Insulin Antagonism of Catecholamine Stimulation of Fatty Acid Transport in the Adipocyte

STUDIES ON ITS MECHANISM OF ACTION*

Nada A. Abumrad, Carroll M. Harmon, Usha S. Barnela, and Richard R. Whitesell
From the Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Insulin at physiological concentrations can suppress catecholamine activation of the membrane transport of long chain fatty acids in the adipocyte. We have previously shown that the stimulatory effect of catecholamines was mediated by a β-receptor interaction and cAMP (Abumrad, N. A., Park, C. R., and Whitesell, R. R. (1986) J. Biol. Chem. 261, 13082-13086). In this study we have investigated the mechanism of insulin action to antagonize transport activation. Fatty acid transport was stimulated using different cAMP derivatives with varying susceptibilities to hydrolysis by the cAMP-degrading enzyme phosphodiesterase. Insulin was effective in antagonizing the effect of cAMP analogs which were good substrates for the phosphodiesterase and failed to suppress the effect of those which were poorly hydrolyzed by the enzyme. Addtion of increasing concentrations (1-100 μM) of the phosphodiesterase inhibitor methylisobutylxanthine (MIX) to norepinephrine (0.1 μg/ml) gradually abolished insulin's antagonism. Insulin was completely ineffective in inhibiting stimulation by norepinephrine and 20 μM methylisobutylxanthine. Also consistent with involvement of cAMP lowering in insulin action was the finding that adenosine removal greatly diminished insulin's responsiveness. Treatment of cells with adenosine deaminase (1 unit/ml) enhanced the effect of norepinephrine by about 30%. A 10-fold higher range of insulin concentrations was then required to produce inhibition of fatty acid transport. The effect of adenosine removal was reversed by addition of phosphorylcholine (500 μM), which is resistant to hydrolysis by the deaminase. Finally, exposure of insulin-treated cells (1 nM for 5 min) to dinitrophenol (1 mM for 5 min) reversed insulin action, consistent with reports of reversal of insulin's activation of the phosphodiesterase. In conclusion, our studies support the involvement of cAMP lowering in insulin's antagonism of fatty acid transport stimulation in the adipocyte.

Experimental Procedures

Methods

Preparation of Fat Cells—Adipocytes were prepared from the epididymal fat of one to two 170-200-g Sprague-Dawley rats (Harlan Industries, Inc.) Cell isolation followed the methodology detailed previously (2). The washed cells were suspended (30%, v/v) in Krebs-Ringer Hepes buffer (KRH) containing 0.2% fatty acid-free albumin (Sigma, fraction V, fatty acid free) and glucose (2 mM) except where indicated. The cell suspension was allowed to cool to room temperature (3-5 min) before the fatty acid transport assay was performed.

Transport Assay—Membrane transport of [14C]oleate (DuPont-New England Nuclear) was measured as described in detail previously (3, 6). Medium (20 μl) containing labeled and unlabeled fatty acid (42 μM, about 3000 cpm/μl) complexed to 10 μM bovine serum albumin (BSA)1 was preincubated onto the bottom of polystyrene tubes. The cell suspension was mixed before sampling with the use of an automatic pipette with the plastic tip enlarged to avoid cell breakage. The assay was started by rapidly ejecting 30 μl of cell suspension onto the 20-μl medium and this was followed by gentle swirling. At the desired time, stop solution (5 ml of ice-cold KRH buffer containing 200 μM phloretin) was added. The cells were separated from medium and washed by filtration as described in detail previously (6). Controls for fatty acid absorption to cells and filters were routinely subtracted. Controls were samples where isotope and stop solution were premixed before cell addition.

Stimulation of Fatty Acid Transport—Cells (30% (v/v) in KRH with 0.2% BSA and 2 mM glucose) were incubated at 37 °C for 10 min in the case of epinephrine and norepinephrine and for 20 min in the case of CAMP analogs. The various hormones or agents were added as a few microliters from a concentrated stock. At the end of the incubation period the cells were allowed to cool to room temperature before assay of fatty acid transport.

