0026-895X/92/040558-05$03.00/0

MOLECULAR PHARMACOLOGY, 42:558–562

ACCELERATED COMMUNICATION

Adipocyte Fatty Acid-Binding Protein: Regulation of Gene Expression *In Vivo* and *In Vitro* by an Insulin-Sensitizing Agent

ROLF F. KLETZIEN, LISA A. FOELLMI, PETER K. W. HARRIS, BEATRICE M. WYSE, and STEVEN D. CLARKE[1]

Metabolic Diseases Research, Upjohn Laboratories, The Upjohn Company, Kalamazoo, Michigan 49001

Received June 5, 1992; Accepted July 7, 1992

SUMMARY

Pioglitazone, a thiazolidinedione, is a novel antidiabetic compound that can lower blood glucose in diabetic rodents by increasing insulin sensitivity in target tissues. We have previously demonstrated that pioglitazone can enhance the insulin- or insulin-like growth factor-1-regulated differentiation of 3T3-L1 cells, a cell line that undergoes morphological and biochemical differentiation to mature adipocytes [*Mol. Pharmacol.* **41**:393–398 (1992)]. In this study, we have examined the effect of pioglitazone on the expression of the adipocyte fatty acid-binding protein (aFABP) in *ob/ob* mice and 3T3-L1 cells. Administration of the drug to mice was observed to cause a dose-dependent increase in aFABP mRNA expression in epididymal fat, which was correlated with a decrease in blood glucose and insulin levels. Treatment of 3T3-L1 cells with pioglitazone enhanced aFABP expression in a time-dependent fashion. To explore a possible direct effect of pioglitazone on aFABP expression, a chimeric gene was constructed containing the aFABP promoter fused upstream of the bacterial reporter gene for chloramphenicol acetyltransferase. After transfection into 3T3-L1 cells and selection of stable transformants, regulation of the chimeric gene was studied. Pioglitazone, in combination with insulin or insulin-like growth factor-1, was observed to elicit a dose-dependent increase in expression, indicating a role for pioglitazone in regulating transcription of the aFABP gene. Several thiazolidinedione analogs were tested for their ability to induce the expression of the chimeric gene, and it was found that activity in this assay paralleled the structure-activity relationships observed for enhancement of 3T3-L1 cell differentiation. These observations on control of aFABP gene expression by pioglitazone suggest possible mechanisms by which cellular sensitivity to insulin may be regulated.

NIDDM is characterized by insulin resistance in target tissues, including muscle, liver, and adipose (1). Thus, blood glucose is elevated in NIDDM as a result of underutilization of glucose by muscle and adipose, and insulin fails to suppress glucose production in liver (1, 2). Recently, novel antidiabetic agents that ameliorate insulin resistance associated with NIDDM have been developed (3). The thiazolidinediones are a class of antidiabetic compounds that lower blood glucose in diabetic rodent models through a mechanism that involves increased insulin sensitivity in target tissues. Pharmacological effects of the drugs in rodents include decreased blood levels of glucose, triglyceride, and insulin (4, 5) and decreased cholesterol absorption (5). Two thiazolidinedione analogs, ciglitazone and pioglitazone, have been shown clearly to enhance insulin stimulation of glucose metabolism in adipose tissue of diabetic rodents (2, 6). Recently, it has been shown that administration of pioglitazone to diabetic animals up-regulates expression of the insulin-regulatable glucose transporter in adipose tissue (7).

The molecular mechanisms by which the thiazolidinediones elicit their effects are not understood, but it is evident that the key mode of action is to increase postreceptor events mediating insulin action in target tissues (reviewed in Ref. 3). In this regard, attention has focused on the possible role that aFABP may play in the insulin signaling process. This protein (also known as aP2 or 422) can be phosphorylated on a tyrosine residue by the insulin receptor tyrosine kinase, a process that is stimulated after the binding of one molecule of fatty acid (8). Two aFABP protein phosphatases have been identified (9), and the suggestion has been made that the aFABP protein plays an intermediary role in insulin signaling (8, 10, 11). In this study, we have used the obese (*ob/ob*) mouse as a model of NIDDM and found that pioglitazone up-regulated aFABP expression in adipose tissue. Therefore, we initiated studies in an *in vitro* adipocyte model to study the mechanism of drug action.

