Biochemical Pharmacology, Vol. 52, pp. 693–701, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00345-0

Induction of Uncoupling Protein in Brown Adipose Tissue

SYNERGY BETWEEN NOREPINEPHRINE AND PIOGLITAZONE, AN INSULIN-SENSITIZING AGENT

Lisa A. Foellmi-Adams, Beatrice M. Wyse,**
David Herron,† Jan Nedergaard† and Rolf F. Kletzien‡*

*Endocrine Pharmacology and Metabolism, Pharmacia & Upjohn Inc., Kalamazoo, MI 49001, U.S.A. and †The Wenner-Gren Institute, The Arrhenius Laboratories F3, Stockholm University, S-106 91 Stockholm, Sweden

ABSTRACT. Insulin resistance and obesity in rodent models of non-insulin-dependent diabetes mellitus have been correlated with ablated or defective brown adipose tissue (BAT) function. The mitochondrial uncoupling protein (UCP) allows BAT to perform its unique role in facultative energy expenditure. In this study, we observed an increase in both BAT mass and the expression of UCP mRNA in BAT from obese diabetic mice and their lean littermates following treatment with the thiazolidinedione pioglitazone, a novel insulin-sensitizing agent. Thus, we wanted to ascertain if pioglitazone directly induces BAT differentiation. We found that treatment for 48 hr with pioglitazone caused a 32-fold increase in UCP mRNA, whereas a 7-hr treatment with norepinephrine caused a 24-fold increase in expression. Cells treated with pioglitazone for 48 hr, with norepinephrine added during the last 7 hr, demonstrated a 59-fold increase in UCP mRNA. However, simultaneous treatment with pioglitazone and repeated treatment norepinephrine for 48 hr yielded a greater than 200-fold increase in UCP mRNA. Examination of UCP protein levels demonstrated a similar time-dependent increase with pioglitazone and/or norepinephrine treatment, as well as a synergistic increase with concurrent pioglitazone and norepinephrine treatment. This study shows that pioglitazone exerts a direct effect on BAT cells *in vitro* by increasing UCP mRNA and protein levels, and that it also synergizes with norepinephrine perhaps by inducing and stabilizing UCP mRNA and/or preventing proteolysis of UCP protein. BIOCHEM PHARMACOL 52;5:693–701 , 1996.

KEY WORDS. brown adipose tissue; uncoupling protein; insulin resistance; pioglitazone; norepinephrine; diabetes

BAT§ is known to be responsible for nonshivering thermogenesis and cold acclimatization [1]. Owing to its high competence for facultative energy expenditure, it has been speculated that this tissue normally functions to prevent obesity [2, 3] and the insulin resistance that can lead to non-insulin-dependent diabetes mellitus [4, 5]. Located in the inner mitochondrial membrane of BAT cells, UCP allows BAT the unique heat-dissipating function and serves as a molecular and biochemical marker of BAT cells. UCP operates by short-circuiting the proton electrochemical gradient generated across the mitochondrial membrane during substrate oxidation, thus promoting energy dispersion as heat [6]. Cold exposure of animals is associated with an enhanced sympathetic nerve activation leading to increased NE release [7], and it has been shown *in vivo* [8, 9] that NE infusion induces UCP mRNA expression in BAT and *in vitro* [10, 11] that treatment of cultured BAT cells with NE increases UCP mRNA and protein levels. Additionally, transgenic mice, which have ablated BAT, demonstrate extreme obesity, hypertriglyceridemia, and insulin resistance, providing strong evidence that BAT may play a critical role in the balance of energy regulation, obesity, and insulin resistance in mice [4].

The thiazolidinediones are a class of compounds that lower blood glucose in diabetic rodent models through a mechanism that increases postreceptor events mediating insulin action in target tissues [12, 13]. Pharmacological effects of the thiazolidinediones in rodents include decreased blood levels of glucose, insulin, triglyceride, and free fatty acids and decreased cholesterol absorption [14–16] as well as enhanced thermogenesis via sympathetic activation of BAT [17]. Although it has been demonstrated

‡ Corresponding author: Rolf F. Kletzien, Ph.D., Endocrine Pharmacology and Metabolism, Pharmacia & Upjohn Inc., 301 Henrietta Street, Kalamazoo, MI 49001. Tel. (616) 833-8747; FAX (616) 833-9763.

§ *Abbreviations:* BAT, brown adipose tissue; UCP, uncoupling protein; NE, norepinephrine; PIO, pioglitazone; ob/ob, C57BL/6J-ob/ob; C57+/+, C57BL/6J-+/+; DMEM, Dulbecco's modified Eagle's medium; Dex, dexamethasone; IBMX, isobutylmethylxanthine; PCR, polymerase chain reaction; and PPAR2γ, peroxisome proliferating activated receptor 2γ.

Received 21 July 1995; accepted 19 March 1996.

that the diminished thermogenic activity of diabetic obese (ob/ob) mice may be related to insulin resistance [5], treatment of these animals with a thiazolidinedione has been shown to ameliorate this insulin resistance and restore defective BAT response [18]. Therefore, an increase in UCP expression in BAT could lead to an improved utilization of glucose and increased insulin sensitivity in this target tissue. In this study, we have examined the effects of NE and the thiazolidinedione PIO, alone and in combination, on the expression of UCP mRNA and protein levels in primary cultures of BAT cells, as well as the physiological effect of PIO on BAT UCP in the ob/ob mouse.

MATERIALS AND METHODS

Reagents

DMEM, fetal bovine serum, gentamicin, trypsin, and the Random Primers DNA Labelling Kit were purchased from Gibco/BRL (Grand Island, NY). Insulin, norepinephrine, BSA, Dex, and IBMX were obtained from Sigma (St. Louis, MO). The GeneAmp DNA Amplification Reagent Kit was purchased from Perkin Elmer Cetus (Foster City, CO). [^{14}C]Sodium acetate and [α-^{32}P]dCTP were from DuPont NEN (Boston, MA). All stock reagents of Tris-glycine SDS running buffer, Tris-glycine transfer buffer, and sample buffer as well as the pre-cast Tris-glycine polyacrylamide gels were obtained from NOVEX (San Diego, CA). Immobilon PVDF membrane was from Millipore (Bedford, MA). The ECL western blotting detection reagents were purchased from Amersham (Arlington Heights, IL). Collagenase was purchased from Boehringer Mannheim (Indianapolis, IN), RNAzol from Cinna/Biotex (Friendwood, TX), and Duralon-UV membrane from Stratagene (La Jolla, CA). Ob/ob, C57+/+, and CF-1 mice were obtained from Jackson Laboratories (Bar Harbor, ME).

