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Enhancement of Adipocyte Differentiation by an Insulin-Sensitizing Agent

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SUMMARY

The thiazolidinediones are a class of novel antidiabetic compounds that enhance the response of target tissues to insulin. Pioglitazone, a thiazolidinedione analog, lowers blood glucose and insulin levels in rodent models of non-insulin-dependent diabetes mellitus. We have studied the effect of pioglitazone on 3T3-L1 cells, a cell line that undergoes differentiation from a preadipocyte fibroblastic morphology to that of an adipocyte. Pioglitazone treatment of preadipocytes enhanced the insulin- or insulin-like growth factor-1 (IGF-I)-regulated differentiation (monitored by the rate of lipogenesis or triglyceride accumulation), whereas treatment of the cells in the absence of insulin or IGF-I resulted in no apparent change in the cellular phenotype. Pioglitazone caused both a leftward shift and enhanced maximum response for the IGF-I-regulated differentiation of the cells, consistent with the idea that the drug enhances the sensitivity of cells to polypeptide hormones. A series of pioglitazone analogs were tested in this system, and variations in activity relative to that of the parent compound were observed. A study of the time required for the drug to exert an effect on differentiation revealed that an increased rate of lipogenesis occurred 16–24 hr after drug treatment in appropriately staged cells. An increased rate of glucose transport and increased activity of lipogenic enzymes were noted in a time frame that correlated with the change in lipogenesis. Analysis of mRNA abundance for Glut-4, lipoprotein lipase, and glucose-6-phosphate dehydrogenase showed that pioglitazone enhanced the insulin induction of these mRNA species. Thus, pioglitazone, in combination with insulin or IGF-I, appears to be exerting effects on the cellular phenotype by eliciting changes in the expression of genes that regulate metabolic pathways leading to the acquisition of the differentiated phenotype.

Pioglitazone, a thiazolidinedione analog, is a novel antidiabetic compound that lowers blood glucose in diabetic rodent models through a mechanism that involves increased insulin sensitivity in the target tissues (1). Administration of pioglitazone to insulin-resistant fatty rats resulted in a dose-dependent decrease in blood levels of glucose, triglycerides, and insulin (2). Improved oral glucose tolerance was also observed in insulin-resistant rats, whereas the amount of insulin released in response to an oral glucose challenge was decreased (2). Several pharmacologic effects of the thiazolidinedione analogs have been described, and it is evident that the key effect of this class of drugs is to increase post-binding events mediating insulin action in target tissues (reviewed in Ref. 1). However, the molecular mechanism by which the thiazolidinediones elicit their effects is not understood. Several studies have demonstrated that glucose metabolism of adipose tissue in animal models of non-insulin-dependent diabetes mellitus is enhanced by the thiazolidinediones (2–4). Therefore, we initiated a series of studies using an *in vitro* culture system for adipocytes, to investigate the mechanism of action.

The 3T3-L1 cell line, in the presence of the appropriate hormonal signals, undergoes differentiation from a fibroblastic adipoblast to a mature adipocyte capable of carrying out insulin-regulated lipogenesis (5, 6). The differentiation pathway in this cell line is a well characterized process, for which many cellular and molecular parameters have been described (7–10). We sought to determine whether the thiazolidinediones, alone or in combination with insulin or IGF-I, could influence any aspect of the differentiation pathway. The results reported here demonstrate that pioglitazone or various analogs markedly enhance the insulin- or IGF-I-regulated differentiation of 3T3-L1 adipocytes.

Materials and Methods

Reagents. Dulbecco's modified Eagle's medium, fetal calf serum, and gentamicin were purchased from GIBCO. DEX and IBMX were from Sigma Chemical Co. Sodium [^{14}C]acetate and 2-deoxy-D-[^{14}C] glucose were purchased from New England Nuclear. Recombinant IGF-I was obtained from Boehringer Mannheim.