The abbreviations used are: BSA, bovine serum albumin; KRH, Krebs-Ringer solution buffered with Hepes; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MIX, methylisobutylxanthine; PIA, phenylisopropyladenosine; 8-CPT cAMP, 8-thiodiphenoxypropyl cAMP; 8-AHA cAMP, 8-aminohexylamino cAMP; NE, norepinephrine; DNP, dinitrophenol.

* This work was supported by National Institutes of Health Grant DK33301 and by Grants 186651 and 187653 from the Juvenile Diabetes Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Molecular Physiology and Biophysics, 602 Light Hall, Vanderbilt University School of Medicine, Nashville, TN 37232.
Insulin Treatment—Insulin was added at the doses indicated, usually at the same time as the stimulatory hormone or agent, unless indicated otherwise. The insulin used was lot F23695 (Schwarz/Mann). The various insulin stocks or dilutions were made only a few minutes before actual use in KRH containing 0.2% BSA.

Treatment with Lipolytic Inhibitor RHC80267—When inhibition of lipolysis by insulin was the objective, the isolated cells were suspended in KRH buffer at 90% (v/v) and were divided into two batches. RHC80267 (15 μM) was added to one batch, and both were incubated at 37 °C for 15 min. Under these conditions RHC80267 inhibits NE-generated glycerol release by 90% (1). Following this incubation, each batch was subdivided into aliquots for the various hormone treatments and processed as indicated for the particular condition.

Lipolysis Measurement—Lipolysis was estimated by assaying the glycerol released into the medium and also by measuring intracellular fatty acids. For these measurements, cells (30% (v/v) in KRH with 0.2% BSA and 2 mM glucose) were suspended and incubated under the same conditions described previously for hormonal treatments. At the desired time, the cell suspensions were mixed and an aliquot (100 μl) of cell suspension was ejected into 5 ml of ice-cold stop solution. This was followed by filtration and washing as described for the assay of fatty acid transport. Filters were immediately transferred to extraction tubes containing 2.4 ml of isopropanol. The tubes were vortexed for 30 s, and then heptane and sulfuric acid (0.05 N) were added in the volumes (0.6 and 0.06 ml, respectively) necessary to bring their concentrations to those present in 3 ml of Dole’s extraction mixture (7). The vials were vortexed again for 30 s, then 2 ml of heptane and 3.5 ml of water were added. The heptane layer was transferred to clear tubes and washed once with an equal volume of 0.05 N sulfuric acid. An aliquot (0.6–1 ml) was then evaporated at 35 °C under nitrogen and resuspended in 125 μl of heptane, 100 μl of which was used for measurement of free fatty acids according to Ho and Meng (8). For measurement of medium fatty acid or glycerol, an aliquot of cell suspension (350–500 μl) was centrifuged for 15 s in microfuge tubes. The infranatant was then transferred to tubes containing 3 ml of Dole’s mixture (for fatty acid extraction and titration) or to chilled tubes for later assay of glycerol. For glycerol estimation, 200 μl of medium were deproteinized with 40 μl of 5% perchloric acid, and the extract was neutralized on ice with 10 N KOH. Glycerol was determined enzymatically (9). Sensitivity of the assay was ±0.905 mmol/ml.

Measurement of Protein Kinase Activity Ratios—Cells were maintained under conditions identical to those used for the assay of fatty acid transport (see legend to Table II). Following incubation at 37 °C with the various agents tested, the cells were added to homogenizing buffer at 23 °C (10 mM potassium phosphate, 0.5 mM MgCl₂, 10 mM EDTA, 0.5 mM DTT, 0.05% BSA). The cells were homogenized (10 strokes) in a glass homogenizer (Kontes) with a Teflon pestle (cleaning B). The homogenate was centrifuged at 4 °C (10,000 rpm for 10 min). Protein kinase activity in the presence or absence of cAMP was assayed in the infranatant below the fat layer using Kemptide (Peninsula Laboratories) as substrate following the methodology of Beebe et al. (10). The protein kinase activity ratio is defined as the ratio of activity measured in the absence and presence of cAMP.

Cellular ATP was measured according to standard procedures (11).