Isolation of Cells

The animals used for the isolation of preadipocytes were male CF-1 mice or male Sprague–Dawley rats 21- to 25-days-old, fed *ad lib.*, and kept at room temperature until decapitation, following the guidelines of the AVMA Panel on Euthanasia [19] for the use of laboratory animals. Preadipocytes were isolated from the interscapular and cervical brown fat tissue as previously described [20], without hypoosmotic shock.

Cell Culture

Mouse preadipocytes were isolated and cultured in DMEM supplemented with 10% fetal bovine serum, 25 μg/mL ascorbate, 10 mM HEPES, 4 nM insulin, and 20 μg/mL gentamicin and incubated at 37° in 10% CO_2. The medium was changed daily until the cells reached confluence. Rat preadipocytes, used for lipogenesis studies due to ease of isolating large numbers of cells, were cultured initially in a 25-cm^2 tissue culture flask containing DMEM supplemented with 10% fetal bovine serum and 20 μg/mL gentamicin and incubated at 37° in 10% CO_2. Eighteen hours after inoculation, the medium was changed. Twenty-four hours later, the cells were trypsinized and transferred to a 75-cm^2 tissue culture flask. After an additional 24 hr, the cells were trypsinized and transferred to 35-mm culture dishes. Confluent cells were staged to differentiate as previously described [12]. Addition of insulin or NE to the medium was accomplished by dissolving the hormone in 10 nM HCl containing 0.2% BSA. Multiple additions of NE were used to maintain hormone levels [11]. Addition of PIO to the medium was accomplished by dissolving the drug in dimethyl sulfoxide and diluting 1000-fold. Dimethyl sulfoxide was present in all control cultures at a concentration of 0.1%.

Animals

All procedures in this study were in compliance with the Animal Welfare Act Regulation, 9 CFR parts 1, 2, and 3 and with the Guide for the Care and Use of Laboratory Animals [21]. Seven-week-old female ob/ob and C57+/+ mice were housed individually at room temperature and fed ground Purina Mouse Chow and water *ad lib.* At 10 weeks of age, the ob/ob and C57+/+ mice were divided into two groups, each containing six mice, based on food intake, body weight, and blood glucose levels. Treatment was started with PIO mixed in the chow at a concentration of 0.3 mg/g chow, and dosage was estimated by normalizing food intake with body weight. Treated ob/ob mice received approximately 22 mg PIO/kg/day and treated C57+/+ mice received approximately 67 mg PIO/kg/day. After treatment with PIO for 6 weeks, blood was obtained from the orbital sinus for determination of plasma glucose and insulin levels, the mice were killed by decapitation, and tissues were removed for analysis. The perirenal and the interscapular brown fat were immediately weighed and homogenized in 1 mL RNAzol. Due to the increase in interscapular BAT mass in animals treated with PIO, the relative abundance of interscapular UCP and aP2 mRNA from each animal was determined by quantifying the amount of radiolabeled probe bound per microgram of RNA dotted and normalizing that value with the weight of the BAT pad and the amount of RNA extracted from the pad. The abundance of UCP and aP2 mRNA from the perirenal fat of each animal was determined by quantifying the amount of radiolabeled probe bound per microgram of RNA dotted.

Molecular Biological and Chemical Assays

The relative abundance of UCP and aP2 mRNA was determined by isolating the total RNA from cells or tissue through the use of RNAzol, and then probing northern or dot blots for these species as previously described [13]. The UCP cDNA probe was derived from rat brown fat tissue RNA using oligonucleotides from bases 417–440 and 591–616 of the published UCP gene sequence [22] as primers in

the PCR [23]. The cDNA was radiolabeled in a subsequent PCR incorporating [α-^{32}P]dCTP. The aP2 probe [24] was radiolabeled using the random primers system. Lipogenesis was determined by following the incorporation of [^{14}C]acetate into the triglyceride fraction as described [25]. Cell protein was determined by the bicinchoninic acid method [26]. RNA was quantified by absorbance at 260 nm. Insulin was assayed by methods reported earlier [16].

Preparation of Cell Extracts and Immunoblotting

Cells were treated as described in figure legends, the medium was removed, and the cells were rinsed twice in ice-cold PBS. Cells were scraped into ice-cold PBS and centrifuged for 5 min at 16,000 *g* at 4°; the resulting pellets were resuspended in sample buffer, denatured in a boiling water bath for 5 min, and then stored at 4°. Immunoblots were prepared as described previously [10, 27]; the antibody used was that characterized earlier [28]. Immunoreactive bands were detected by submersing the blot in ECL western blotting reagents, and then exposing to Kodak Xomat film.

RESULTS

PIO Effects In Vivo

The effect of PIO on UCP mRNA levels in interscapular and perirenal brown fat of ob/ob and C57+/+ mice was examined (see Tables 1 and 2, respectively). Additionally, we followed changes in physiological parameters. The mice were treated with PIO for 6 weeks at which time the relative abundance of UCP and aP2 mRNA from the two fat depots was determined. Plasma glucose and insulin levels were measured as well as the total weight of the interscapular BAT pad. In both ob/ob and C57+/+ mice, PIO increased BAT mass as well as interscapular BAT UCP and aP2 mRNA levels 2- to 3-fold. Perirenal fat was affected in a similar manner in both species. PIO treatment caused a 2-fold decrease in plasma glucose and a 5.5-fold decrease in insulin levels in ob/ob mice, but no change in these parameters in the C57+/+ mice. The increase in UCP and aP2 (a gene shown to be adipose differentiation dependent [29, 30]) mRNA levels in both fat depots due to PIO treatment, as well as the proportionate changes in the measured physiological parameters, prompted us to examine the consequence of PIO treatment of BAT cells in culture to determine if it was directly affecting BAT precursor cells.

