulin shows a dose dependent increase in cyclic AMP phosphodiesterase (PDE) activity. However, when fat cells are pre-exposed to either guinea pig antiserum against insulin, rabbit antiserum against glutathione-insulin transhydrogenase (GIT), or immunogamma globulin against GIT, each antibody preparation totally or almost totally abolished the insulin-dependent increase in PDE activity. In control experiments, appropriate normal (non-immune) sera, normal gamma globulin, or the GIT-antiserum or the GIT-immunogamma globulin which had been previously neutralized with purified rat liver GIT were found to be completely ineffective in abolishing the insulin-dependent PDE activity of fat cells. These results suggest that the GIT-catalyzed sulfhydryl-disulfide interchange reaction with insulin might be part of the mechanism by which insulin regulates the intracellular cyclic AMP concentration.

There is ample evidence in the literature that adenosine 3', 5'-cyclic monophosphate participates in many cellular reactions and is probably the intracellular agent which mediates the actions of a number of hormones, including insulin (1). Insulin lowers the intracellular concentration of cAMP in liver and adipose tissue (2,3). This effect of insulin appears to be, at least in part, the result of a stimulatory action of the hormone on the catabolizing enzyme, adenosine 3', 5'-cyclic monophosphate phosphodiesterase (E.C. 1.1.4.17; PDE) (4-9). The mechanism by which insulin exerts its effect on this enzyme, however, remains unknown.

Glutathione-insulin transhydrogenase (GIT, thiol: protein disulfide oxidoreductase, E.C. 1.8.4.2) catalyzes sulfhydryl-disulfide interchange between simple thiol compounds and certain disulfide-containing proteins (see references 10,11 for more details concerning GIT); among the naturally occurring substrates, insulin is the most preferred substrate (11). Although the reaction with insulin is reversible, in the presence of excess GSH in vitro the enzyme catalyzes the cleavage of disulfide bonds of the hormone to form A and B chains.

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GIT occurs ubiquitously (12). The level of GIT activity in various tissues is inversely related to the *in vitro* sensitivity of the tissues to insulin (12), suggesting that muscle, heart, fat, etc., exhibit *in vitro* insulin-dependent effects because they contain the lowest level of GIT activity, whereas liver, kidney, etc., exhibit little or no *in vitro* insulin-dependent effects because they contain the highest levels of GIT activity. However, considering that *in vivo* liver shows the most rapid and dramatic response to insulin, another opposite interpretation might be that aside from the function of disposing of insulin, GIT might also serve to regulate the action of insulin on various types of cells (12). Consistent with this possibility are also the findings (13-17) that the hepatic concentration of GIT is under feedback control by the circulating insulin level, i.e., its concentration in liver fluctuates in parallel to the insulin level in the blood.

There have been reports, from time to time, implicating the disulfide interchange reaction in the action of insulin. The involvement of sulfhydryl groups in the activity of the insulin-dependent particulate bound fat cell PDE (9) has also been recently noted. We, therefore, wondered as to whether the GIT-catalyzed disulfide interchange reaction with insulin is involved in the expression of the insulin effect on the intracellular cAMP concentration. The effect of antiserum against rat liver GIT on the ability of insulin to stimulate the PDE activity of isolated fat cells was therefore investigated. An abstract reporting some of the data has appeared previously (18).

MATERIALS AND METHODS: Cyclic AMP and 5'-nucleotidase (Grade IV) were purchased from Sigma, [^{3}H] cyclic AMP (37.7 Ci/nmole) and Aquasol from New England Nuclear, anion exchange resin (AG 1-X8, 200-400 mesh) from Bio-Rad, and collagenase from Worthington Corp. Porcine amorphous insulin was kindly donated by the Lilly Company. The preparations of purified rat liver GIT (19), and of antisera against insulin (20) and against rat liver GIT (19) have been described previously. One ml of antiserum against GIT was capable of neutralizing 140 units of GIT activity. Gamma globulin fractions of whole sera were prepared by ammonium sulfate precipitation (21). The immunogamma globulin fraction was dissolved in one-fourth the volume of original serum, i.e., it was capable of neutralizing 560 units of GIT activity per ml.

Preparation of Isolated Fat Cells. Epididymal fat pads were obtained from Holtzman male rats (weighing 140 to 190 g) that were deprived of food for about 18 hours before decapitation. Isolated fat cells were prepared essentially by the method of Rodbell (22) using a 45 min incubation period with collagenase

(1 mg/ml) in Krebs-Ringer bicarbonate buffer containing 2% bovine serum albumin and 1 mg of glucose/ml. The isolated cells were washed two times with the incubation buffer, once with Krebs-Ringer bicarbonate buffer containing 4% bovine serum albumin, and then suspended in the latter buffer.