Cell culture. The 3T3-L1 cell line was from the American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle's medium containing 5% fetal calf serum and 10 μg/ml gentamicin. Cells were staged to differentiate by incubation for 48 hr in medium

ABBREVIATIONS: IGF-I, insulin-like growth factor-1; IBMX, isobutylmethylxanthine; G6PDH, glucose-6-phosphate dehydrogenase; Glut-4, the insulin-regulated glucose transporter isoform; DEX, dexamethasone.

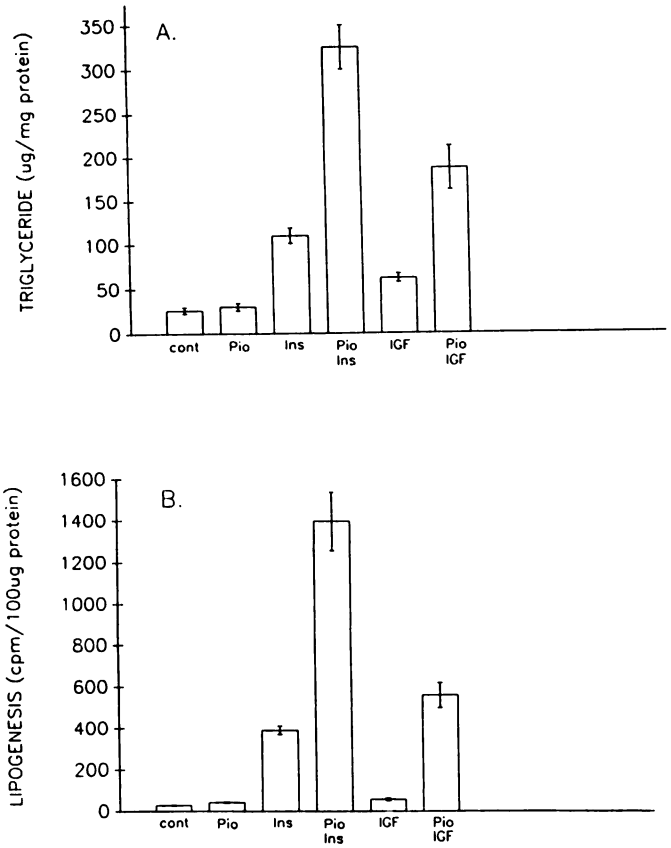

Fig. 1. Triglyceride accumulation (A) and rate of lipogenesis (B) in 3T3-L1 cells pretreated with IBMX/DEX before incubation with insulin (*Ins*), IGF-I, or pioglitazone (*Pio*). Confluent 3T3-L1 cells were incubated with IBMX (0.5 mM) and DEX (1 μM) for 48 hr. Cultures were then incubated in Dulbecco's modified Eagle's medium/5% fetal calf serum for 4 days with the indicated additions of insulin (150 nM), IGF-I (100 ng/ml), and pioglitazone (25 μM). The concentrations of hormone used produced the maximum response, as determined by triglyceride accumulation or lipogenesis. Cultures were incubated with [^{14}C]acetate for 1 hr to determine the rate of lipogenesis, as described in Materials and Methods. After the harvest of cells, lipids were extracted and analyzed for triglyceride and acetate incorporation. Each value represents the mean ± standard error of six determinations from three experiments.

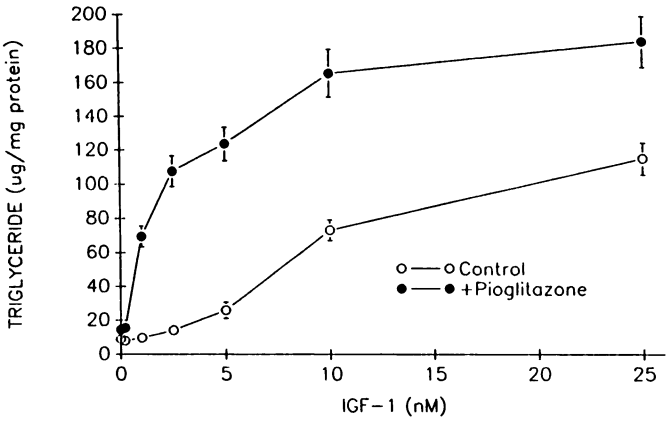

Fig. 2. Dose response for IGF-I effect on triglyceride accumulation. Confluent 3T3-L1 cells were staged for the experiment as described in Fig. 1. Cells were incubated with IGF-I with or without pioglitazone (12.5 μM), for 4 days before analysis of triglyceride content. Each value represents the mean of three separate experiments.

