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The Effect of Phenformin and Other Adenosine Triphosphate (ATP)-Lowering Agents on Insulin Binding to IM-9 Human Cultured Lymphocytes

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In the present study, we investigated the mechanism by which the antidiabetic drug phenformin increases insulin binding to its receptors in IM-9 human cultured lymphocytes. After a 24-hr preincubation, phenformin induced a twofold increase in specific ^{125}I-insulin binding, and removal of phenformin was followed 6 hr later by a return in binding to control levels. This effect of phenformin on insulin binding was not a consequence of either inhibition of cell growth, changes in cellular cyclic adenosine monophosphate (AMP) levels, or changes in guanosine triphosphate (GTP) content. Since phenformin is known to inhibit various aspects of cellular energy metabolism, the relationship between ^{125}I-insulin binding and energy metabolism in IM-9 cells was investigated. The phenformin-induced increase in insulin binding to IM-9 cells was related to a time- and dose-dependent decrease in ATP levels. Other agents that lowered ATP levels, including antimycin, dinitrophenol, and 2-deoxyglucose, also raised insulin binding. These studies indicated, therefore, that phenformin enhances insulin binding to receptors on IM-9 cells and that this effect on insulin receptors may be related to alterations in metabolic functions that are reflected by a lowering of ATP levels.

Key words: phenformin, biguanides, insulin, insulin receptor, ATP

The insulin receptor is under the regulation of a variety of hormones [1,2], substances [3,4], and pharmacological agents [5,6]. Recently, we have reported that the biguanides phenformin and metformin enhance the binding of insulin to its receptors in several lines of human and rat cells in tissue culture [7–9]. In the present study, to probe the mechanisms of action of phenformin, we have studied IM-9 human

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cultured lymphocytes. This cell type has well-characterized insulin receptors [10] and shows the greatest increase in insulin binding when treated with phenformin [9].

It is well established that phenformin has multiple effects on cellular energy metabolism and ATP [11–13]. Recently, it has been shown that hormone receptors in target cells are influenced by changes in the metabolic environment, including energy levels [14–16]. Moreover, there are data from our laboratory and others [17,18] indicating that ATP may influence the binding of insulin to its receptor. The present studies were carried out, therefore, to determine whether a relationship exists between the effect of phenformin and other agents that lower ATP, and their effects on insulin receptors in IM-9 cells.

MATERIALS

Crystalline porcine insulin (27.3 U/mg) was purchased from Elanco Products Co (Indianapolis, IN), and cycloheximide, antimycin, dinitrophenol, and 2-deoxy-D-glucose were purchased from Sigma Chemical Co (St Louis, MO). The following were gifts: phenformin from Boehringer Biochemia, Milan, Italy; and mycophenolic acid from Dr B. Ullman, University of California, San Francisco. ^{125}I-insulin was prepared as previously described [19].

METHODS

IM-9 human cultured lymphocytes were grown in continuous suspension culture in Eagle's minimal essential media with 10% fetal calf serum, nonessential amino acids, 2 mM glutamine, and antibiotics, as previously described [20]. Prior to study, cells were centrifuged at 200 g for 10 min and suspended at 0.9×10^6/ml in the aforementioned growth medium supplemented with 20 mM HEPES, pH 7.4 (incubation medium). Various agents were then added, and the cells were incubated in plastic tissue culture flasks at 37°C in a humidified incubator under 95% air/5% CO_2. At appropriate times, the cells were centrifuged as described above and placed at 0.9×10^6/ml into bicarbonate-free Eagle's minimal essential media with 10% calf serum, nonessential amino acids, 2 mM glutamine, and 20 mM HEPES, pH 7.4 (binding medium). ^{125}I-insulin, 100 pM, was then added in either the absence or presence of 10 μM unlabeled insulin, and the incubation was continued at 24°C in air. Typically, after 60 min, 400-μl aliquots of cells were centrifuged 3 min at 8,000 g in a Beckman model B microcentrifuge. The supernatants were aspirated and the cell pellets washed once with 0.4 ml of 154 mM NaCl at 4°C followed by recentrifugation for 15 sec. The tips of the tubes containing the bound hormone were then excised and counted. Total binding was determined with ^{125}I-insulin only: nonspecific binding was determined with labeled plus unlabeled hormone, whereas specific binding was the difference between total and nonspecific binding. Specific binding to cells preincubated in both the absence and presence of phenformin was at steady state after 60 min (Fig. 1). All data are reported as specifically bound ^{125}I-hormone/free hormone per 100 μg cellular protein.