Procedure for Incubation. Aliquots of the fat cell suspension (0.5 ml) and 1.4 ml of Krebs-Ringer bicarbonate buffer with 4% bovine serum albumin were placed in polyethylene vials (Packard Instrument Co.). In experiments in which sera or gamma globulin were included in the incubation media, the buffer volume was decreased accordingly to accommodate the addition of the appropriate serum or gamma globulin solution. Antiserum or immunogamma globulin was neutralized with the appropriate amount of purified rat liver GIT by preincubation for 5 min in a 37° shaker bath before addition of the fat cell suspension. An atmosphere of O_2/CO_2(95:5) was then introduced and each vial was capped and placed in a 37° shaker bath (60 cycles/min). After 15 min, 0.1 ml of either buffer or insulin was added to each vial and the incubation was continued for 20 min more. The cell suspensions were transferred quantitatively with plastic dropper pipets to individual 3 ml Potter-Elvehjem homogenizer tubes. The tubes were centrifuged at 400 x g for 30 sec, and the infranatant incubation media discarded. Each fat cell float was suspended in 2 ml of cold 10 mM Tris-0.25 M Sucrose, pH 7.4, cooled in ice, and homogenized by 8 strokes of a motor driven Teflon pestel (425 rpm). In early experiments the homogenates were subjected to centrifugation at 400 x g for 4 min; the fat float was discarded and the remaining infranatant extract (designated as E) was used for the assay of PDE activity. In later experiments, the centrifugation was carried out at 10,000 x g for 7 min at 4°; both the fat layer and supernatant were discarded. The centrifuge tube was carefully dried with a cotton swab without disturbing the protein pellet (designated as particulate fraction P). The P pellets thus obtained were suspended in 0.5 ml of Tris-sucrose buffer and used for the assay of PDE activity.

Assay of Cyclic AMP Phosphodiesterase. This was carried out with the coupled enzyme procedure using 5'-nucleotidase essentially as described by Zinman and Hollenberg (7). The final concentration of cAMP including [^{3}H]-cAMP ($\sim$ 2.5 pmole) was 0.05 µM. [^{3}H] Adenosine, the end product in this assay scheme, was separated using Dowex 1-X8 resin by a modification of the procedure described by Boudreau and Drummond (23). One milliliter aliquots of a Dowex suspension (1:6 w/v) in 60 mM acetic acid were added to each assay tube, suspended with a Vortex mixer, and separated by centrifugation (100 x g, 10 min, 20°). A 0.5 ml sample of each supernatant, which contains [^{3}H]-adenosine, was mixed with 10 ml of Aquasol for liquid scintillation counting. Zero time blanks were routinely included in each series of assays.

The triglyceride content of fat cell preparations was determined by measuring the amount of hydroxymate-reactive ester (24) in hexane extracts of the cells (25), with tripalmitin as a standard.

All observations reported for any series of experiments were replicated with at least two different preparations of fat cells. All PDE activities are expressed as a percent of the basal activity measured with cells incubated without added insulin; each experiment contained incubations without (i.e., basal) and with insulin in addition to the other indicated experimental incubations. The basal PDE activity expressed as pmoles of cAMP hydrolyzed/µmole of triglyceride ester, averaged (+ SEM) 3.57 $\pm$ 0.34 (n=25) for the E preparations and 0.63 $\pm$ 0.03 (n=42) for the P preparations.

RESULTS: The exposure of isolated fat cells to insulin produced a dose-dependent increase in PDE activity in both the infranatant extracts (E) and the particulate fractions (P) (Fig. 1). Maximal insulin stimulation occurred at