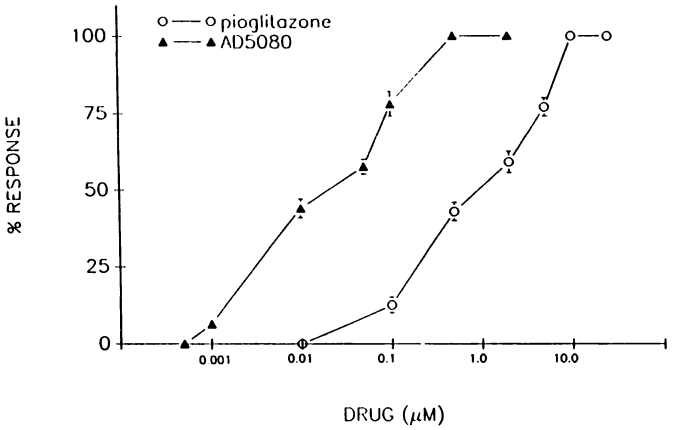

Fig. 3. Dose response for pioglitazone and AD5080 effect on triglyceride accumulation. Confluent 3T3-L1 cells were staged for the experiment as described in Fig. 1. Cells were incubated with the indicated drug concentrations and insulin (150 nM) for 4 days before harvest and analysis of triglyceride accumulation. The percentage response is based on the maximum triglyceride accumulation observed. Maximum triglyceride accumulated for cells treated with pioglitazone was 369 ± 37.6 μg/mg, whereas that for cells treated with AD5080 was 358 ± 43.2 μg/mg. Each value represents the mean ± standard error of eight determinations in two experiments.

containing 1 μM DEX and 0.5 mM IBMX. Additions of insulin or IGF-I to the medium were accomplished by dissolving the hormone in 3 mN HCl containing 0.2% bovine serum albumin. Addition of drugs to the medium was accomplished by dissolving the drug in dimethyl sulfoxide and diluting the drug 1000-fold. Dimethyl sulfoxide was present in all control cultures at a concentration of 0.1%.

Chemical assays. Cell protein was determined by the method of Lowry *et al.* (11), and DNA was quantified by fluorescence enhancement with Hoechst dye 33258. Cellular triglycerides were extracted with isopropanol and assayed by the acetylacetone method. Lipogenesis was determined by following the incorporation of [^{14}C]acetate into the triglyceride fraction, as described (12).

Enzyme and molecular biologic assays. G6PDH was assayed by following the reduction of NADP and glyceraldehyde-3-phosphate dehydrogenase, as described previously (13). The glucose transport activity of the cells was estimated by measuring the uptake of 0.5 mM 2-deoxy-D-[1-^{14}C]glucose in 4 min, as described earlier (14). The uptake of this glucose analog was linear for >20 min under these conditions. The relative abundance of mRNA species encoding G6PDH, lipoprotein lipase, and Glut-4 was determined by isolating the total RNA from cells through the use of RNAzol (Cinna/Biotecx) and probing Northern and dot blots for these species, as described previously (15, 16). The abundance of actin mRNA was determined as a hybridization control, because a previous study (17) had demonstrated that actin decreased during adipocytes differentiation. Probes used in these studies were cDNAs and/or antisense oligonucleotides, as described earlier (15, 16).