The 3T3-L1 cell line is capable of undergoing differentiation from a fibroblastic adipoblast to a mature adipocyte (12, 13). We have recently demonstrated that pioglitazone enhances the insulin- or IGF-1-regulated differentiation of 3T3-L1 cells (14).

[1] Current address: Department of Food Science and Human Nutrition, Colorado State University, Ft. Collins, CO 80523.

ABBREVIATIONS: NIDDM, non-insulin-dependent diabetes; IGF-1, insulin-like growth factor-1; aFABP, adipocyte fatty acid-binding protein; CAT, chloramphenicol acetyltransferase; DEX, dexamethasone; IBMX, isobutylmethylxanthine; Kb, kilobase(s).

The orderly expression of genes during the differentiation of these cells is well documented (15–18). Recent studies have demonstrated that regulatory proteins such as the nuclear transcription factors C/EBP and c-Fos are elevated early in the differentiation pathway (15, 19–21). Enzymes responsible for the metabolism of glucose and synthesis of fatty acids, such as acetyl-coenzyme A carboxylase and fatty acid synthase, are induced later (17, 18). Several groups have focused attention on the differentiation-dependent expression of aFABP, because the gene encoding aFABP is transcriptionally activated during differentiation of 3T3-L1 cells (22, 23). The aFABP promoter contains multiple regulatory *cis* elements, including sites for C/EBP, cAMP, c-Fos/c-Jun, glucocorticoids, and fatty acid-specific elements within the proximal 250 bases from the transcription start site (15, 19, 23, 24). In addition, an element at −4.9 to −5.4 kb appears to be responsible for tissue-specific and differentiation-dependent expression of the gene (23). In view of the potential role of aFABP in mediating insulin action (8, 10, 11), we focused this study on the regulation by pioglitazone of aFABP mRNA expression *in vivo* and *in vitro*.

Experimental Procedures

Materials. Dulbecco's modified Eagle's medium, fetal calf serum, G418, and gentamicin were purchased from GIBCO. DEX was obtained from Sigma Chemical Co. [^{14}C]Chloramphenicol was purchased from Amersham. Recombinant IGF was purchased from Boehringer Mannhein. RNAzol was from Cinna/Biotecx. Mice (*ob/ob*) were obtained from The Jackson Laboratory. Duralon-UV membranes were from Stratagene.

Cell culture. The 3T3-L1 cell line (American Type Culture Collection) was maintained in Dulbecco's modified Eagle's medium containing 10% calf serum and 10 μg/ml gentamicin. Cells were staged to differentiate by incubation in medium containing 10% fetal calf serum, 1 μM DEX and 150 nM insulin. Additions of insulin or IGF-1 to the medium were made by dissolving the hormone in 3 mN HCl containing 0.2% bovine serum albumin. Pioglitazone was added to the medium by dissolving the drug in dimethylsulfoxide and diluting the drug 1000-fold.

Animal use. Male *ob/ob* mice at a starting age of 3–3.5 months received pioglitazone in normal laboratory chow, at doses ranging from 0 to 71 mg/kg/day, for 6 weeks. Blood samples were taken from the orbital sinus for glucose and plasma insulin determinations. Animals were sacrificed by decapitation over a course of 3 days, with equal numbers taken from each treatment group each day. Both epididymal fat pads were removed and homogenized in 2 ml of RNAzol.