TABLE 1. Influence of PIO on ob/ob mice

	-PIO	+PIO	+PIO/ -PIO
Interscapular UCP mRNA (cpm/animal)	4,495 ± 760	7,590 ± 1,360	1.7
Interscapular aP2 mRNA (cpm/animal)	16,420 ± 2,430	48,850 ± 2,630	3.0
Interscapular BAT weight (g)	0.81 ± 0.05	1.54 ± 0.09	1.9
Perirenal UCP mRNA (cpm/2 μg RNA)	6.7 ± 0.9	12.8 ± 1.4	1.9
Perirenal aP2 mRNA (cpm/2 μg RNA)	170.9 ± 21.5	438.1 ± 36.4	2.6
Glucose (mg/dL)	221 ± 36.7	120 ± 15	0.5
Insulin (μU/mL)	1,135 ± 365	207 ± 43	0.2

Ob/ob mice were treated with 22mg/kg/day PIO for 6 weeks. Relative abundance of UCP and aP2 mRNA from interscapular and perirenal BAT was determiend as described in Materials and Methods. Plasma glucose and insulin were measure as described in Materials and Methods. Each value is the mean ± SEM for six animals.

TABLE 2. Influence of PIO on C57+/+ mice

	-PIO	+PIO	+PIO/ -PIO
Interscapular UCP mRNA (cpm/animal)	4,530 ± 720	9,340 ± 1,165	2.1
Interscapular aP2 mRNA (cpm/animal)	9,310 ± 1,530	21,170 ± 1,170	2.3
Interscapular BAT weight (g)	0.153 ± 0.010	0.268 ± 0.013	1.8
Perirenal UCP mRNA (cpm/2 μg RNA)	11.5 ± 1.3	32.8 ± 2.2	2.9
Perirenal aP2 mRNA (cpm/2 μg RNA)	93.2 ± 7.5	189.9 ± 14.4	2.0
Glucose (mg/dL)	138 ± 6	139 ± 7	1.0
Insulin (μU/mL)	19 ± 3	14 ± 1	0.7

C57+/+ mice were treated with 67 mg/kg/day PIO for 6 weeks. Relative abundance of UCP and aP2 mRNA from interscapular and perirenal BAT was determined as described in Materials and Methods. Plasma glucose and insulin were measured as described in Materials and Methods. Each value is the mean ± SEM from six animals.

PIO Effects In Vitro

MORPHOLOGY. Brown fat preadipocytes in culture will grow to confluence and differentiate, accumulating fat in a multilocular manner [20]. The light photomicrographs in Fig. 1 were obtained with primary cultures of BAT preadipocytes that had been staged to differentiate, and then treated with or without insulin and PIO for 3 days. Treatment with insulin alone or PIO alone (Fig. 1, b and c, respectively) caused cells to differentiate as demonstrated by the distinct accumulation of fat in the form of small lipid droplets, previously described [20]. However, simultaneous treatment with insulin and PIO resulted in nearly 100% of the cells exhibiting a differentiated morphology (Fig. 1d).

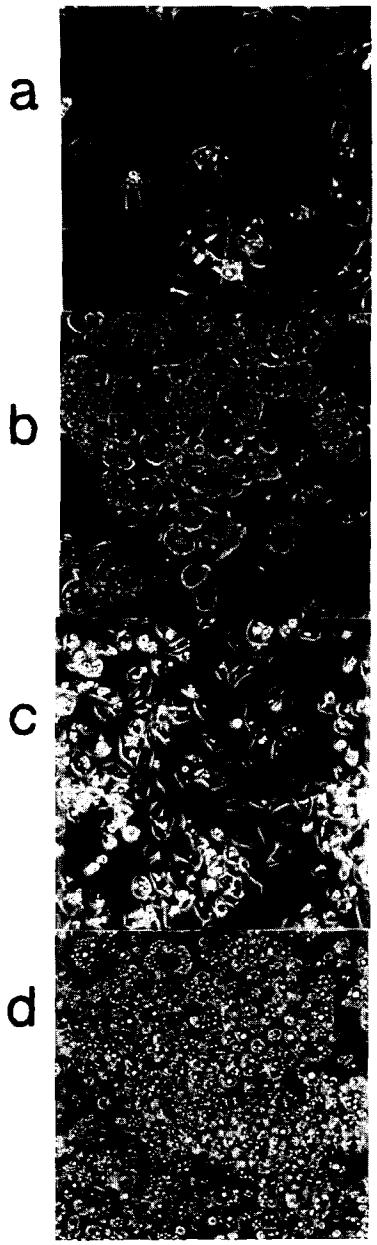

FIG. 1. Light photomicrographs (Nikon Inverted Phase Microscope) of rat BAT precursor cells. Original magnification = x182. Cells were isolated and maintained as described in Materials and Methods. Confluent cells were staged to differentiate in 0.25 mM IBMX and 1 μM Dex for 48 hr. The medium was changed and the cells were treated for 3 days as follows: (a) control, (b) 25 nM insulin, (c) 5 μM PIO, and (d) insulin + PIO.

LIPOGENESIS. The ability to carry out insulin-stimulated lipogenesis is a distinguishing characteristic of differentiated adipocytes. The experiment in Fig. 2 demonstrates that primary cultures of preadipocytes isolated from interscapular and cervical brown fat and staged to differentiate are capable of carrying out lipogenesis. Treatment of the cells with insulin alone increased lipogenesis 6-fold, whereas treatment with PIO alone caused a 13-fold increase; simultaneous treatment with insulin and PIO increased lipogenesis 18-fold.