Results

The presence of insulin in the culture medium is typically required for induction of the differentiated phenotype in 3T3-L1 cells (5, 6). In the experiment shown in Fig. 1, cells were staged to differentiate by incubation in medium containing IBMX/DEX for 48 hr; the medium was then replaced and the indicated hormone/drug additions were made. Triglyceride accumulation and the rate of lipogenesis were measured as indices of the differentiated adipocyte phenotype. Incubation of cells with pioglitazone alone failed to elevate either triglyceride accumulation or lipogenesis above that observed in the control cultures, whereas, as expected (6), incubation with insulin caused a marked increase in both. However, the simultaneous

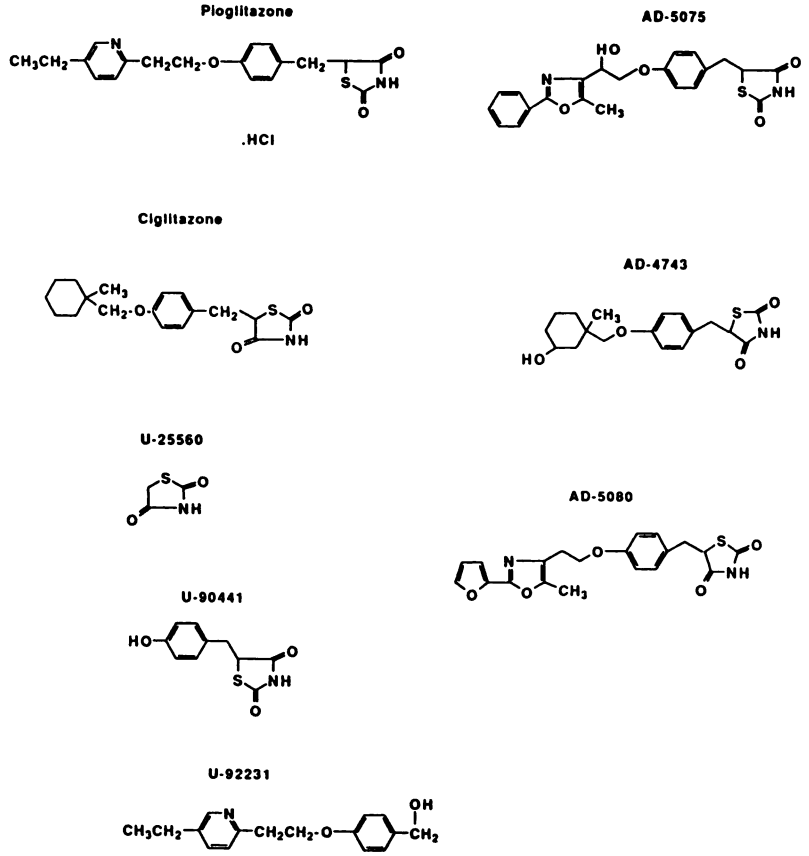

Fig. 4. Structures of pioglitazone and related analogs.

TABLE 1

Triglyceride accumulation in 3T3-L1 cells after treatment with pioglitazone analogs

Confluent cells were staged for the experiment as described in Fig. 1, and the culture medium contained 150 nM insulin. The drug was dissolved in each case in dimethyl sulfoxide and diluted 1000-fold into the culture medium. Cultures were harvested for triglyceride analysis after 4 days of incubation. Each value represents the mean ± standard error for four to six determinations from two experiments.

Analog	Dose	Triglyceride
	μM	μg/mg of protein
Control		34.6 ± 2.9
Pioglitazone	0.1	72.2 ± 5.7
	5.0	495.3 ± 41.1
AD5075	0.1	480.7 ± 50.3
	5.0	504.9 ± 34.7
AD5080	0.1	487.1 ± 47.9
	5.0	605.5 ± 55.2
Ciglitazone	0.1	36.1 ± 2.5
	5.0	32.6 ± 3.1
AD4742	0.1	33.3 ± 2.0
	5.0	93.4 ± 7.5
AD4743	0.1	31.9 ± 2.1
	5.0	191.2 ± 18.7
U25560	12.5	33.6 ± 4.1
U90441	12.5	36.5 ± 1.9
U92231	12.5	37.6 ± 2.9

incubation of cells with pioglitazone and insulin resulted in a 3–3.5-fold enhancement in both parameters above that observed with insulin alone. Pioglitazone also enhanced the effect of insulin on both the cell size (accumulation of fat) and number of cells that differentiated (data not presented). The simultaneous addition of pioglitazone and IBMX/DEX did not increase triglyceride accumulation or lipogenesis unless insulin or IGF-I was subsequently added to the cultures.