Chemical, enzyme, and molecular biological assays. Cell protein was determined by the bicinchoninic acid method (25) and RNA by absorbance at 260 nm. Glucose and insulin levels were assayed as described previously (6). CAT activity was measured by a modification of the method of Gorman *et al.* (26). The relative abundance of mRNA species encoding aFABP and β-actin was determined by isolating the total RNA from cells or tissues through the use of RNAzol and probing Northern and dot blots for these species, as described previously (14, 27). The abundance of actin mRNA was determined as a hybridization control, because a previous study (28) had demonstrated that this mRNA decreased during adipocyte differentiation. The cDNA probe used in these studies was gel purified and represented 510 bases of coding sequence. Stable transformants of the 3T3-L1 cells were produced by transfecting cells with a 10:1 molar ratio of aP2(7Kb)/CAT or aP2(−100)/CAT and pWLNeo (Stratagene), by using Lipofectin (BRL). Cells were selected and maintained in medium containing 400 μg/ml G-418.

Plasmid construction. A *Hind*III-*Pst*I fragment containing the proximal 7 kb of the aFABP promoter was placed upstream of the CAT reporter gene in plasmid pBASIC (Promega), creating plasmid aP2(7Kb)/CAT. A *Hind*III/*Pst*I polymerase chain reaction product, extending from −100 to +21 bp of the aFABP promoter, was placed upstream of the CAT reporter gene in plasmid pBASIC, creating control plasmid aP (−100)/CAT.

Results

The expression of aFABP in epididymal fat of *ob/ob* mice was studied after administration of pioglitazone. Hyperinsulinemia appears in the *ob/ob* mouse at about 1 month of age and peaks at about 6 months. Hyperglycemia and marked resistance to exogenous insulin appear after the onset of hyperinsulinemia and reach a peak by 12 weeks (29). Animals were treated with various doses of pioglitazone for 6 weeks, at which time the relative abundance of aFABP mRNA in the epididymal fat was determined and the blood levels of glucose and insulin were analyzed (Fig. 1). A 3-fold increase in the relative abundance of the aFABP mRNA was observed at the highest dose (71 mg/kg/day), whereas the lowest dose (2.2 mg/kg/day) elicited a slightly less than 2-fold increase (Fig. 1). Blood levels of both insulin and glucose were proportionately decreased by the drug, as expected from previous studies with diabetic rodents (6). The dramatic decrease in insulin levels and concomitant diminution of blood glucose reflect an increase in sensitivity to insulin (1). In other studies we have observed a drug concen-