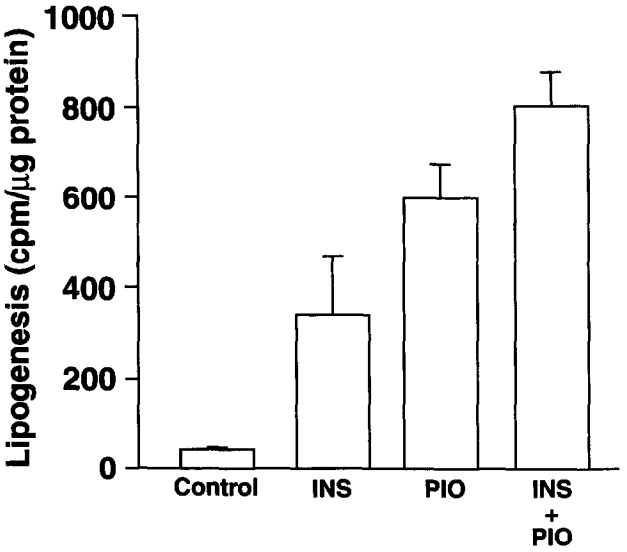

FIG. 2. Lipogenesis in rat BAT precursor cells. Cells were isolated and cultured as described in Materials and Methods. Confluent cells were staged to differentiate in 0.25 mM IBMX and 1 μM Dex for 48 hr. The medium was changed and the cells were treated for 3–4 days with 25 nM insulin (INS), 5 μM PIO, or both. [^{14}C]Acetate incorporation into triglyceride was determined as described in Materials and Methods. Each value is the mean + SEM of 6–7 determinations from 3 experiments.

aP2 mRNA INDUCTION. In earlier studies, it has been shown that the gene encoding aP2 is transcriptionally activated during differentiation of 3T3-L1 adipocytes [29, 30], and we have demonstrated that PIO appears to directly effect aP2 gene expression [12]. As a positive control of adipocyte differentiation, we determined the ability of PIO to increase aP2 mRNA expression in cultured BAT cells staged to differentiate. We found that although insulin treatment alone increased aP2 mRNA levels approximately 3-fold, treatment with PIO alone increased expression almost 12-fold. Simultaneous treatment with insulin and PIO did not enhance aP2 expression above that seen with PIO alone (see Fig. 3). Interestingly, treatment with NE had no effect on aP2 gene expression (data not shown).

UCP mRNA INDUCTION. The only qualitatively discriminating difference between white and brown fat cells in culture is expression of the UCP gene. To determine if NE or PIO, alone or in combination, could induce UCP in BAT cells, we carried out the following experiment. Cultures were incubated with NE for 7 hr or with PIO for 48 hr (maximal effects of these agents on gene expression have been noted in these time frames [11, 13]), and mRNA was isolated. Sister cultures were incubated with PIO for 48 hr with NE present for the last 7 hr of incubation. Northern blot analysis of RNA isolated from primary cultures of BAT cells revealed that treatment with either PIO or NE can induce UCP mRNA expression. However, simultaneous treatment caused a greatly enhanced expression (see Fig. 4). Further quantitative analysis (Fig. 5, top panel) showed that treatment of cultured BAT cells with PIO for 48 hr

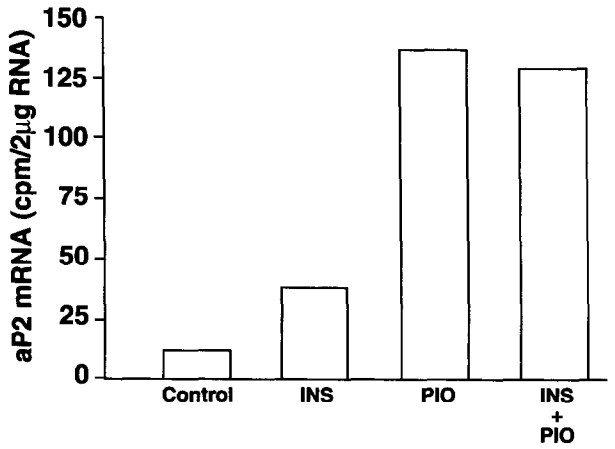

FIG. 3. Induction of aP2 gene expression in BAT precursor cells. Cells were isolated and cultured as described in Materials and Methods. Confluent cells were staged to differentiate in 0.25 mM IBMX and 1 μM Dex for 48 hr. The medium was changed and the cells were treated for 3 days with 25 nM insulin and/or 5 μM PIO. RNA was isolated, then blotted on Duralon-UV membrane, and hybridized with a radiolabeled aP2 cDNA probe as described in Materials and Methods. This is a representative experiment where each value depicts the mean counts per minute from duplicate dots where each dot represents 2 μg total RNA.

caused a 32-fold induction of UCP mRNA over control, whereas treatment with NE for 7 hr caused a 24-fold increase. Cells treated with PIO for 48 hr, with NE added for the last 7 hr, demonstrated a 59-fold increase in expression. However, cells treated simultaneously with PIO and NE for 48 hr showed approximately the same UCP mRNA levels as cells treated with PIO alone unless they were repeatedly treated with NE, approximately every 10 hr, during the 48-hr period. Under conditions of multiple NE treatments, UCP mRNA levels were increased 230-fold over that seen in control cells (see Fig. 5, top panel). In the experiment depicted in the bottom panel of Fig. 5, we demonstrated the time–course of UCP mRNA induction by PIO and NE. NE caused a rapid expression of UCP by 7 hr which returned nearly to control levels by 24 hr. Induction of UCP mRNA by PIO treatment remained quite low until after 24 hr of treatment. However, simultaneous treatment with PIO and

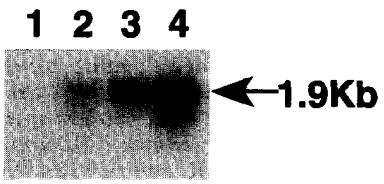

FIG. 4. Northern blot analysis of UCP mRNA. Mouse BAT precursor cells were isolated and maintained as described in Materials and Methods. Confluent cells were treated as follows: (lane 1) control, (lane 2) 0.1 μM NE 7 hr, (lane 3) 5 μM PIO 48 hr, and (lane 4) PIO and NE 48 hr. Total RNA was isolated, then separated by electrophoresis in a formaldehyde-agarose gel, transferred to Duralon-UV membrane, and hybridized to the UCP cDNA probe as described in Materials and Methods. Each lane contains 30 μg total RNA.