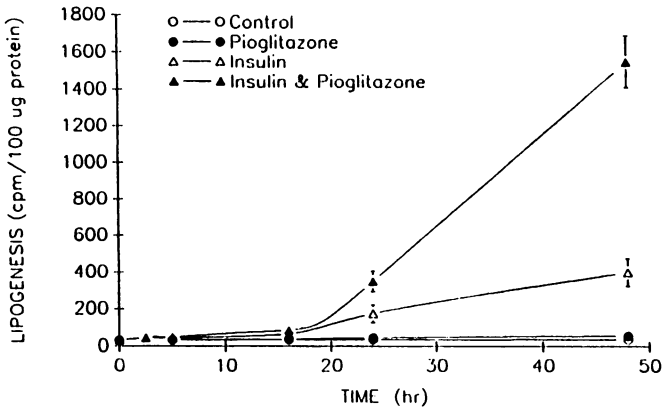

Fig. 5. Time course of the effect of pioglitazone and/or insulin treatment on the rate of lipogenesis. Confluent cells were staged for the experiment as in Fig. 1. At time zero, the indicated additions were made, and the cultures were pulsed with [^{14}C]acetate for 1 hr immediately before harvest and analysis of the radiolabel in the triglyceride fraction. Each value represents the mean ± standard error of four determinations in two experiments.

Previous studies (18, 19) have demonstrated that IGF-I can replace insulin for evoking differentiation of 3T3-L1 adipocytes. Furthermore, evidence has accumulated showing that, when insulin is causing differentiation of these cells, it is signaling through the IGF-I receptor (18, 19). Therefore, we carried out a series of experiments to determine whether pioglitazone could similarly enhance IGF-I-regulated differentiation of 3T3-L1 cells. The experiment in Fig. 1 demonstrates that pioglitazone enhanced the response of cells to IGF-I, although the magnitude of the response was not as great as that observed with insulin. The greater relative effect of insulin

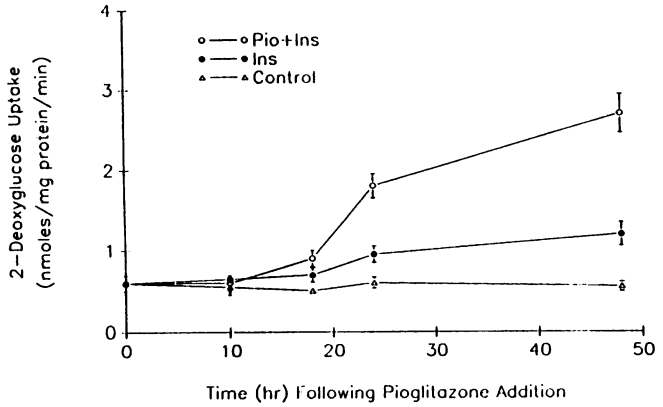

Fig. 6. Glucose transport activity as a function of time after pioglitazone (*Pio*) treatment. Cells were staged for the experiment as described in Fig. 1. At time zero, pioglitazone and/or insulin (*Ins*) was added to the cultures, and 2-deoxyglucose transport activity was determined at the indicated times. Cultures treated with pioglitazone alone were not significantly different from control. Each value represents the mean ± standard error of six determinations from two experiments.

versus IGF-I on triglyceride accumulation and lipogenesis, both of which are late indices of differentiation, may result from the expression of insulin receptors during the course of differentiation. Thus, the final stage (lipid accumulation) of adipocyte differentiation is more responsive to insulin than IGF-I (18). The response of 3T3-L1 cells to varying doses of IGF-I in the presence or absence of pioglitazone is shown in Fig. 2. Pioglitazone enhanced the maximal response of cells to IGF-I and caused a leftward shift in the dose-response curve.