To measure ATP, cells were centrifuged, and then the pellets were extracted with 0.5 M perchloric acid. The supernatants containing the ATP were neutralized with 0.6 M $NaHCO_3$, and ATP was measured by the luciferase method [21]. To measure both ATP and GTP simultaneously, the cells were centrifuged as above and

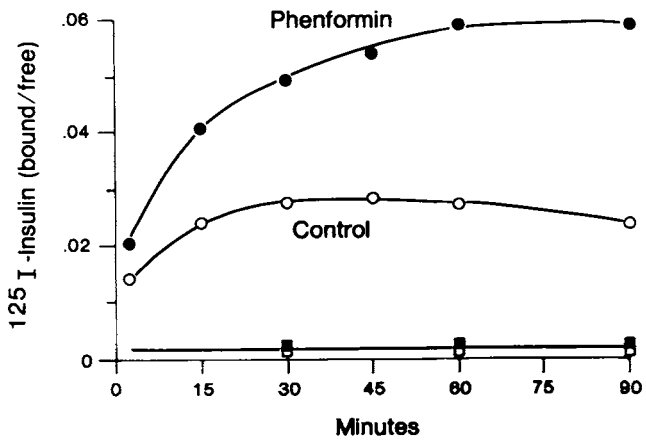

Fig. 1. Time course of ^{125}I-insulin binding to IM-9 cells preincubated 24 hr in the absence or presence of 20 μM phenformin. Each value is the mean of triplicate determinations. ●---●, specific binding, phenformin-treated; O---O, specific binding, control; ■---■, nonspecific binding, phenformin-treated; □---□, nonspecific binding, control. A representative of six experiments is shown.

the pellets extracted with 1.5 M trichloroacetic acid. The supernatants were neutralized with 1 N KOH and the nucleotides measured by high-pressure liquid chromatography [22]. The high-pressure liquid chromatography method yielded slightly lower values for ATP content than did the luciferase method. However, the marked fall in ATP levels after phenformin treatment was seen with both methods. Cyclic AMP was measured by radioimmunoassay [23].

RESULTS

Effect of Phenformin on ^{125}I-Insulin Binding

As previously reported, the effect of phenformin on insulin receptors in IM-9 lymphocytes and other cells is not immediate but requires 18 to 24 hr to become maximal [8]. After a 24-hr preincubation in the absence and presence of a maximally effective concentration of phenformin (20 μM), the specific binding of insulin to both control and phenformin-treated IM-9 cells reached a plateau after 60 min of incubation (Fig. 1). In 11 separate experiments phenformin increased specific insulin binding to IM-9 lymphocytes by 101.3 $\pm$ 11.5% over control cells (mean $\pm$ SE). Nonspecific binding of ^{125}I-insulin, which was a very small fraction of specific binding, was unchanged by phenformin treatment (Fig. 1). In addition, the degradation of ^{125}I-insulin in the incubation medium under these conditions was less than 5% of total and was not affected by phenformin.

To determine whether phenformin induced a permanent alteration of insulin receptors, cells were preincubated with phenformin for 24 hr and subsequently placed into a phenformin-free medium for 6 hr. After the second preincubation in phenformin-free medium, specific insulin binding approached that of control cells, indicating that the effect of phenformin was reversible (Table I).

It was also observed that phenformin reduced the growth of IM-9 cells, as evidenced by both decreased cellular protein content (Table II) and cell counts, and

TABLE I. Reversibility of the Effect of Phenformin of ^{125}I-Insulin Binding to IM-9 Lymphocytes*

	I-insulin (bound/free)		
First preincubation (24hr)		Second preincubation (6hr)	
A) No phenformin	0.030 ± 0.001	A) No phenformin	0.031 ± 0.003
B) Phenformin (20 μM)	0.066 ± 0.009	B) No phenformin	0.039 ± 0.005

*IM-9 cells were first preincubated 24 hr in the presence and absence of 20 μM phenformin, and then specific ^{125}I-insulin binding measured. The cells were then preincubated for a second time in fresh phenformin-free media for 6 hr, and ^{125}I-insulin binding was again measured. Each value is the mean ± SE of five separate experiments.