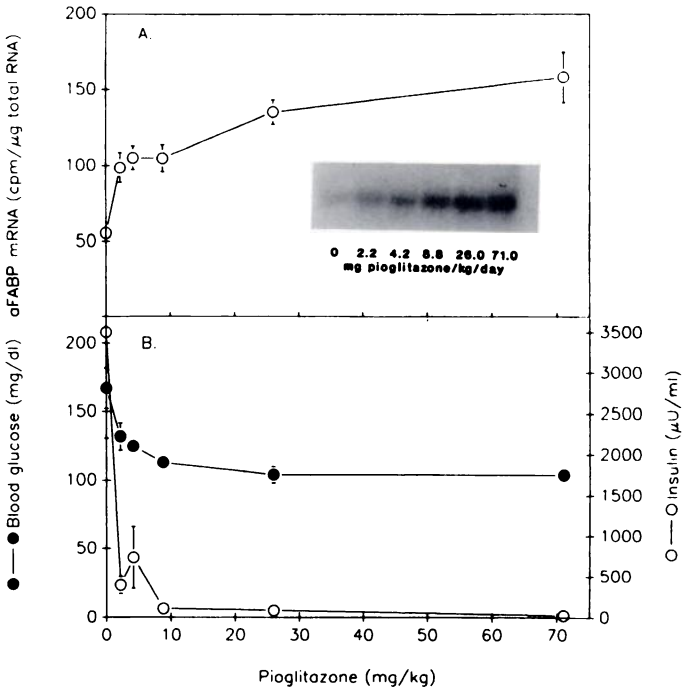

Fig. 1. Effect of pioglitazone on aFABP mRNA and blood levels of glucose and insulin in *ob/ob* mice. Animals were dosed with the indicated amounts of pioglitazone for 6 weeks. A, Total RNA was isolated from the epididymal fat pads, fractionated by denaturing electrophoresis, and transferred to Duralon-UV membrane using standard procedures. The Northern blots were probed with ^{32}P-labeled cDNA obtained by random priming of inserts corresponding to 510bases of coding sequence for aFABP. Inset, relevant portion (0.6–0.7 kb) of a resulting autoradiogram. Each *lane* contains 5 μg of total RNA from one animal of each treatment group. Each value for aFABP mRNA represents the mean ± standard error for six animals per treatment group. Radioactivity was quantified using an Ambis radioanalytical imaging system. B, Insulin and glucose values were determined at the time of sacrifice. Each value represents the mean ± standard error for six animals per treatment group.

tration in the peripheral blood of 1–3 μM at a dose of 10 mg/kg of body weight (data not presented). Therefore, we have used drug concentrations of 1–12.5 μM in our *in vitro* studies of pioglitazone action.

To determine whether the thiazolidinediones could influence the expression of aFABP in a cell culture model of adipocytes, we studied the effect of pioglitazone on 3T3-L1 cells, because we have previously shown that pioglitazone enhances the insulin- or IGF-1-regulated differentiation of this cell line (14). The time course of aFABP mRNA expression elicited by pioglitazone treatment of 3T3-L1 cells was studied in the experiment depicted in Fig. 2. Cells were staged to differentiate by incubation of confluent cultures with DEX and insulin, in the presence or absence of the drug. Pioglitazone increased the relative abundance of aFABP mRNA 10-fold in 48 hr, compared with that observed for DEX and insulin alone. After 4–5 days of incubation, the cultures incubated with pioglitazone had accumulated many terminally differentiated adipocytes, as evidenced by the appearance of fat droplets within the cells, and the drug enhanced the expression of aFABP mRNA by 20–28-fold (Fig. 2). Incubation of cultures with the drug for 48 hr followed by incubation for an additional 3 days in the absence of drug increased aFABP mRNA 10–12-fold (data not shown). The data in Table 1 establish that pioglitazone also enhances the expression of aFABP mRNA induced by IGF-1. In addition, it was found that the pioglitazone enhancement of aFABP expression (Table 1) occurred in the same concentration range as did enhancement of cellular differentiation (14). Thus, the presence of the drug clearly resulted in a substantial increase in aFABP mRNA, although one cannot distinguish from these experiments an effect of pioglitazone on transcription of the aFABP gene versus stabilization of the mRNA.

To determine whether the aFABP gene could be regulated by pioglitazone, a chimeric gene was constructed by fusing the proximal 7 kb of the aFABP promoter to the CAT reporter gene. Analysis of this construct [aP2(7Kb)/CAT] using transient expression in 3T3-L1 cells, with 48-hr incubation with

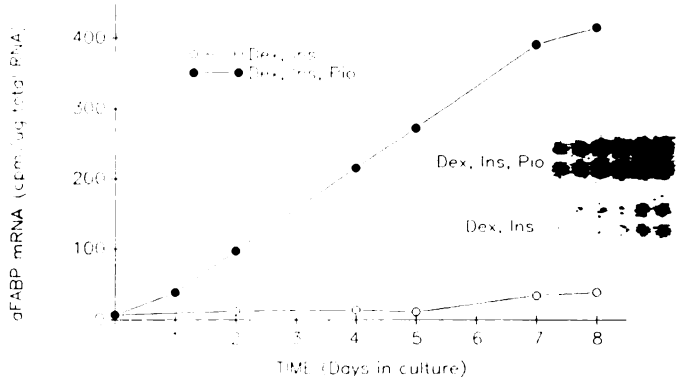

Fig. 2. Induction of aFABP mRNA in 3T3-L1 cells after treatment with pioglitazone. Preadipocyte 3T3-L1 cells were staged for the experiment by treatment of confluent cultures with the indicated additions [DEX, 1 μM; insulin (*Ins*); 150 nM; pioglitazone (*Pio*), 5 μM] in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Total RNA was isolated and analyzed as indicated in Experimental Procedures. Each value represents the mean for three separate determinations. The hormones/drugs were added at time zero, and cultures were harvested at the appropriate times. Each dot (*inset*) represents the signal from 2 μg of total RNA from one culture dish after hybridization with the aFABP probe. The RNA samples were analyzed for β-actin mRNA sequence abundance as a control, and it was found that a 40% reduction occurred during the 8 days of the experiment.