The dose response for enhancement of 3T3-L1 differentiation by pioglitazone and a related analog, AD-5080, is shown in Fig. 3. The data presented here show that AD-5080 (EC_{50} = 0.03 μM) was substantially more active than pioglitazone (EC_{50} = 1 μM). The morphologic characteristics of the cells incubated with either drug were identical (data not presented). Several other thiazolidinedione analogs (see Fig. 4) were selected for study here, to determine whether they could enhance insulin-regulated differentiation of the cells. Ciglitazone did not cause a significant increase in triglyceride content at the concentrations used; however, the metabolites (AD4742 and AD4743) were active, although not as potent as pioglitazone (Table 1). The major chemical moieties (U25560, U90441, and U92231) of the pioglitazone structure were tested in this system, and none were found to be active. Thus, the cells appear to exhibit fairly precise chemical structure requirements for this class of drugs.

The effect of pioglitazone on triglyceride accumulation and lipogenesis, as a function of time of drug treatment, was determined. Pioglitazone and insulin, alone or in combination, were added at time zero, and the effect on triglyceride content was determined at various times after addition. Pioglitazone alone failed to increase triglyceride content versus control at any time measured, whereas treatment of cells with pioglitazone and insulin resulted in an increase in triglyceride accumulation above that seen with insulin alone, which could first be detected at 24 hr and which was significantly increased at 36 hr (data not presented). The rate of lipogenesis was measured during the hour immediately before harvest of the cells in the experiment shown in Fig. 5. This demonstrates that a difference between pioglitazone and insulin treatment versus insulin alone was first observed at 16 hr, and significant differences were apparent by 24 hr. A clear effect on the maximum rate of lipogenesis was also brought about by the drug in combination with insulin. Thus, within this time period of drug treatment, changes have been elicited in the cell that result in enhancement of insulin action.

The change in the cellular phenotype brought about by differentiation of 3T3-L1 cells is the result of numerous changes in gene expression (7, 8). Prominent among these changes are those associated with enzymes involved in the uptake and conversion of glucose to fatty acid. To determine the time required for pioglitazone to exert an effect on this process, the experiment shown in Fig. 6 was carried out. Simultaneous treatment of cells with pioglitazone and insulin caused an increase in the glucose transport activity before that observed in those cultures treated with insulin alone, and the maximum response was elevated. A comparable time course was observed when G6PDH, the rate-limiting enzyme in the pentose phosphate pathway, was assayed in 3T3-L1 cells staged as described above (Fig. 7). We have observed similar results for other

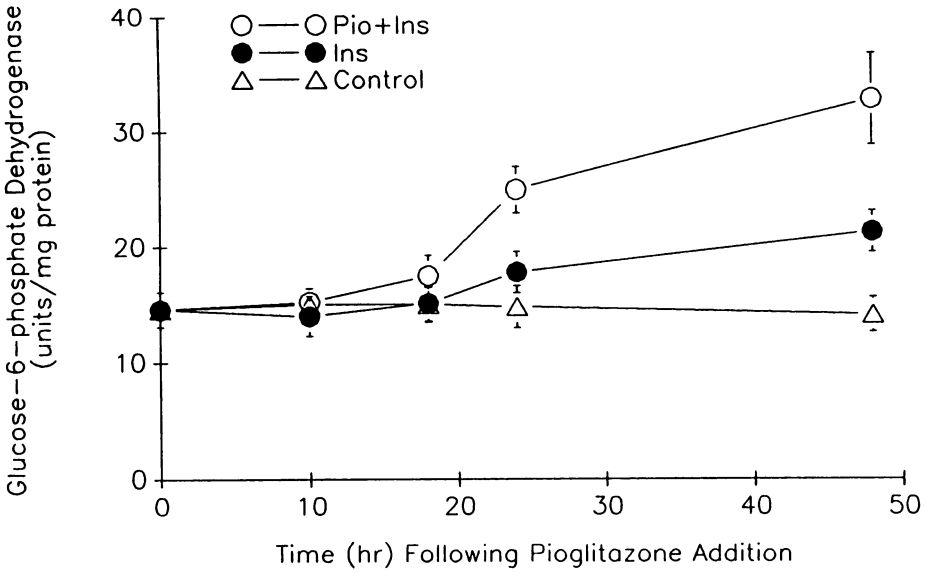

Fig. 7. G6PDH activity as a function of time after pioglitazone (*Pio*) treatment. Cells were staged for the experiment as described in Fig. 7. Cultures treated with pioglitazone were not significantly different from control. Each value represents the mean ± standard error of four determinations from two experiments. *Ins*, insulin.