NE led to a rapid, sustained, and enhanced expression of UCP mRNA.

UCP IMMUNOBLOT. Since both PIO and NE were able to induce the expression of UCP mRNA, we assessed the influence of PIO and NE on UCP protein levels in lysates from BAT cells treated with both agents. The data presented in Fig. 6 demonstrate that although 48-hr NE treatment was able to increase the amount of UCP over control, 48-hr PIO treatment yielded approximately 6-fold more protein. In addition, simultaneous treatment of PIO and NE produced 3.5-fold more protein than PIO alone, indicative of synergistic actions.

DISCUSSION

In most animal models of diabetes, there is a defect in control of BAT thermogenesis which results in a greatly increased metabolic efficiency, thus promoting the development of obesity [3]. Transgenic mice, which have ablated BAT, demonstrate extreme obesity, insulin resistance, hypertriglyceridemia, and hypercholesterolemia [4]. These data suggest that proper BAT function may be crucial in preventing obesity that leads to the diabetic state. It is well known that expression of UCP is physiologically stimulated by cold exposure and diet in animals, and it is assumed that NE released from sympathetic nerves regulates the physiologic stimulation [11]. Studies have indicated that in the obese diabetic ob/ob mouse, the defective acute cold-induced activation of thermogenesis, which develops at approximately 35 days of age, coincides with the appearance of hyperinsulinemia and hyperglycemia [5]. Although the molecular mechanism of action is unknown, adipose tissue is a known target tissue for the antihyperglycemic action of the thiazolidinediones [12–14, 17, 25] and, in the ob/ob mouse, thiazolidinedione treatment not only ameliorated insulin resistance but also normalized cold-induced thermogenesis [18]. In this study, we have demonstrated that PIO, a thiazolidinedione, increases BAT mass and UCP mRNA expression *in vivo*, and induces UCP mRNA and protein expression in BAT cells *in vitro* where it exerts a synergistic effect with NE.

We have demonstrated that treatment of ob/ob mice with PIO not only normalized their plasma glucose and caused a 5-fold decrease in insulin levels, but also increased BAT mass and UCP and aP2 mRNA levels in interscapular and perirenal BAT (see Table 1). The same changes in mRNA levels were determined in their lean littermates, the C57+/+ mice (see Table 2). These results prompted closer examination of PIO action in primary cultures of BAT cells. Preadipocytes isolated from interscapular BAT and grown *in vitro* differentiated in the presence of insulin or PIO; however, concurrent treatment with both insulin and PIO induced nearly 100% of the preadipocytes to differentiate, as shown in the photomicrographs in Fig. 1. Since the ability to carry out insulin-stimulated lipogenesis is a hallmark of differentiated adipocytes, we performed experiments to determine if PIO could stimulate lipogenesis in