Materials
All [³H]oleate (9 Ci/mmol), [³P]ATP (protein kinase assay), and [⁶⁷Ni] fatty acid measurements were obtained from Du Pont-New England Nuclear. Analogs of cAMP were generously donated by Dr. Jackie Corbin at Vanderbilt University or were purchased from Sigma. RHC80267 was a generous gift from Dr. Charles Sutherland of the Revlon Care Group, Hershey, PA.

RESULTS
Insulin Antagonism of Fatty Acid Transport Stimulation by cAMP Analogs—As reported previously (2) permeable cAMP analogs were added to cell suspension to determine the effects of various hormone treatments (4). A correlation between insulin’s effect and susceptibility of the analogs to hydrolysis would suggest that insulin’s antagonistic effect on fatty acid transport is mediated by a lowering of cAMP via stimulation of its degradation. As shown in Fig. 1 insulin at the optimally effective dose of 1 nM (1) was unable to antagonize the stimulatory effect of N⁶-benzoyl cAMP and 8-aminohexyloxamino cAMP (AHA cAMP) (Fig. 1, A and B, respectively). However, pretreating cells with the lipolytic inhibitor RHC80267 did not modify the results, indicating that insulin’s unresponsiveness was not related to the potency of the analog in increasing intracellular fatty acid (data not shown). As determined by Beebe et al. (4), N⁶-benzoyl and 8-AHA cAMP are poor substrates for the low Kₗ phosphodiesterase (4). On the other hand, insulin inhibited fatty acid transport stimulation by 8-thioethyl cAMP (not shown), 8-bromo cAMP, and 8-CPT cAMP (Fig. 1, C and D, respectively). These analogs are good substrates for the phosphodiesterase. In all three cases insulin was able to suppress fatty acid transport, when stimulated by the analogs by 8- to 10-fold, down to the basal rate. However, when the concentration of each analog was increased above its optimal dose, there was a decrease in insulin’s ability to antagonize transport stimulation which was similar in the absence or presence of RHC80267. The data are consistent with insulin exerting its effect through activation of the low Kₗ phosphodiesterase and consequently decreasing cAMP levels. When the levels of the nucleotide become too high, the antagonistic effect of insulin is partially lost. It is completely lost when the levels of cAMP analog cannot be lowered, as in the case of 8-aminohexyloxamino- and N⁶-benzoyl-cAMP which are resistant to the action of the phosphodiesterase. Insulin’s effectiveness as an antagonist was not related to whether the cAMP derivatives were specific for site 1 (8-modified derivatives) or site 2 (6-modified derivatives) on the cAMP-dependent protein kinase.

Effect of Adenosine Deaminase and Adenosine—Londos and co-workers (12) provided evidence recently to support the view that the effective concentrations of insulin as an antilipolytic hormone were greatly dependent on the level of adenine in the cell suspension. Adenosine is an inhibitor of adenylyl cyclase and, as such, it would be expected to alter effects which are mediated by changes in cAMP levels. We examined the effects that adenosine removal by treatment with adenosine deaminase (ADA), and subsequent adenosine addition, in the form of the nonhydrolyzable phenylisopropyladenosine (PIA), had on the regulation of fatty acid transport, by norepinephrine plus insulin. Enhancement of insulin’s effectiveness by adenosine would further support involvement of cAMP lowering in insulin action. Addition of adenosine deaminase (1 unit/ml) did not significantly stimulate basal fatty acid transport (not shown), but it produced a 30% enhancement of the stimulatory effect of norepinephrine (Fig. 2). Sensitivity to insulin was decreased. For example 100 μM insulin was only 25% inhibitory and 1–5 nM insulin reduced the effect of NE by 65%. Pretreatment of cells with the lipolytic inhibitor RHC80267 (15 μM) before exposure to adenosine deaminase did not improve insulin’s effectiveness (data not shown) although RHC80267 decreased intracellular fatty acid by 65% (Table I). In control experiments it was determined that RHC did not alter the protein kinase activity ratios (Table II) nor did it affect the activity of brain adenylyl cyclase measured in vitro (data not shown). The effect of adenosine deaminase was due to adenosine removal since it was reversed by addition of PIA, which is resistant to hydrolysis. PIA addition to adenosine deaminase-treated cells, returned insulin sensitivity (Fig. 2) almost fully to the levels we had reported earlier (1). More specifically, insulin at the concentration of 50 pm was 70% inhibitory in the presence of...
Fatty Acid Transport in the Adipocyte

