BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **251,** 374–378 (1998)
ARTICLE NO. RC989479

In Vivo Effects of Pioglitazone on Uncoupling Protein-2 and -3 mRNA Levels in Skeletal Muscle of Hyperglycemic KK Mice

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Received September 8, 1998

Pioglitazone is a thiazolidinedione drug (TZD) which potently and specifically stimulates peroxisome proliferator-activated receptor γ (PPAR γ) and sensitizes cells to insulin. Since TZDs are thought to increase energy expenditure, changes in mitochondrial thermogenesis uncoupling protein-2 and -3 mRNA levels in response to pioglitazone treatment were measured in mouse skeletal muscle. Normally hyperglycemic and hyperinsulinemic KK/Ta mice were given pioglitazone for 2 weeks to treat this non-insulin dependent diabetes-like condition. During treatment, UCP2 mRNA levels increased to 185% of normal untreated control levels in soleus muscle. In contrast, UCP3 mRNA levels significantly decreased, up to 67% of normal untreated control levels. Interestingly, UCP3 mRNA levels correlated quite strongly with blood glucose levels, with r = 0.82 for gastrocnemius tissue and r = 0.92 for soleus tissue. These results may indicate that pioglitazone increases glucose catabolism by direct upregulation of muscle *UCP2* gene expression *in vivo*. Therefore, *UCP3* gene expression is controlled by a different mechanism than *UCP2* expression.

Non-insulin dependent diabetes mellitus (NIDDM) is characterized by peripheral insulin resistance, increased hepatic glucose production, and defects in insulin secretion from pancreatic β -cells (1). Thiazolidinediones (TZDs), including pioglitazone, sensitize peripheral tissues to insulin, thereby ameliorating hyperglycemia and hyperinsulinemia (2). These drugs are believed to act by stimulating the peroxisome proliferator-activated receptor γ (PPAR γ), which belongs to its own class in the nuclear receptor superfamily of ligand-activated transcription factors (3). Strong evidence indicating PPAR γ is the molecular target which affects adipogenesis is the antidiabetic actions of TZDs. Formerly, TZDs were thought to alter adipocyte differentiation and metabolism, resulting in lower serum lipid levels and decreased secretion of humoral factors such as TNF-α from adipocytes. This, in turn, alleviated insulin resistance (4–6). However, recent evidence indicates that the antidiabetic actions TZDs occurs independently of their effects on adipose tissue (7).

Skeletal muscle, one of the most abundant tissues in the body, is the major organ for glucose use and development of peripheral insulin resistance (1). Recent efforts led to the discovery of 2 additional uncoupling proteins, called UCP2 and UCP3 (8–10). The tissue distribution of UCP3 is primarily in skeletal muscle (10, 11). In contrast, the major expression sites of UCP2 and UCP1 are quite different from UCP3. UCP2 is ubiquitous, including distribution in skeletal muscle (9, 12). However, UCP1 is specifically expressed in brown adipose tissue (BAT) (13). Recent evidence indicates that these three proteins uncouple mitochondrial ATP synthesis, indicating their important role in theromogenesis (14, 15). The control of expression of these uncoupling proteins has been studied in various tissues and animals using several stimuli. Fasting upregulates UCP2 and UCP3 mRNAs in skeletal muscle (12, 15, 16). Exposure to cold stimulates *UCP1* and *UCP3* gene expressions in BAT, but does not stimulate *UCP3* expression in skeletal muscle (11, 15, 17). Thyroid hormone upregulates *UCP3* gene expression in skeletal muscles (12, 17, 18). Although, *in vitro* exper-

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Abbreviations: BAT, brown adipose tissue; NIDDM, non-insulin dependent diabetes mellitus; PPAR, peroxisome proliferator-activated receptor; RT-PCR, reverse transcription-polymerase chain reaction; TNF-α, tumor necrosis factor-α ; TZD, thiazolidinediones; UCP, uncoupling protein; WAT, white adipose tissue.

0006-291X/98 $25.00

TABLE 1

Summary of Primer Pairs and TaqMan Probes

Genes	Primer (Forward)	TaqMan Probe	Primer (Reverse)
UCP2	5′-$^{-85}$GTTCCTCTGTCTCGTCTTGC^{-66}-3′	5′-$^{-29}$CTTCTGGGAGGTAGCAGGAAATCAG^{-5}-3′	5′-^{18}GGCCTTGAAACCAACCA^{2}-3′
UCP3	5′-^{392}TGACCTGCGCCCAGC^{406}-3′	5′-^{408}CACGGATGTGGTGAAGGTCCGATTT^{432}-3′	5′-^{452}CCCAGGCGTATCATGGCT^{435}-3′
18S rRNA	5′-TGGTTGATCCTGCCAGTAG-3′	5′-CCGGTACAGTGAAACTGCGAATG-3′	5′-CGACCAAAGGAACCATAACT-3′

Note. Superscripts indicate the nucleotide number from cDNA and the nucleotide #1 is the A of the ATG codon that encodes the initiator methionine.

iments indicate that TZDs stimulate expression of *UCP2* gene in cell lines representing WAT, BAT and skeletal muscle (19). UCP3 response was not measured.