TABLE II. Cell Growth Inhibitors and ^{125}I-Insulin Binding*

	Cell protein (μg/ml)	^{125}I-insulin (bound/free)
A. Phenformin		
No incubation	65 ± 7	0.031 ± 0.002
24-hr incubation		
0	125 ± 11	0.030 ± 0.002
5 μM	108 ± 4	0.036 ± 0.003
20 μM	87 ± 5	0.066 ± 0.006
B. Thymidine		
No incubation	91 ± 3	0.022 ± 0.003
24-hr incubation		
0	171 ± 18	0.024 ± 0.004
2 mM	156 ± 12	0.023 ± 0.004
8 mM	128 ± 3	0.024 ± 0.021
C. Cycloheximide		
No incubation	102 ± 5	0.022 ± 0.002
24-hr incubation		
0	170 ± 11	0.023 ± 0.004
100 μM	88 ± 3	0.019 ± 0.003

*Protein concentration and specific ^{125}I-insulin binding to IM-9 cells were measured immediately, and again after a 24-hr incubation with the above agents. After this 24-hr incubation, protein concentration and specific ^{125}I-insulin binding were measured again. Values are the mean ± SD of triplicate determinations of three representative experiments.

that this effect was also reversible (data not shown). Furthermore, this inhibition of cell growth occurred over concentrations of phenformin that increased ^{125}I-insulin binding (Table II). To determine whether inhibition of cell growth per se increased ^{125}I-insulin binding, IM-9 cells were preincubated 24 hr with either cycloheximide or thymidine. Both agents, like phenformin, inhibited cell growth, but neither increased ^{125}I-insulin binding (Table II). Cycloheximide at 100 μM, a concentration that markedly inhibits protein synthesis in IM-9 cells, actually decreased ^{125}I-insulin binding.

Relationship of the Phenformin Effects on Insulin Binding to Changes in ATP Levels

Since phenformin is known to influence glucose metabolism in many tissues, we studied the effect of glucose on the action of phenformin in IM-9 cells. When the

glucose concentration in the preincubation medium was increased, the phenformin effect on insulin binding decreased. At 5.5 mM glucose, 20 μM phenformin increased binding over control by 87.4 ± 14.1% (mean ± SE of eight experiments), at 11 mM glucose it increased binding by 42.7 ± 9.5%, and at 16.5 mM glucose it increased binding by only 10.3 ± 4.0%.

It is known that phenformin interferes with several aspects of intracellular glucose utilization by diverting glucose metabolism through nonoxidative pathways, and that phenformin reduces the production of phosphorylated nucleotides including ATP [12,13]. When IM-9 cells were preincubated with phenformin in the presence of 5.5 mM glucose, the levels of the nucleotide were significantly reduced. In contrast, preincubation with phenformin in the presence of 11.5 mM glucose prevented this fall in ATP (Table III).

We investigated, therefore, the time and dose relationships between the phenformin effect on ^{125}I-insulin binding to IM-9 cells and its effects on ATP levels. Preincubation of IM-9 cells with phenformin lowered ATP levels in a time-dependent manner; an effect of the drug became evident after 3 hr and was maximal at 18–24 hr. After 24 hr, cellular ATP levels decreased to 40% ± 6% of control (mean ± SE of nine experiments) (Fig. 2). This fall in ATP levels was mirrored by a rise in ^{125}I-insulin binding.

Preincubation with phenformin lowered ATP levels in a dose-dependent manner. An effect of phenformin on ^{125}I-insulin binding was observed at 3 μM (Fig. 3), and at this concentration a small fall in ATP levels was detected. At 30 μM, where the effect of phenformin on ^{125}I-insulin binding was maximal, ATP levels had fallen to more than one half of control.