**Fig. 1.** Insulin antagonism of fatty acid (FA) transport stimulation by various cAMP analogs. Adipocytes suspended (30%, v/v) in KRH containing 0.2% BSA and 2 mM glucose were treated with the indicated concentrations of cAMP analogs ±1 nM insulin for 30 min at 37°C. The cells were then cooled to room temperature for 4 min before assay of fatty acid transport. Fatty acid uptake expressed per ml of packed cells/min was computed from 2-12-s measurements as described under "Methods." The basal rate is indicated by the intersection of the curves with the y axis. The final molar ratio of fatty acid to BSA after correction for the BSA contributed by the cell suspension is 1. Total fatty acid concentration is 18 μM. The figure shows insulin’s ability to antagonize fatty acid transport stimulation by N'-benzoyl cAMP (A), 8-aminohexylamino cAMP, 8-AHA (B), 8-bromo cAMP (C), and 8-thioparachlorophenyl cAMP, 8-CPT (D). The data shown are a composite of four experiments.

**Fig. 2.** Effect of adenosine removal and subsequent addition on regulation of fatty acid transport by NE and insulin. Adipocytes were incubated for 10 min at 37°C with the indicated agent. Adenosine deaminase (ADA) concentration was 1 unit/ml, that of NE was 0.1 μg/ml or 0.3 μM. PIA was added to a final concentration of 200 or 500 nM. Insulin concentrations are indicated on the x axis. Fatty acid transport is expressed as percent of maximally stimulated rates in the presence of NE + ADA were about 30% higher than those measured with NE alone. Conditions for assay of fatty acid transport are similar to those described in the legend to Fig. 1.

excess PIA while it was only 15% effective in its absence. Maximal inhibition (95–98%) could be obtained with 100 pm insulin. Addition of excess PIA or adenosine to cells which had not been treated with adenosine deaminase did not increase insulin sensitivity tested over a range of insulin concentrations (data not shown). This is consistent with the finding that our methods of cell handling result in minimal activation of adenylate cyclase as shown by the measurement of low protein kinase activity ratios (Table II).

**Effect of Methylisobutylxanthine (MIX) on Insulin’s Antagonism—**MIX is an inhibitor of the cAMP-degrading enzyme phosphodiesterase. It has been shown previously to potentiate stimulation of fatty acid transport by suboptimal concentrations of catecholamines (2). We tested the effect of cell treatment with MIX on insulin’s antagonism of fatty acid

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**Table I**

| Incubation conditions | Cellular free fatty acids | Intracellular free fatty acids before and after treatment with NE, insulin, and RHC80267 |
|-----------------------|--------------------------|-----------------------------------------------------------------------------------|
|                       | Basal                    | 5 min after NE + insulin*                                                         |
| 0.2% BSA              | 1.4 ± 0.4                | 4.4 ± 0.8                                                                         |
| 0.2% BSA + RHC80267   | 0.49 ± 0.07              | 1.6 ± 0.05                                                                        |
| 4% BSA                | 0.6 ± 0.1                | 0.9 ± 0.2                                                                         |