TABLE 1

Regulation of endogenous aFABP mRNA levels

Cells were staged for the experiment as described for Fig. 2. IGF-1 was 25 nM when present. Cells were harvested after 6 days of treatment, and the RNA was isolated and analyzed for aFABP mRNA abundance as indicated for Fig. 2. Values in experiment A represent the mean ± standard error of six determinations, whereas values in experiment B represent the average of duplicate determinations.

Treatment	aFABP mRNA abundance
	cpm/μg of total RNA
Experiment A	
Control	76 ± 12
DEX	115 ± 18
DEX/pioglitazone (5 μM)	127 ± 14
DEX/IGF-1	408 ± 35
DEX/IGF-1/pioglitazone (5 μM)	1220 ± 83
Experiment B	
DEX	12
DEX/insulin	58
DEX/insulin/pioglitazone (0.05 μM)	65
DEX/insulin/pioglitazone (0.5 μM)	285
DEX/insulin/pioglitazone (1 μM)	620
DEX/insulin/pioglitazone (5 μM)	675

IBMX/DEX to stage the cells for differentiation, proved intractable because of high levels of expression of the chimeric gene during treatment with IBMX/DEX (data not presented). Other investigators have also observed direct effects of IBMX (presumably acting through cAMP) and DEX on expression of the aFABP gene in transient expression systems (24). Therefore, stable transformants (G418 resistant) were selected and cells were differentiated in the protocol used in Fig. 2 above. Cultures were harvested and assayed for CAT activity after 6 days of incubation with hormones/pioglitazone. The results in Fig. 3 establish that this chimeric gene is regulated by pioglitazone in a dose-dependent manner. The induction of the chimeric gene by pioglitazone was observed to occur in the same concentration range as that required for differentiation of 3T3-L1 cells (14) or induction of the endogenous aFABP mRNA (see Table 1). It is evident that the presence of DEX in the culture medium elevates the background expression of the aP2(7Kb)/CAT gene. When the background level of expression (DEX-alone treatment) was subtracted from the other values, a 25-fold increase in expression of CAT was observed, which is similar to that seen for the endogenous aP2 gene under the same culture conditions (see Fig. 2).

We have previously shown that pioglitazone can enhance either insulin- or IGF-1-regulated differentiation of 3T3-L1 cells (14). The data in Table 2 establish that pioglitazone can up-regulate the expression of the aP2(7Kb)/CAT gene if cells are simultaneously incubated with the drug and insulin or IGF-1. However, in the absence of insulin or IGF-1, pioglitazone was ineffective in elevating expression, a result consistent with the effect of the drug on cellular differentiation (14). If the cultures were incubated with the indicated hormone/drug combinations but in the presence of DEX for just the initial 72 hr, overall expression of CAT was decreased by about 15%, although the effect of pioglitazone treatment was the same (data not shown). A control chimeric gene containing the proximal 100 bases of the aFABP promoter fused to the CAT gene failed to respond to drug treatment, establishing that the pioglitazone response element is not located here. This region (bases −1 to −100) of the aFABP promoter is devoid of regulatory sequences but does contain the correct elements for RNA polymerase loading and initiation of transcription (23).