Skeletal muscle is classified into two types of fibers according to their physiological characteristics: red-colored (type 1) and white-colored (type 2) (20, 21). Type 1 fibers, which predominate in soleus muscle, and type 2 fibers, which predominate in gastrocnemius muscle, are quite different anatomically, biochemically, and physiologically (20).

To better understand *UCP2* and *UCP3* gene expression, mRNA levels in gastrocnemius and soleus muscles were studied after treating KK mice with pioglitazone, which reduces their normal diabetic-like condition. The results obtained show that in soleus skeletal muscle, UCP2 mRNA was upregulated by *in vivo* pioglitazone treatment. However, UCP3 mRNA expression level was downregulated, indicating this gene in skeletal muscles is not under positive regulation by PPAR γ ligand.

MATERIALS AND METHODS

Reagents. Pioglitazone hydrochloride was synthesized at Yamanouchi Pharmaceutical (Tokyo). HPLC analysis confirmed the purity was greater than 98%. The total RNA extraction reagent Isogen and Deoxyribonuclease (DNase, RT grade) were purchased from Nippon Gene (Toyama, Japan). TaqMan probe and oligonucleotide primers were purchased from Perkin-Elmer Applied Biosystems (Tokyo, Japan) and Genset K. K. (Tokyo, Japan), respectively. The TaqMan EZ RT-PCR Core Reagents were from Perkin-Elmer Applied Biosystems. All other reagents were commercially obtained from standard sources.

Animal experiment. Male hyperglycemic and hyperinsulinemic KK/Ta Jcl mice (8 weeks old) obtained from Japan Clea (Tokyo, Japan) were fed a high calorie diet (CMF, Oriental Yeast Co., Ltd., Tokyo, Japan) and given water *ad libitum*. Each mouse was caged separately to prevent fights due to violent moods which occur in mice housed under pathogen-free conditions. At 15 weeks old, blood samples (10 μ 1) were obtained from a tail vein and blood glucose levels determined using an "Autopack A glucose kit" (Boehringer Mannheim; Tokyo, Japan). Mice were divided into 2 groups determined by mean body weight and blood glucose level. After grouping (n=8 per each group) at week 0, either pioglitazone hydrochloride (10 mg/kg/day) or saline as a control were injected subcutaneously at 4:00 pm during the test period. After 1 week and 2 weeks of treatment, blood glucose levels were determined at 10:00 am. After 2 weeks of the treatment, whole blood was drawn by cardiac puncture into syringes. Blood insulin level was determined according to the manufacturer's protocol using "Phadeseph insulin" (Pharmacia&Upjohn, Diagnostics). The hind limb gastrocnemius and soleus muscles were excised and separated, and other tissues such as adipose stuck to the muscle tissues were removed. The muscles were rinsed with ice-cold saline. Muscle tissue was immediately frozen in liquid nitrogen and pulverized in an earthenware mortar. Total RNA was extracted with Isogen and treated with DNase according to the manufacturer's protocol.

A real time quantitative RT-PCR analysis for mRNA determination using ABI PRISM 7700 system. mRNA levels were determined using a quick, accurate, and highly sensitive method employing real time quantitative RT-PCR analysis as described previously (22). Oligonucleotide primers (forward, reverse) and TaqMan probes were designed using Primer Express, version 1.0 (Perkin-Elmer Applied Biosystems) from the *GenBank* database as follows: mouse UCP2 (Accession# U69135), mouse UCP3 (AB013132) (22), and mouse 18S rRNA (X00686) (23) (Table 1).

Quantitative RT-PCR analysis was carried out according to the manufacturer's protocol (24–26). Briefly, each reaction mixture contained 1× TaqMan EZ buffer, 3 mM $Mn(OAc)_2$, 300 μM each dA/dC/dG/dUTP, 2.5 unit rTth DNA polymerase, 200 nM primers (Forward and Reverse), 100 nM TaqMan probe, 50 ng total RNA in 25 μ1. RT reaction conditions were 55°C for 50 min, 60°C for 10 min, 95°C for 2 min for 1 cycle; PCR conditions were 95°C for 15 sec, 58°C for 1.5 min for 40 cycles on an ABI PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems). Relative mRNA levels were determined as an average from 8 replicates/mouse × 8 mice and normalized to 18S rRNA level in each sample. Linearity of the standard curve for target gene mRNAs was confirmed for the 20-500 ng total RNA range using total RNA prepared from C57BL/6J Jc1 mouse skeletal muscles.

Analysis of data. Statistical analyses were carried out using the paired *t*-test and the simple regression test.