*Insulin inhibited fatty acid transport effectively (80–90%) under all conditions tested.
Transport stimulation. Fig. 3 shows fatty acid transport stimulation by various concentrations of MIX by itself or with norepinephrine and the ability of 1 nM insulin to antagonize the stimulatory effects. MIX by itself stimulated fatty acid transport significantly at concentrations equal to or higher than 20 μM. Insulin (1 nM) was effective in antagonizing the effect of MIX at all concentrations tested (1–100 μM). Low concentrations of MIX (1–10 μM) had a potentiating effect when added to an optimal dose of NE (0.1 μg/ml). Under these conditions, insulin’s effectiveness was completely abolished at a combination of 20 μM MIX and 0.1 μg/ml NE. Although MIX when added to NE produced the same magnitude of transport stimulation at 3 and 20 μM doses, insulin was 50% effective against the first dose and completely ineffective in the second case. On the other hand, under comparable experimental conditions insulin (1 nM) was effective in antagonizing epinephrine and norepinephrine (Fig. 4) at extremely high doses (10–1000 μg/ml). This could indicate that in our assay system of a concentrated cell suspension and low BSA in the medium there is early negative feedback of accumulated fatty acid (Table II) or of adenosine 3′:5′ cyclase which prevents further production of cAMP as the catecholamine concentration is increased. Pretreatment of cells with RHC80267 did not modify the effectiveness of insulin in spite of a 70% decrease in intracellular fatty acid in both basal and NE-treated cells. The increase in intracellular free fatty acid generated by NE was antagonized by insulin when it inhibited transport irrespective of the initial concentration of intracellular fatty acid. For example, when cells were incubated in 4% BSA instead of the usual 0.2%, intracellular fatty acid dropped significantly (Table I). Under both conditions, however, insulin was equally effective in inhibiting stimulation of fatty acid transport and lipolysis.

Effect of Dinitrophenol on Fatty Acid Transport in Cells Pretreated with Insulin—We have previously shown that pretreatment of cells with dinitrophenol (DNP) abolishes the effects on fatty acid transport of both catecholamines and insulin (3). In this study we show that addition of DNP following the hormonal treatments had a different effect. DNP did not affect transport stimulation by catecholamines. Furthermore, it prevented the return of transport rates to basal levels upon withdrawal of the hormone. In contrast, DNP treatment completely eliminated insulin’s antagonistic effect. This was observed when DNP was added 5 min following exposure to insulin. As shown in Fig. 5, fatty acid transport rates in cells exposed to norepinephrine plus insulin for 5 min and then to dinitrophenol for 5 min were comparable to those

![Table II](image)

**Table II**

Effect of RHC80267 and NE in the absence or presence of MIX or adenosine deaminase (ADA) on protein kinase activity ratios in rat adipocytes

| Incubation conditions | Control | RHC80267 | NE | NE + 3 μM MIX | NE + 100 μM MIX | 100 μM MIX | NE + ADA |
|-----------------------|---------|----------|----|--------------|----------------|-----------|----------|
| Protein kinase activity ratio | 0.063 ± 0.005 | 0.065 ± 0.006 | 0.077 ± 0.009* | 0.085 ± 0.01 | 0.19 ± 0.01 | 0.057 ± 0.006 | 0.15 ± 0.030 |

*The effect of NE was significant when the paired t test was employed (p < 0.025).
measured in cells treated with norepinephrine alone. DNP was less effective, however, when added at 10 min following insulin addition. This pattern was similar to that described for DNP's effect on insulin activation of the phosphodiesterase (13). As discussed by Kono (13), it is one of the distinguishing factors between the mechanisms of action of insulin on the phosphodiesterase versus its effect on glucose transport. Glucose transport, once stimulated by insulin, is not altered by DNP treatment. In addition, DNP blocks reversal of the glucose transport stimulation which would otherwise occur upon washout of insulin.

**DISCUSSION**

We have previously shown that a variety of cAMP analogs can stimulate long chain fatty acid transport in the rat adipocyte (2). Beebe *et al.* (4) recently determined the susceptibility of the various analogs to degradation by the low $K_m$ phosphodiesterase. Furthermore, they showed in the case of a large number of cAMP analogs that insulin's ability to antagonize their antilipolytic effect generally correlated with phosphodiesterase susceptibility. Our findings show a similar pattern to that reported previously (4, 17, 18). Insulin (1 nM) was 100% effective at shutting off glycerol release after 0.1 and 1 μg/ml NE. It was 68 and 33% effective at 50 and 500 pg/ml NE, respectively, and completely ineffective at 1000 pg/ml NE. Although insulin effects on fatty acid transport and lipolysis correlated, there did not appear to be any general correlation between absolute levels of intracellular fatty acid and the magnitude of insulin's effectiveness on fatty acid transport. For example, insulin was equally effective when the cells were suspended in 0.2 or 4% albumin in spite of different levels of intracellular fatty acid before and after incubation with NE (Table I). A lowering of intracellular fatty acid by insulin never occurred in the absence of an effect on fatty acid transport, suggesting that the two effects were a result of the same preceding event. In contrast, insulin stimulated glucose transport independently of the effects on fatty acid transport (data not shown).