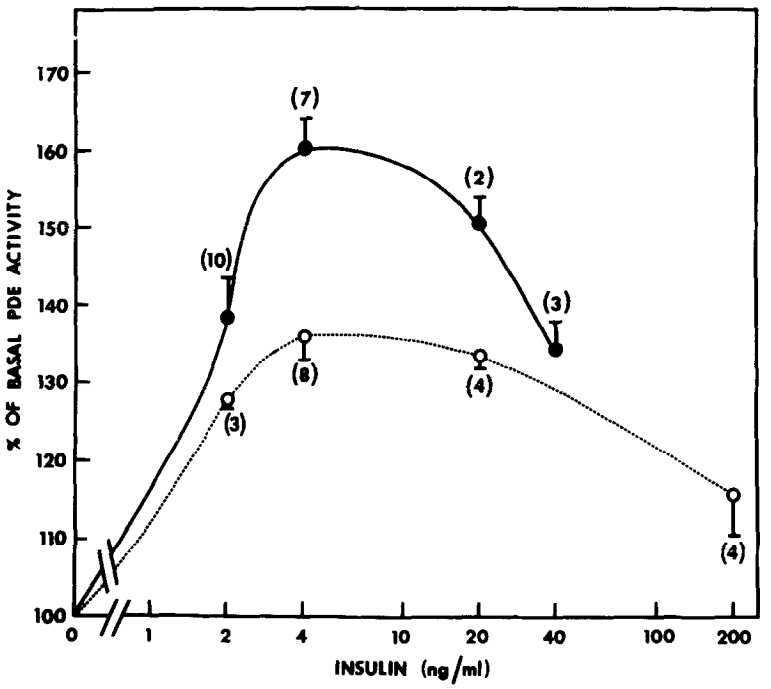

Fig. 1. Effect of varying insulin concentration on fat cell PDE activity. Isolated fat cells were pre-incubated in Krebs-Ringer-bicarbonate-BSA buffer at 37° for 15 min, followed by another period of incubation for 20 min in the presence of indicated concentrations of insulin. The fat cells were freed from the incubation medium, suspended in Tris-sucrose and homogenized; the extracts E or the particulate fractions P were prepared and tested for PDE activity. See text for details. Activities are expressed as an average percentage ± SEM of the basal activity measured with cells incubated in the absence of the added hormone; the number of determinations carried out are shown in parentheses. O·····O, E extract; ●—●, P fraction.

4 ng/ml of the hormone (0.7 nM), with a 20-40% increase for E, and 40-60% increase for P. The magnitude of the insulin effect in these experiments is in the same range as that reported by several other workers (5-7), with the exception of Kono, et al., (9), who reported a 2 to 3-fold stimulation by insulin. Despite several attempts, we have not been able to duplicate the magnitude of the insulin effect reported by these workers (9). In qualitative terms, however, the pattern of stimulation by insulin obtained by us is in excellent agreement with that reported by Kono, et al., (9). Pre-exposure of fat cells to guinea pig antiserum (1:4000 final dilution) against insulin almost completely neutra-

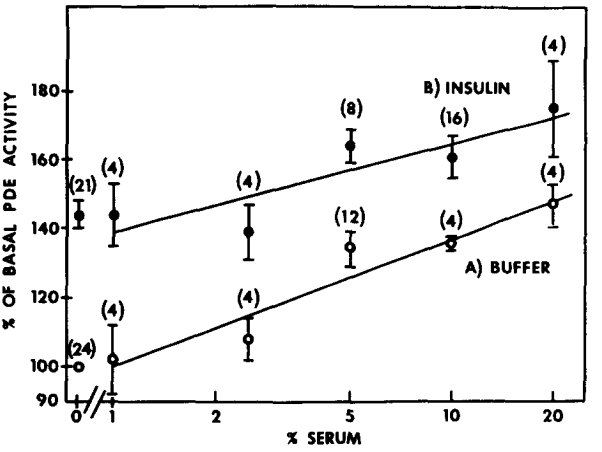

Fig. 2. Effect of rabbit normal serum on basal and insulin-stimulated particulate PDE activity of fat cells. Isolated fat cells were preincubated at 37° for 15 min in Krebs-Ringer-bicarbonate-BSA buffer containing the indicated final concentrations of normal serum; then buffer (curve A) or insulin (4 ng/ml, curve B) was added and the incubations were continued for another 20 min. See text and legend to Fig. 1 for details. The lines were fitted by linear least squares analyses of the data; correlation coefficient for curve A is +0.96 and for curve B +0.88. Note the logarithmic scale of the obcissa..

lized the stimulatory effects produced by insulin. Guinea pig normal serum at 1:4000 final dilution did not significantly alter the stimulatory effect of insulin on fat cell PDE activity. The addition of insulin to E or P preparations showed no detectable effect on the enzyme activity, indicating that intact fat cells are required for demonstrating the effect of insulin on the PDE activity.

The incubation of fat cells with serum itself produced a dose-dependent stimulation of the basal PDE activity, reaching a value at 20% serum almost equivalent to that produced by insulin in the absence of serum (Fig. 2). However, the addition of insulin to normal serum produced a further increase in the fat cell PDE activity, although the stimulation produced by insulin in the presence of 2-20% serum (final concentration) was about half that observed in the absence of serum. The ability of sera to enhance the PDE activity was not altered by an overnight dialysis against physiological saline. Whether the serum activation of fat cell PDE is related to a change in the number of forms of the enzyme, as recently reported for the PDE activity of BHK cultured cells