Effect of Other ATP Lowering Agents on ^{125}I-insulin Binding

Since the phenformin induced a rise in both ^{125}I-insulin binding and a fall in ATP levels, other agents known to lower ATP levels were preincubated with IM-9 cells. At concentrations sufficient to produce a 50–75% fall in ATP levels, dinitrophenol, 2-deoxyglucose, and antimycin all increased the binding of ^{125}I-insulin to its receptor (Table IV). There was, however, no strict correlation for these drugs between lowered ATP levels and increased insulin binding.

Effect of Phenformin on GTP and Cyclic AMP Levels

In addition to ATP levels, it is known that phenformin reduces the cellular level of other phosphorylated nucleotides, including GTP [13]. When GTP was measured

TABLE III. Effect of High and Low Glucose Concentrations of ^{125}I-Insulin Binding and ATP Levels*

Condition	^{125}I-insulin (bound/free)	ATP (nmol/100 μg protein)
5.5 mM glucose	0.020 ± 0.004	5.27 ± 0.25
5.5 mM glucose + phenformin	0.038 ± 0.004	1.83 ± 0.45
16.5 mM glucose	0.019 ± 0.003	5.11 ± 0.07
16.5 mM glucose + phenformin	0.023 ± 0.002	5.00 ± 0.27

*Cells were preincubated for 18 hr in 5.5 and 16.5 mM glucose in the presence and absence of 20 μM phenformin. Each value is the mean ± SE of three separate experiments.

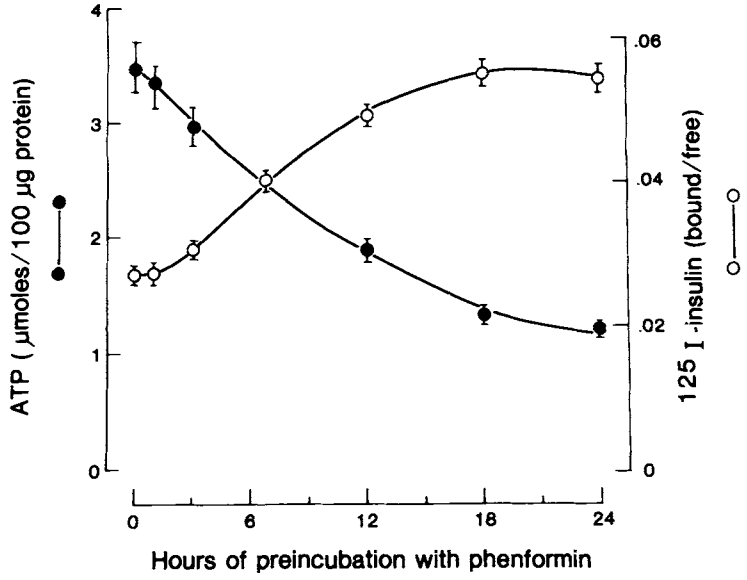

Fig. 2. Effect of duration of incubation on specific ^{125}I-insulin binding and ATP levels. Cells were incubated with 20 μM phenformin for up to 24 hr and at the specified times, specific ^{125}I-insulin binding and ATP levels were measured. Each value is the mean ± SE of four separate experiments.

TABLE IV. Effect of Inhibitors of ATP on ^{125}I-Insulin Binding and ATP Levels*

Inhibitor	ATP (nmol/100 μg protein)	^{125}I-Insulin (bound/free)
None	5.28 ± 0.32	0.020 ± 0.002
Phenformin (20 μM)	2.55 ± 0.69	0.037 ± 0.004
Antimycin (2 μM)	1.40 ± 0.20	0.032 ± 0.003
2-Deoxyglucose (20 mM)	2.24 ± 0.19	0.033 ± 0.003
Dinitrophenol (100 μM)	2.77 ± 0.29	0.036 ± 0.002

*Cells were incubated with the above drugs for 18 hr, and ATP levels and specific ^{125}I-insulin binding were then measured. Values for ATP and ^{125}I-insulin binding are the mean ± SE of three separate experiments.