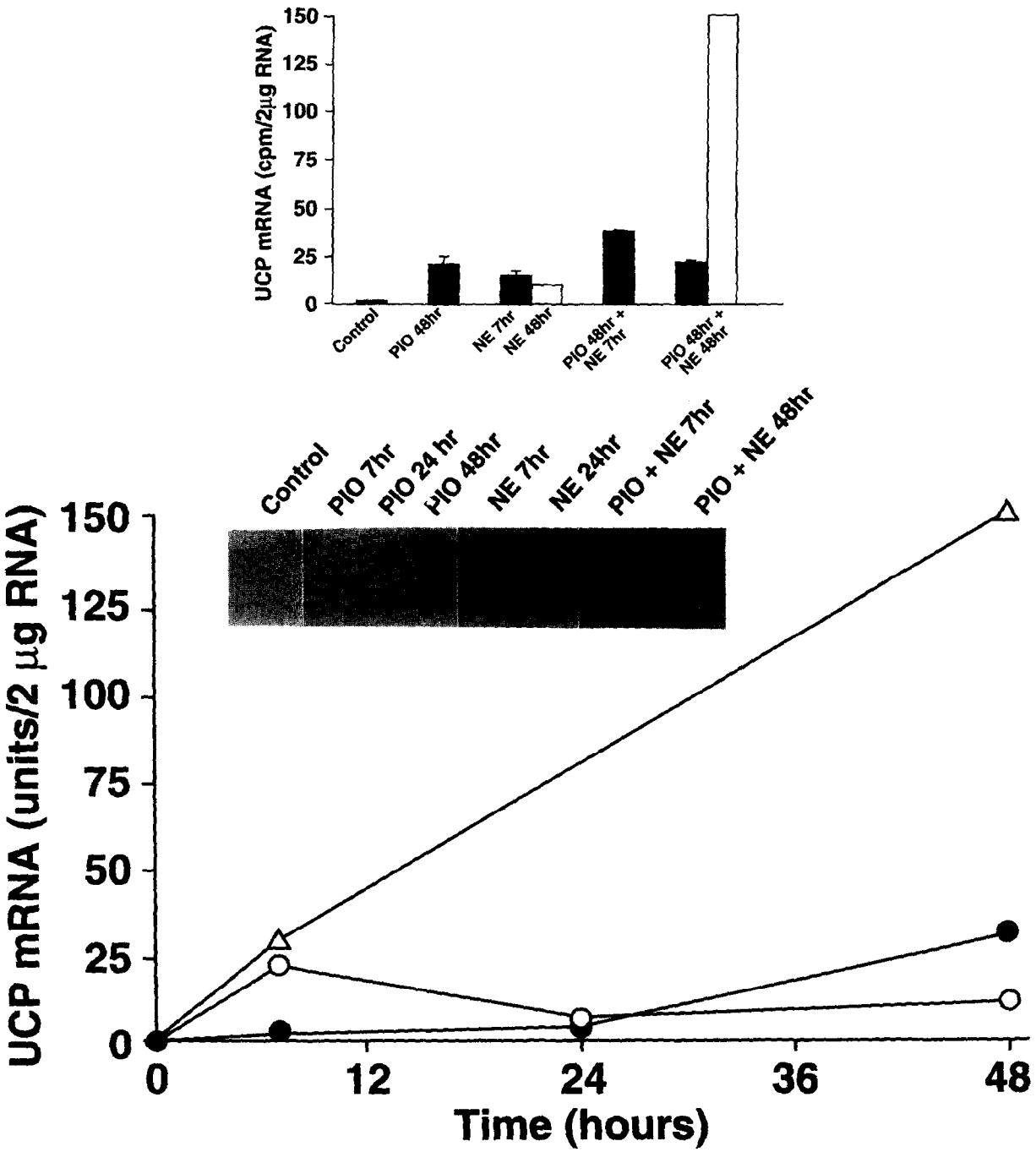

FIG. 5. Time-course of UCP mRNA induction. Mouse BAT precursor cells were isolated and maintained as described in Materials and Methods. RNA was isolated, then blotted on Duralon-UV membrane, and hybridized with a radiolabeled UCP cDNA probe as described in Materials and Methods. Top panel: Confluent cells were treated with 5 μM PIO and/or 0.1 μM NE for the amount of time indicated. Each value for the filled bars is the mean ± SEM of 4–5 determinations from 3–4 experiments. Open bars indicate multiple NE treatments (approximately every 10 hr), and each value is the mean of 2 determinations from one experiment. Bottom panel: Confluent cells were treated with (●) 5 μM PIO, (○) 0.1 μM NE, or (△) PIO and NE at time zero, and cultures were harvested at the appropriate times. Cells treated with NE received multiple doses, approximately every 10 hr. This is a representative experiment where each value is the mean of the signal from duplicate 2 μg dots of total RNA (inset) from one culture dish after hybridization with the UCP probe.

these cells. PIO increased lipogenesis in BAT cells staged to differentiate, and this increase was approximately additive to that of insulin (see Fig. 2). PIO also induced aP2 gene expression, a gene that is regulated in a differentiation-dependent manner in adipocytes, although the amount of aP2 mRNA was not increased further with simultaneous insulin treatment (see Fig. 3). These results correlated well with previously reported increases in aP2 mRNA levels by

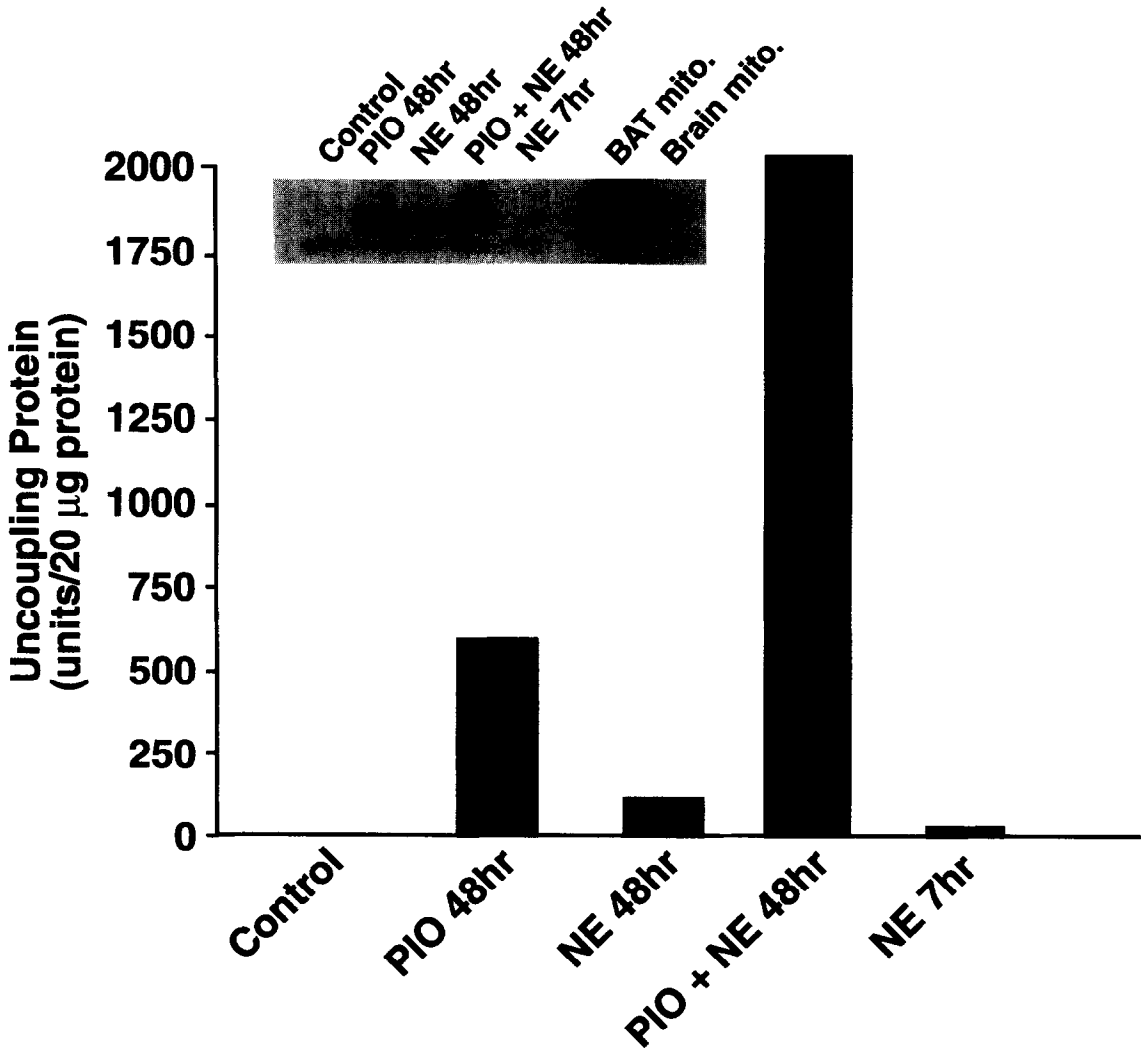

FIG. 6. Immunoblot analysis of UCP. Mouse BAT precursor cells were isolated and maintained as described in Materials and Methods. Confluent cells were treated with 5 μM PIO and/or 0.1 μM NE for the time indicated. Cells treated with NE received multiple doses, approximately every 10 hr. Whole cell lysates were prepared and immunoblotted as described in Materials and Methods. This is a representative experiment where each value and each band (inset) represent the signal from 20 μg protein from one culture dish after immunoblotting with the UCP antibody.