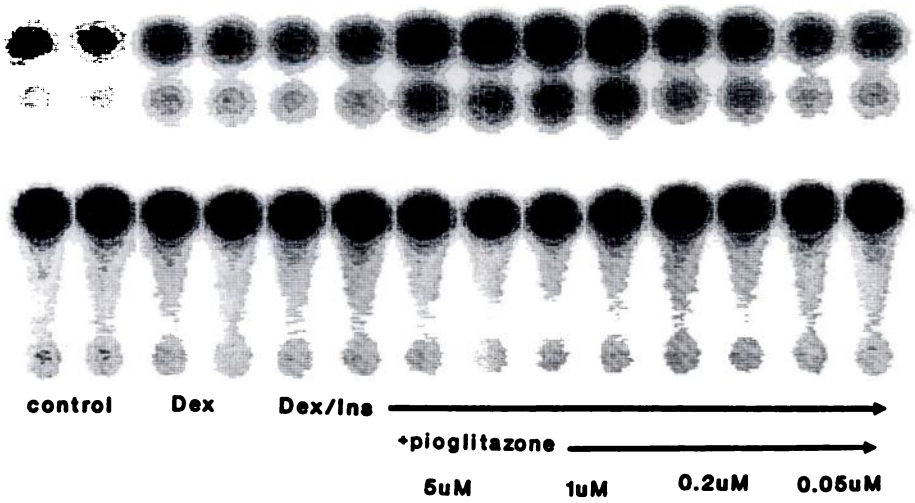

Fig. 3. Induction of the aP2(7Kb)/CAT chimeric gene by pioglitazone. Cells that were stably transfected with the aP2(7Kb)/CAT gene were grown to confluence and then treated, as indicated for Fig. 2, with DEX, insulin (*Ins*), and pioglitazone. Six days later the cultures were harvested and assayed for CAT activity. Each *lane* represents an extract prepared from a separate culture dish. The radioimage presented is a scan (Ambis) of a representative experiment.

TABLE 2

Regulation of aP2(7Kb)/CAT by pioglitazone and insulin/IGF-1

Preadipocytes (3T3-L1) stably transformed with either the aP2(7Kb)/CAT or aP2(-100) CAT chimeric genes were staged for the experiment as described for Fig. 3. Cultures were treated with the hormones (DEX, 1 μM; insulin, 150 nM; IGF-1, 25 nM) and pioglitazone (12.5 μM) at the time confluent cultures were placed in medium containing fetal calf serum; cultures were harvested 6 days later. CAT assays were carried out as described for Fig. 3. Values represent the mean ± standard error for three determinations, except for cultures treated with IGF-1, for which the values represent the average from duplicate samples.

Chimera	Treatment	CAT activity[a]
		pmol
aP2(7Kb)/CAT	None	9.8 ± 0.6
aP2(7Kb)/CAT	DEX	35.4 ± 2.7
aP2(7Kb)/CAT	DEX/insulin	38.5 ± 2.9
aP2(7Kb)/CAT	DEX/insulin/pioglitazone	157.5 ± 5.2
aP2(7Kb)/CAT	DEX/pioglitazone	44.3 ± 3.7
aP2(7Kb)/CAT	DEX/IGF	65.3
aP2(7Kb)/CAT	DEX/IGF/pioglitazone	109.9
aP(-100)/CAT	DEX/insulin	3.9 ± 0.2
aP(-100)/CAT	DEX/insulin/pioglitazone	3.6 ± 0.3

[a] Activity is expressed in pmol of acetylated chloramphenical produced in 2 hr per cell extract.

TABLE 3

Regulation of aP2(7Kb)/CAT by pioglitazone analogs

Preadipocytes (3T3-L1) stably transformed with the aP2(7Kb)/CAT chimeric gene were staged for the experiment and assayed for CAT activity as described for Fig. 3. The concentration of the thiazolidinedione analogs was 12.5 μM. Each value represents the average of duplicate cultures from a representative experiment.

Treatment	CAT activity[a]
	pmol
DEX/insulin	38.7
DEX/insulin + pioglitazone	141.2
DEX/insulin + ciglitazone	36.1
DEX/insulin + AD4533	70.9
DEX/insulin + AD5080	189.7
DEX/insulin + U25560	26.7
DEX/insulin + U90441	30.8

[a] Activity is expressed in pmol of acetylated chlormaphenicol produced in 2 hr per cell extract.