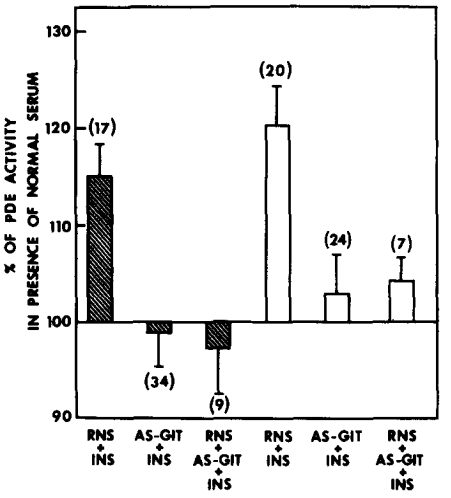

Fig. 3. Effects of antisera to GIT on the insulin-dependent PDE activity of fat cells. Isolated fat cells were pre-incubated for 15 min at 37° in the presence of 0.2 ml of each indicated serum; insulin (4 ng/ml) or buffer was then added and the incubations were continued for another 20 min. See text and legend to Fig. 1 for other details. Activities are expressed as an average percentage ± SEM of the activity measured with cells that had been incubated with the corresponding normal serum but without added insulin. Striped boxes, extract E; clear boxes, fraction P. INS, insulin; RNS, rabbit normal serum, AS-GIT, rabbit antiserum against GIT.

(26), or is due to other factors, although of interest, was not investigated since this was not the objective of the current study.

When fat cells were exposed to 5-20% rabbit antiserum against GIT, and then to insulin, the hormone was no longer capable of stimulating the PDE activity in the E extracts above the level of activity in the presence of normal serum alone (Fig. 3). This effect of the antiserum was not altered by the presence of normal serum. Although the antibody did not completely abolish the insulin stimulation of PDE activity in the P preparation, the effect of insulin in the presence of the antibody was significantly less ($p < 0.001$) than that observed in the presence of normal serum; in fact, the actual quantitative decrease in the enzymic activity caused by the antibody was about the same for both the E and P preparations (Fig. 3).

In order to provide further evidence for the specificity of the antiserum

TABLE I

NEUTRALIZATION BY PURIFIED GIT OF THE ABILITY OF GIT ANTIBODIES TO BLOCK THE STIMULATION BY INSULIN OF FAT CELL PARTICULATE PDE ACTIVITY.

Experimental conditions were identical with those described in the legend to Fig. 3, except that antiserum (0.1 ml) or immunogamma globulin (0.05 ml) was first neutralized by incubation at 37° for 5 min with 14 and 28 units of GIT activity, respectively, prior to the addition of the fat cells. Insulin was present at 4 ng/ml. NgG, gamma globulin from rabbit normal serum; IgG, gamma globulin from rabbit antiserum against rat liver GIT; other abbreviations are explained in the legned to Fig. 3.

Conditions	n	PDE Activity (% Basal ± SEM)
NgG + buffer	20	100%
NgG + Insulin	20	126 ± 4%
IgG + Insulin	15	105 ± 3%
Neutralized IgG + Insulin	12	124 ± 8%
RNS + Buffer	16	100%
RNS + Insulin	16	121 ± 3%
AS-GIT + Insulin	27	105 ± 2%
Neutralized AS-GIT + Insulin	9	119 ± 2%

effect, these experiments were repeated using immunogamma globulin against GIT. As can be seen from the data shown in Table I, immunogamma globulin against GIT also blocked the stimulation of fat cell PDE activity by insulin. Furthermore, preincubation with purified rat liver GIT was found to effectively neutralize the capacity of both the GIT antiserum and immunogamma globulin to block the insulin stimulation of fat cell PDE activity (Table I).

It should be noted that even though the net insulin effect in the presence of sera and gamma globulin is small, we feel that, considering the total number of experiments performed, the data obtained are a reliable index.

DISCUSSION: The nullification by GIT specific antibody of the insulin-mediated increase in the PDE activity of isolated fat cells and the inability of neutralized GIT-antibody (prepared by treatment with purified GIT) to abolish the insulin effect clearly indicate that insulin-GIT interactions might be involved in the expression of insulin effects on cAMP metabolism. GIT might mediate the insulin effect on intracellular cAMP metabolism either via its products, i.e., A and B chains, or via its sulfhydryl-disulfide exchange reaction with insulin.

We have found that although isolated insulin A and B chains do stimulate

the fat cell PDE activity, the stimulation with the chains is, on a molar basis, much smaller than that observed with intact insulin (data not shown). If A and B chains were the "active fragments" for the insulin action, they should have produced effects that are equal to or greater than that of intact insulin. The possibility exists however, that the lower effects of the chains might be due either to their more rapid degradation than insulin (27), or to a requirement for the production of A and B chains at the site of insulin action.

The recent findings that GIT in intact isolated liver cells either is located on the cell surface, or is readily accessible to the cell surface (28) support the idea that GIT may be located proximally to the "insulin receptor protein" (see also 29,30). It is possible that the GIT molecules that are accessible at the cell surface (28-30) adjust the sulfhydryl-disulfide status of the cell by reacting with insulin added externally and, thus, distally stabilize (i.e., activate) the activity of intracellularly located cAMP phosphodiesterase (9). Such a mechanism of action for GIT would be consistent with the effects of externally added insulin and antibodies observed in the present study. Work is currently in progress to determine the validity of the mechanisms discussed above.

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