PIO in white fat *in vivo* and *in vitro* [13]. Interestingly, treatment of BAT cells with NE had no effect on aP2 mRNA levels, indicating that NE is not involved with the expression of the differentiation-dependent aP2 gene in these cells *in vitro*. Once we had established that in BAT cells PIO was exerting the same effects on such parameters as lipogenesis and aP2 gene expression previously reported [12, 13], we wanted to determine if BAT was a specific target tissue of PIO action. We did this by examining changes in UCP, the only unique molecular marker of brown fat, in cultured BAT cells treated with PIO in the absence and presence of NE. As shown in the top panel of Fig. 5, we found that both NE and PIO treatments increased UCP mRNA levels. Although cells treated with PIO for 48 hr and exposed to NE for the last 7 hr demonstrated a non-additive increase in UCP mRNA, cells treated for 48 hr with PIO and which were repeatedly treated with NE exhibited a greatly enhanced induction of UCP mRNA. The time–course of UCP mRNA induction by PIO and NE differed notably (see Fig. 5, bottom panel). NE treatment caused a rapid increase in UCP mRNA by 7 hr which returned to near control levels by 24 hr, whereas PIO treatment did not start to induce an increase until after 24 hr. The most interesting data, however, are the rapid, sustained, and enhanced UCP mRNA expression obtained via simultaneous PIO and NE treatment. These data suggest distinctly different mechanisms by which PIO and NE are increasing UCP mRNA levels, either by inducing transcription or by mRNA stabilization. These studies were followed up with western blot analysis to determine if PIO in the absence or presence of NE was exerting the same influence on UCP protein levels as we had seen on mRNA levels. As illustrated in Fig. 6, NE treatment increased the amount of UCP over control values; however, treatment of

the cells with PIO increased this amount 6-fold over the NE value. Simultaneous treatment with PIO and NE clearly demonstrated a synergistic effect.

What is the mechanism by which PIO is exerting its influence on UCP? It has been shown recently [31, 32] that stabilization/destabilization of UCP mRNA plays an integral role in UCP expression both in the animal and in cultured BAT cells. It was observed that both transcriptional and translational blockade stabilized UCP mRNA, indicating that gene products, probably nuclease(s), are necessary for the degradation of mRNA rather than for protection from degradation. Since there is a discrete delay in PIO-induced UCP expression, perhaps PIO is causing a transcriptional inhibition of this putative nuclease. Alternatively, PIO could be directly up–regulating UCP gene transcription perhaps through a PPAR2γ receptor. Recent work [33–35] has demonstrated that thiazolidinediones enhance transcription of the aP2 gene through a PPAR2γ receptor. We speculate that the UCP promoter may likewise possess a PPAR2γ response element. Finally, PIO could be provoking the activation of a key signaling protein which, depending on stimulation of the β-adrenergic signaling pathway, could activate different regulatory transcription factors. Similar results have demonstrated the activation of two divergent mitogen-activated protein kinase cascades, differing only in the substrate of key proteins in the cascades, that contribute to transcriptional regulation [36]. At the present time, it is not possible to delineate the detailed molecular mechanism by which PIO increases UCP expression. In addition, the relationship of increased UCP expression to the enhanced sensitivity to insulin is not clear, although we suggest that PIO-elevated UCP and BAT mass results in more efficient utilization of carbohydrate and fatty acids.