TABLE 2

G6PDH, Glut-4, and lipoprotein lipase (LPL) mRNA abundance in 3T3-L1 cells treated with insulin and pioglitazone

Cells were staged for the experiment as described in Fig. 1, and incubation with insulin and pioglitazone was carried out for 2 days. RNA was isolated and analyzed as indicated in Materials and Methods. Each value represents the mean ± standard error for three or four determinations.

Treatment	mRNA abundance		
	Glut-4	LPL	G6PDH
	cpm/μg of total RNA		
Control	319 ± 45	651 ± 61	1200 ± 115
Pioglitazone	375 ± 52	722 ± 41	1158 ± 153
Insulin	449 ± 38	975 ± 93	1650 ± 147
Pioglitazone/insulin	1575 ± 137	2173 ± 193	2300 ± 205

TABLE 3

Acute and chronic pioglitazone treatment of preadipocytes and mature adipocytes

Preadipocytes (3T3-L1) were staged as described in Fig. 1, with the control values representing cells at the end of the IBMX/DEX treatment. After removal of IBMX/DEX, cultures were treated with insulin (150 nM) and pioglitazone (10 μM) for 4 or 20 hr. Mature adipocytes were produced from 3T3-L1 cells treated for 48 hr with IBMX/DEX and insulin (1 μg/ml). Cells were then placed in medium containing insulin (1 μg/ml) for 6 days, after which drug treatment was initiated.

Time of drug treatment	G6PDH[a]	GAPDH[b]	Glucose transport[a]
hr	*μmol/mg*	*μmol/mg*	*nmol/mg/min*
Preadipocytes			
None	14.3 ± 1.0	710	0.66 ± 0.05
4	14.5 ± 0.4	687	0.64 ± 0.04
20	20.9 ± 1.1	1793	1.15 ± 0.02
Adipocytes			
None	38.3 ± 3.2	4791	3.5 ± 0.29
4	36.9 ± 2.3	4827	3.5 ± 0.11
20	42.9 ± 3.9	5397	3.9 ± 0.15

[a] Values represent the mean ± standard error for six to eight determinations.
[b] GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Values represent the average from two experiments.

lipogenic enzymes (glyceraldehyde-3-phosphate dehydrogenase, malic enzyme, and acetyl-CoA carboxylase) in these cells (data not presented). Thus, the time course and maximal expression of the enzymes that participate directly in the glucose to fatty acid conversion require both the drug and insulin (or IGF-I) and are consistent with the notion that the drug is active at the level of gene expression.

Experiments were carried out to determine whether pioglitazone, alone or in combination with insulin, caused changes in the level of mRNA encoding G6PDH, Glut-4, and lipoprotein lipase in 3T3-L1 cells. Cells were treated with pioglitazone and insulin for 48 hr, and the abundance of the specific mRNA was determined (Table 2). In each case, pioglitazone by itself did not increase mRNA abundance. However, the drug enhanced the insulin-regulated expression of each species, and the effect on the Glut-4 and lipoprotein lipase mRNA was >2-fold.

To determine whether pioglitazone could acutely influence the activity of enzymes regulated by insulin, the experiment described in Table 3 was carried out. The 3T3-L1 cells were staged such that the preadipocyte or mature adipocyte phenotype was expressed. The cultures were treated with pioglitazone for either 4 or 20 hr, and the indicated enzymatic activities were assayed. It was found that acute drug treatment (4 hr) had no effect on enzymatic activity in either cell type. The longer treatment significantly increased activity in the preadipocytes and to a lesser extent in the mature adipocytes. The extended time requirement needed to observe an effect of the drug on these enzymes is consistent with action of the drug at the level of gene expression.