Analogs of pioglitazone were tested for their ability to induce the aP2(7Kb)/CAT gene. Ciglitazone, the prototypical thiazolidinedione, was found to be inactive, whereas a hydroxyl metabolite of ciglitazone, AD4533, was active (Table 3). Similar results were found when ciglitazone and its metabolites were tested for their ability to enhance insulin/IGF-1-regulated differentiation of 3T3-L1 cells (14). An analog, AD5080, known to be more potent than pioglitazone (14) was found to induce expression strongly, whereas chemical entities representing only limited regions of the thiazolidinedione structure were inactive (Table 3). Thus, the ability of the thiazolidinediones to induce the expression of aP2(7Kb)/CAT closely parallels the activity of this series of compounds to enhance adipocyte differentiation (14).

Discussion

It is well established that the expression of the aFABP gene in 3T3-L1 cells is regulated in a differentiation-dependent fashion (22, 23). We have demonstrated that pioglitazone can regulate the expression of aFABP *in vivo* and that the increase in aFABP expression in epididymal fat of *ob/ob* mice was correlated with effects of the drug on physiological parameters indicative of increased insulin sensitivity. Although it is difficult to relate this observation in the intact animal to a direct effect of the drug on adipose tissue, our work with the 3T3-L1 cells shows that the drug elicits a striking increase in aFABP mRNA in cultures that are staged to differentiate into adipocytes. Thus, it is apparent from this work and an earlier study (14) that the drug directly influences phenotypic expression in adipocytes. Although the increase in aFABP mRNA could result from either increased transcription or stabilization of the mRNA, we suggest that increased transcription is involved, based on previous studies (22–24) on the differentiation-dependent expression of this gene. Support for this view was bolstered by our construction of a pioglitazone-regulated chimeric gene. The aFABP promoter must contain a *cis* element capable of responding to the presence of pioglitazone, although it is clear from the data in Tables 1 and 2 that the drug by itself cannot trigger expression. The presence of insulin or IGF-1 is required for the effects on aFABP expression to be noted or for differentiation of the 3T3-L1 cells (14).

Virtually nothing is known regarding the receptor for this drug, but it is possible that the drug is interacting with a protein that is a member of the steroid superfamily of receptors. The drug/receptor complex could be interacting directly with a site in the aFABP promoter to enhance expression of this gene. The site of this interaction could be in the proximal 250 bases of the promoter, because this region is known to contain several

important regulatory elements (15, 19, 23, 24). Alternatively, the region of the promoter at −5 kb is a candidate for the location of the pioglitazone-responsive element, because this region appears to be important in tissue-specific and differentiation-dependent expression of the gene (23).

The molecular basis for insulin resistance is not understood, nor is the molecular mechanism by which pioglitazone can influence cellular sensitivity to insulin. Because pioglitazone ameliorates insulin resistance *in vivo* and increases cellular sensitivity to insulin *in vitro* (7), understanding of the mechanism of action for pioglitazone should lead to a better understanding of insulin action. In this regard, the suggestion has been made that aFABP, a cytoplasmic protein present at high levels in terminally differentiated adipocytes, is involved in mediating some of the actions of insulin (8, 10, 11). The protein is a target for the insulin receptor tyrosine kinase, and a protein phosphatase with substrate specificity for the tyrosyl phosphate has been described (8, 9). If this is the case, then the induction of aFABP by pioglitazone could facilitate the action of insulin. Additionally, pioglitazone may be inducing the expression of genes whose promoter contains a response element similar to that of aFABP. In this scenario, a cadre of genes induced by pioglitazone may be involved in mediating insulin action and, thus, coordinately influencing cellular sensitivity to insulin.

Acknowledgments

The authors are grateful to Drs. Robert Christy and Dan Lane for the clone of the aFABP promoter. The excellent assistance of Linda Rogers in preparing this manuscript is gratefully acknowledged.

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Send reprint requests to: Rolf F. Kletzien, Metabolic Diseases Research, 7250–126–3, The Upjohn Company, Kalamazoo, MI 49001.