References

1. Smith RE and Horwitz BA, Brown fat and thermogenesis. *Physiol Rev* **49:** 330–352, 1969.
2. Rothwell NJ and Stock MJ, A role for brown adipose tissue in diet-induced thermogenesis. *Nature* **281:** 31–35, 1979.
3. Himms-Hagen J, Brown adipose tissue thermogenesis and obesity. *Prog Lipid Res* **28:** 67–115, 1989.
4. Lowell BB, Susulic VS, Hamann A, Lawitts JA, Himms-Hagen J, Boyer BB, Kozak LP and Flier JS, Development of obesity in transgenic mice after genetic ablation of brown adipose tissue. *Nature* **366:** 740–742, 1993.
5. Mercer SW and Trayhurn P, The development of insulin resistance in brown adipose tissue may impair the acute cold-induced activation of thermogenesis in genetically obese (ob/ob) mice. *Biosci Rep* **4:** 933–940, 1984.
6. Nicholls DG and Locke RM, Thermogenic mechanisms in brown fat. *Physiol Rev* **64:** 1–64, 1984.
7. Ricquier D, Bouillaud F, Toumelin P, Mory G, Bazin R, Arch J and Penicaud L, Expression of uncoupling protein mRNA in thermogenic or weakly thermogenic brown adipose tissue. *J Biol Chem* **261:** 13905–13910, 1986.
8. Bouillaud F, Ricquier D, Mory G and Thiabult J, Increased level of mRNA for the uncoupling protein in brown adipose tissue in rats during thermogenesis induced by cold exposure or norepinephrine infusion. *J Biol Chem* **259:** 11583–11586, 1984.
9. Bianco AC, Sheng X and Silva JE, Triiodothyronine amplifies norepinephrine stimulation of uncoupling protein gene transcription by a mechanism not requiring protein synthesis. *J Biol Chem* **263:** 18168–18175, 1988.
10. Herron D, Rehnmark S, Néchard M, Lončar D, Cannon B and Nedergaard J, Norepinephrine-induced synthesis of the uncoupling protein thermogenin (UCP) and its mitochondrial targeting in brown adipocytes differentiated in culture. *FEBS Lett* **268:** 296–300, 1990.
11. Rehnmark S, Néchad M, Herron D, Cannon B and Nedergaard J, α- and β-Adrenergic induction of the expression of the uncoupling protein thermogenin in brown adipocytes differentiated in culture. *J Biol Chem* **265:** 16464–16471, 1990.
12. Kletzien RF, Clarke SD and Ulrich RG, Enhancement of adipocyte differentiation by an insulin-sensitizing agent. *Mol Pharmacol* **41:** 393–398, 1992.
13. Kletzien RF, Foellmi LA, Harris PK, Wyse BM and Clarke SD, Adipocyte fatty acid-binding protein: Regulation of gene expression *in vivo* and *in vitro* by an insulin-sensitizing agent. *Mol Pharmacol* **42:** 558–562, 1992.
14. Ikeda H, Taketomi S, Sugiyama Y, Shimura Y, Shoda T, Meguro K and Fujita T, Effects of pioglitazone on glucose and lipid metabolism in normal and insulin resistant animals. *Arzneimittelforsschung* **40:** 156–162, 1990.
15. Colca JR, Dailey CF, Palazuk BJ, Hillman RM, Dinh DM, Melchoir GW and Spilman CH, Pioglitazone hydrochloride inhibits cholesterol absorption and lowers plasma cholesterol concentrations in cholesterol-fed rats. *Diabetes* **40:** 1669–1674, 1991.
16. Chang AY, Wyse BM, Gilchrist BJ, Peterson T and Diani AR, Ciglitazone, a new hypoglycemic agent. I. Studies in ob/ob and db/db mice, diabetic Chinese hamsters, and normal and streptozotocin-diabetic rats. *Diabetes* **32:** 830–838, 1983.
17. Rothwell NJ, Stock MJ and Tedstone AE, Effects of ciglitazone on energy balance, thermogenesis and brown fat activity in the rat. *Mol Cell Endocrinol* **51:** 253–257, 1987.
18. Mercer SW and Trayhurn P, Effects of ciglitazone on insulin resistance and thermogenic responsiveness to acute cold in brown adipose tissue of genetically obese (ob/ob) mice. *FEBS Lett* **195:** 12–16, 1986.
19. Smith AW, Houpt KA, Kitchell RL, Kohn DF, McDonald LE, Passaglia M Jr, Thurmon JC and Ames ER, 1986 Report of the AVMA Panel on Euthanasia. *J Am Vet Med Assoc* **188:** 252–268, 1986.
20. Néchad M, Kuusela P, Carnheim C, Bjorntorp P, Nedergaard J and Cannon B, Development of brown fat cells in monolayer culture: Morphological and biochemical distinction from white fat cells in culture. *Exp Cell Res* **149:** 105–118, 1983.
21. Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences, *Guide for the Care and Use of Laboratory Animals*, DHEW Publication (NIH) **No. 86–23.** National Institutes of Health, Bethesda, MD, 1985.
22. Ridley RG, Patel HV, Gerber GE, Morton RC and Freeman KB, Complete nucleotide and derived amino acid sequence of cDNA encoding the mitochondrial uncoupling protein for rat brown adipose tissue: Lack of mitochondrial targeting presequence. *Nucleic Acids Res* **14:** 4025–4035, 1986.
23. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB and Erlich HA, Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239:** 487–491, 1988.
24. Hunt CR, Ro HS, Dobson DE, Mia HY and Spiegelman BM, Adipocyte P2 gene: Developmental expression and homology to 5′-flanking sequences among fat cell-specific genes. *Proc Natl Acad Sci USA* **83:** 3786–3790, 1986.

25. Sugiyama Y, Taketomi S, Shimura Y, Ikeda H and Fujita T, Effects of pioglitazone on glucose and lipid metabolism in Wistar fatty rats. *Arzneimittelforschung* **40:** 263–267, 1990.
26. Smith PK, Krohn RI, Hernamson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC, Measurement of protein using bicinchoninic acid. *Anal Biochem* **150:** 76–85, 1985.
27. Harlow E and Lane D, *Antibodies; A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1988.
28. Cannon B, Hedin A and Nedergaard J, Exclusive occurrence of thermogenin antigen in brown adipose tissue. *FEBS Lett* **150:** 129–132, 1982.
29. Yang VW, Christy RJ, Cook JS, Kelly TJ and Lane MD, Mechanism of regulation of the 422(aP2) gene by cAMP during preadipocyte differentiation. *Proc Natl Acad Sci USA* **86:** 3629–3633, 1989.
30. Ross SR, Graves RA, Greenstein A, Platt KA, Shyu H-L, Mellovitz B an dSpiegelman BM, A fat-specific enhancer is the primary determinant of gene expression for adipocyte Ps *in vivo*. *Proc Natl Acad Sci USA* **87:** 9590–9594, 1990.
31. Pico C, Herron D, Palou A, Jacobsson A, Cannon B and Nedergaard J, Stabilization of the mRNA for the uncoupling protein thermogenin by transcriptional/translational blockade and by noradrenaline in brown adipocytes differentiated in culture: A degradation factor induced by cessation of stimulation? *Biochem J* **302:** 81–86, 1994.
32. Jacobsson A, Muhleisen M, Cannon B and Nedergaard J, The uncoupling protein thermogenin during acclimation: Indication for pretranslational control. *Am J Physiol* **267:** R999–R1007, 1994.
33. Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM and Kliewer SA, An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). *J Biol Chem* **270:** 12953–12956, 1995.
34. Harris PKH and Kletzien RF, Localization of a pioglitazone response element in the adipocyte fatty acid binding protein gene. *Mol Pharmacol* **45:** 439–445, 1994.
35. Tontonoz P, Hu E, Graves RA, Budavari AI and Spiegelman BM, mPPAR2γ: Tissue specific regulator of an adipocyte enhancer. *Genes Dev* **8:** 1224–1234, 1994.
36. Minden A, Lin A, McMahon M, Lange-Carter C, Dérijard B, Davis RJ, Johnson GL and Karin M, Differential activation of ERK and JNK mitogen-activated protein kinases by raf-1 and MEKK. *Science* **266:** 1719–1723, 1994.