Discussion

We have demonstrated that pioglitazone enhances the insulin- or IGF-I-regulated differentiation of 3T3-L1 adipocytes. The drug clearly sensitizes cells to IGF-I, in that the dose-response curve is shifted to the left and the maximum effect is enhanced after treatment (Fig. 2). A similar experiment with insulin was not carried out, because the cells do not express insulin receptors early in the differentiation pathway and, thus, insulin must be present at pharmacologic levels for effects on differentiation to be observed. A previous study (20) using a thiazolidinedione metabolite suggested that the adipogenic effect of the drug was largely independent of insulin. However, the authors (20) had used a high level of fetal calf serum in the culture medium, making it difficult to discern an independent effect of the drug versus an interaction of the drug with growth factors or hormones present in the serum. By reducing the level of fetal calf serum in our culture system, we have demonstrated that pioglitazone enhances the effect of insulin or IGF-I on differentiation, but we cannot rule out the possible influence of other factors in the serum. Experiments with serum depleted of hormones and growth factors (18) need to be carried out to examine this point.

The enhancement of insulin or IGF-I action by pioglitazone appears to be fairly specific for preadipocyte cell lines and primary cultures of preadipocytes. A survey of standard cell lines, including several hepatoma lines and two primary culture systems (cardiac myocytes and liver parenchymal cells), failed to demonstrate any drug enhancement of hormone or growth factor activities (data not shown). The lack of pioglitazone effects on hepatocyte function (glycogen metabolism and amino acid transport) was surprising, because *in vivo* studies showed that the drug directly and reversibly influenced liver metabolism (1, 2, 4). This suggests that, *in vivo*, the effects of the drug on other insulin target tissues may be indirect, perhaps the result of interaction of the drug with adipose tissue.

Two earlier studies (20, 21) have shown that a thiazolidinedione metabolite will enhance the differentiation of several preadipocyte cell lines. The work reported here demonstrates that the adipogenic activity is observed in several thiazolidinedione parent compounds, although no activity was observed with ciglitazone (parent drug from which the metabolite used in the earlier study was derived) (20, 21). We have found that the relative potency in the 3T3-L1 cells follows that observed for antidiabetic activity *in vivo*. Ciglitazone was found to be 3–6-fold less potent than pioglitazone in *in vivo* antidiabetic activity (1). Thus, there may exist a need for ciglitazone to be metabolized *in vivo* in order to exert antidiabetic activity.

Pioglitazone, in the presence of insulin or IGF-I, clearly accelerates the appearance of the differentiated phenotype in appropriately staged 3T3-L1 cells. We have shown that several mRNA species are elevated in conjunction with this change, although we cannot at this juncture determine whether the drug is having a direct or indirect effect on gene expression. The change in cellular phenotype in going from a fibroblastic preadipocyte to an adipocyte results in alterations in the levels of at least 100 protein species (reviewed in Ref. 7). Many of the induced proteins are directly involved in the function of the differentiated adipocyte, such as the synthesis and storage of

fatty acids, whereas some of the repressed proteins, such as B-actin, are characteristic of only the fibroblastic state. The induction/repression of these proteins is temporally related to the acquisition of the differentiated state, with the end result being the synthesis and storage of fatty acids as triglyceride. Early in the differentiation process, genes are activated that encode regulatory proteins, which then direct the expression of other proteins directly involved in synthesis and storage of lipids. One view of the effect of pioglitazone on this process is that the drug could function as a coinducer (along with the insulin or IGF-I second messenger) of the genes encoding the enzymes involved with metabolism, e.g., fatty acid synthase, Glut-4, etc. An alternative view is that the drug is inducing a regulatory protein(s) whose function is to mediate or enhance the action of insulin. In the latter case all actions of insulin would be amplified, whereas in the former only selected actions would be. Molecular analysis of the effects of pioglitazone on 3T3-L1 cells should provide insight into the mechanism by which the thiazolidinediones sensitize cells to insulin.

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