TABLE V. Effect of Mycophenolic Acid Preincubation on ATP and GTP Levels and ^{125}I-Insulin Binding*

Incubation time	Mycophenolic Acid (μM)	ATP (μmol/100 μg protein)	GTP (μmol/100 μg protein)	^{125}I-insulin (bound/free)
2 hr	0	1.9	0.70	0.022
	1	1.7	0.39	0.023
	5	1.6	0.30	0.023
5 hr	3	1.5	0.03	0.024
9 hr	5	1.6	0.02	0.024

*Cells were placed into incubation media with the above concentrations of mycophenolic acid, and specific insulin binding was measured after the times listed. Each value is the mean of duplicate determinations. A representative of two experiments is shown.

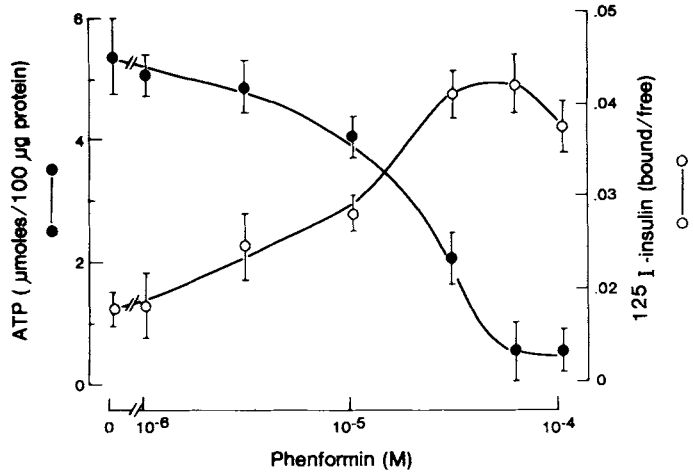

Fig. 3. Effect of increasing phenformin concentrations on specific ^{125}I-insulin binding and ATP levels. IM-9 cells were preincubated with increasing concentrations of phenformin for 24 hr, and then ATP levels and specific ^{125}I-insulin binding were measured. Each value for ATP levels is the mean ± SE of four separate experiments and for ^{125}I-insulin binding is the mean ± SE of seven separate experiments.

in IM-9 cells preincubated 24 hr with 20 μM phenformin, cellular GTP content decreased from 6.2 to 1.0 nmol/100 μg protein. Since GTP is known to influence the affinity of receptors for certain polypeptide hormones [24], the possibility was considered that GTP depletion might be responsible for the increased binding of insulin to IM-9 cells treated with phenformin. Accordingly, we investigated whether lowering GTP in IM-9 cells would reproduce the effect of phenformin on ^{125}I-insulin binding. When cells were preincubated with mycophenolic acid, an agent known to selectively block GTP synthesis without major effects on ATP [25], no significant increase of insulin binding was observed (Table V).

Elevations of cyclic AMP are known to increase the binding of insulin to its receptors [26]. Phenformin, however, did not elevate cyclic AMP levels in IM-9 cells: control levels of cyclic AMP were 555 ± 53 fmol/100 μg protein (mean ± SE of four measurements), whereas cyclic AMP levels in IM-9 cells preincubated 24 hr with 20 μM phenformin were 490 ± 95 fmol/100 μg protein.

DISCUSSION

Previously we found that phenformin, in vitro, increased the binding of ^{125}I-insulin to several lines of human and animal cells, including IM-9 lymphocytes [8], MCF-7 breast cancer cells [7], H35 hepatoma cells [9], and fibroblasts [9]. This action of phenformin was not seen with other hormone receptors, since, under similar conditions, phenformin decreased growth-hormone binding [27]. In addition, biguanides are effective in increasing the insulin binding to its receptors in vivo [28,29]. In view of the observation that phenformin and other biguanides potentiate the action of insulin [11], we studied the mechanisms involved in the action of phenformin on insulin receptors.