The effectiveness of insulin in antagonizing fatty acid transport stimulation by the phosphodiesterase inhibitor MIX is similar to the finding with caffeine (3). A possible explanation is that these agents do not completely inhibit all the phosphodiesterase present in the cell so that an inhibitory effect of insulin can still be observed. On the other hand, 1 μM insulin was unable to block the combined effects of MIX plus NE, in cells pretreated with RHC80267, to rule out deleterious effects of fatty acid. This would suggest that cAMP levels which are significantly raised under these conditions (Table II) cannot be lowered by insulin to levels below those producing stimulation of fatty acid transport. Consistent with this interpretation was the observation that transport stimulation was only increased by 18% when 3 μM MIX is included with the 0.1 μg/ml NE. However, inhibition by insulin was reduced by MIX from 97 to 59%, suggesting a critical range of cAMP levels which insulin can antagonize. Increasing MIX from 10 to 20 μM in the presence of NE reduced insulin inhibition from 69 to 0% but did not result in a proportional increase in the magnitude of fatty acid transport stimulation in the absence of insulin.

The ability of insulin in our studies to antagonize transport stimulation by high concentrations of NE (1–1000 μg/ml) is at variance with what has been reported for its antagonism of NE-stimulated lipolysis (4, 17, 18). In our studies the effectiveness of insulin on NE-stimulated glycerol production was also observed over a wider range of NE concentrations than that reported previously (4, 5, 18). Insulin (1 nM) was 100% effective at shutting off glycerol release after 0.1 and 1 μg/ml NE. It was 68 and 33% effective at 50 and 500 μg/ml NE, respectively, and completely ineffective at 1000 μg/ml NE (data not shown). The discrepancy between our results and previous ones could be explained by the use in our incubations of concentrated cell suspensions and of low BSA (0.2%) in the medium. Both conditions should favor low cellular cAMP. High cell concentration favors adenosine accumulation which inhibits adenylate cyclase, and low BSA accelerates the increase in the molar ratio of fatty acid to albumin which would feed back to inhibit cAMP accumulation and lipolysis (19–21). This interpretation is consistent with the low range of activity ratios for cAMP-dependent protein kinase that we measure in our cell homogenates (Table II) and would suggest

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2 N. A. Abuamrad, C. M. Harmon, U. S. Barnela, and R. R. Whitesell, unpublished observations.
insulin’s effectiveness is enhanced by low cellular cAMP. Under such conditions insulin can lower cAMP and protein kinase to the levels necessary for deactivation of catecholamine-sensitive pathways including fatty acid transport.

The ability of dinitrophenol to block insulin’s action when added 5 min after insulin treatment is consistent with mediation of the insulin effect by activation of the low K_m phosphodiesterase. As shown by Kono (13), phosphodiesterase activation by insulin is abolished by subsequent addition of DNP in contrast to insulin-activated glucose transport which is actually preserved by DNP.

In summary, insulin appears to inhibit catecholamine stimulation of fatty acid transport by accelerating hydrolysis of cAMP through activation of the phosphodiesterase. 1) Insulin can antagonize the effect of up to 1000 µg/ml NE or epinephrine when experimental conditions prevent build up of CAMP, suggesting that the lack of an antilipolytic effect of insulin previously described at high concentrations of NE was related to CAMP accumulation and not to that of fatty acid. 2) Insulin becomes ineffective when cAMP levels are raised by the use of nonhydrolyzable cAMP analogs, by treatment with adenosine deaminase, or by MIX in the presence of NE. Insulin activation of the phosphodiesterase could still be occurring under such conditions but the resultant lowering of cAMP levels is not enough to produce deactivation. Finally, although our studies implicate CAMP lowering in insulin’s inhibition of fatty acid transport, they do not rule out the participation of other events in the insulin effect.

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