Several lines of evidence suggested that the effect of phenformin on IM-9 insulin receptors did not require the synthesis of new cellular proteins. First, cycloheximide pretreatment of these cells, a procedure which by itself causes a small decrease in the binding of insulin to receptors, did not block the stimulatory actions of phenformin [9]. Second, Scatchard analyses of the binding data revealed that phenformin markedly increased the affinity of the insulin receptor without markedly changing its capacity [9]. Finally, in accordance with these Scatchard data, dissociation studies indicated that phenformin acted to increase receptor affinity by slowing the rate of hormone dissociation. These data suggest, therefore, that in IM-9 cells phenformin acts to enhance the ability of the existing insulin receptors to bind insulin, rather than increasing the number of receptors.*

Although phenformin slowed cell growth, studies indicated that inhibition of cell growth was not the cause of increased insulin binding to IM-9 cells. Other agents that inhibit cell growth, such as thymidine and cycloheximide, slowed the growth of IM-9 cells but did not mimic the increase of insulin binding produced by phenformin. Both endogenous and exogenous cyclic AMP are known to decrease cell growth in tissue culture [26]. Further, it has been reported that agents that elevate cyclic AMP levels increase insulin binding to cultured cells, including IM-9 lymphocytes [30]. We found, however, that phenformin had no significant effect on cyclic AMP levels in IM-9 cells, suggesting that phenformin was not acting via cyclic AMP.

It is known that hormones acting via adenylcyclase have an obligatory requirement for the nucleotide GTP [24]. Further, GTP regulates the affinity of the receptors for certain hormones [24]. Since phenformin influences the affinity of the insulin receptor in IM-9 lymphocytes, we investigated the effect of phenformin on GTP levels in these cells and found that phenformin caused a marked fall in cellular GTP content. However, when GTP was selectively lowered by mycophenolic acid, insulin binding to its receptor was not increased. These studies suggested, therefore, that insulin receptors and GTP levels were affected independently by phenformin.

Phenformin has a number of effects on the intracellular metabolism of glucose, including inhibition of both the pentose monophosphate shunt and the Krebs cycle [11–13]. As a consequence of this metabolic inhibition, cellular ATP levels are decreased [13]. In the present study with IM-9 cells, we found that phenformin lowered ATP levels in a dose- and time-dependent manner. Several lines of evidence in IM-9 cells suggested a potential relationship between phenformin's stimulation of insulation binding and phenformin's lowering of ATP levels. First, the time course of the phenformin-stimulated increase of insulin binding was related to the time course of the decrease in ATP levels. Second, both the rise in insulin binding and the fall in ATP levels occurred over similar concentrations of phenformin. Third, other agents that lowered ATP levels also raised insulin binding. Finally, increasing the

*Previously we reported that in MCF-7 cells phenformin increased the binding capacity of the insulin receptor rather than its binding affinity [7]. These data, however, do not necessarily conflict with the present data. For instance, acute fasting of obese subjects initially induces a change in the affinity of the insulin receptors in monocytes, and if fasting is prolonged a change in binding capacity is then observed [31]. It is possible, therefore, that phenformin treatment, like fasting, has multiple effects on insulin receptors that depend on the duration of the study, the cell type investigated, and the metabolic state.

glucose concentration from 5.5 mM to 16.5 mM blocked both the effect of phenformin on insulin binding and its effect on cellular ATP levels. Preliminary studies in H35 and MCF-7 cells (two cell lines in which phenformin also increases insulin binding) indicate that phenformin also decreases ATP levels, but the effect is not as pronounced as that seen in IM-9 lymphocytes (unpublished observations of R. Vigneri and I.D. Goldfine). These observations suggest, therefore, that if ATP regulates the insulin receptor, either a specific pool of ATP may be responsible for this regulation or insulin receptors in different cells have a different sensitivity to ATP depletion.

We have recently observed that ATP, when directly added to either rat liver plasma membranes or human placenta particles, inhibits the binding of insulin to its receptor [17]. ATP has its major effect on binding affinity without significantly influencing binding capacity. With liver plasma membranes and placenta particles, an effect of ATP is seen at 1.0 to 2.5 mM. ATP is also effective in purified insulin receptors from placenta and in this preparation ATP is effective at concentrations as low as 10 μM. In contrast, 5′ adenylylimidodiphosphate (AMP-PNP), an ATP analogue that cannot readily phosphorylate proteins, has reduced activity. Thus it is likely that ATP and related substances can interact directly with the insulin receptor to change its binding affinity. It is possible, therefore, that the present effects of phenformin on ATP levels and insulin binding in IM-9 cells may be related to this direct effect of ATP on the insulin receptor.

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