RESULTS

KK mice are an animal model of non-insulin dependent diabetes mellitus (NIDDM) due to their moderate hyperglycemia and hyperinsulinemia (27). Therefore, this model was used to study thiazolidinediones (TZDs) treatment and its effect on UCP mRNA levels. KK mice were treated for 2 weeks with or without pioglitazone hydrochloride, a peroxisome proliferator-activated receptor γ (PPAR γ ligand. Both body weights and blood glucose levels in control group did not change throughout the treatment (Table 2). In contrast, body weights in the pioglitazone treatment group slightly increased while blood glucose levels showed a significant decrease (−52% at 1 week, −59% at 2 weeks). However, the blood glucose levels in the pioglitazone treatment

TABLE 2

Effect of Pioglitazone on Body Weight, Blood Glucose and Insulin Levels in KK Mice

	Weeks	Control	Pioglitazone[a]
Body weight (g)	0	49.4 ± 0.5	51.0 ± 0.8
	1	49.7 ± 0.5	53.6 ± 0.6*
	2	49.8 ± 0.5	54.1 ± 0.8*
Glucose level (mg/dl)	0	393.5 ± 7.6	392.0 ± 10.0
	1	423.8 ± 14.4	187.4 ± 17.2**
	2	343.2 ± 26.9	159.5 ± 7.7**
Insulin level (μ U/ml)	2	423.0 ± 41.8	40.9 ± 9.5**

[a] Statistical significance vs. control: *: $p<0.01$; **: $p<0.001$. Values (means ± S.E.) are averages of n=8 mice.

group after 2 weeks of treatment were still slightly higher than levels in C57BL/6J Jcl mice (122.68±12.3 mg/dl, at 15 weeks old, male). During the treatment period, food consumption rates were the same for both pioglitazone and control groups (control 5.52±0.62 vs. pioglitazone 4.86±0.41 (g/day/mouse), $p=0.0997$). Treatment with pioglitazone for 2 weeks caused a significant decrease of blood insulin level by 90% (Table 2). Additionally, pioglitazone treatment decreased serum triglyceride (control 318±64 vs. pioglitazone 102±23 (mg/dl), $p<0.01$) and free fatty acid levels (control 2756 ± 406 vs. pioglitazone 1664±388 (mg/dl), $p<0.01$), indicating amelioration of hypertriglyceridemia as described previously (28). These *in vivo* results indicate that the pioglitazone treatment ameliorates hyperlipidemia, hyperglycemia and hyperinsulinemia in KK mice.

Skeletal muscles are among the most abundant tissues in the whole body. Therefore, proper function of these tissues are important to maintain normal blood glucose levels (1). Since skeletal muscles are classified into two types of fibers which are very different physiologically and biochemically, both types were examined (20, 21). Fig. 1 shows the mRNA levels of UCP2 and UCP3 in both gastrocnemius and soleus muscle tissues after 2 weeks of pioglitazone treatment. Fig. 1a shows UCP2 mRNA level: soleus but not gastrocnemius UCP2 mRNA levels showed a dramatic increase (185% vs. control). However, UCP3 mRNA level showed a significant decrease (−32% in gastrocnemius, −67% in soleus; Fig. 1b). These results indicate that the PPAR γ ligand pioglitazone stimulates UCP2 mRNA expression in soleus muscle, which has abundant myoglobin and oxidative enzymes, but not gastrocnemius. In contrast, UCP3 mRNA level was down-regulated in both muscle types. Additionally, both *UCP2* and *UCP3* genes expressed in skeletal muscles appear to be differentially regulated by pioglitazone *in vivo*.

The correlation between blood glucose levels and mRNA levels of UCP2 and UCP3 in KK mouse skeletal muscles was examined (data not shown). The decrease of UCP3 mRNA level in both muscles after pioglitazone treatment showed a strong positive correlation with blood glucose levels [gastrocnemius, $f(X)=0.162X+46.65$, $r=0.817$, $p=0.0001$; soleus, $f(X)=0.437X-30.36$, $r=0.923$, $p=0.0001$]. However, the relationship between UCP2 mRNA levels and blood glucose levels showed no correlation [gastrocnemius, $f(X)=-0.030X+107.9$, $r=0.215$, $p=0.423$; soleus, $f(X)=-0.251X+350.5$, $r=0.259$, $p=0.334$]. Additionally, our data obtained from normoglycemic C57BL/6J Jcl mice and hyperglycemic KK/Ta mice showed no correlation between blood glucose levels and UCP3 mRNA levels among these animals [gastrocnemius, $f(X)=0.207X+76.57$, $r=0.471$, $p=0.0894$; soleus, $f(X)=0.179X+108.9$, $r=0.193$, $p=0.509$].

DISCUSSION

KK mice were treated with pioglitazone hydrochloride, a thiazolidinedione (TZD). This treatment ameliorated the normal hyperglycemia, hyperlipidemia and hyperinsulinemia present in KK mice with a slight increase of body weight as described previously (27, 28). Several reports indicate that insulin sensitizers such as TZD are ligands of peroxisome proliferator-activated receptor γ (PPAR γ), are adipogenic, reduce cytokines such as TNF-α and leptin, and stimulate energy expenditure by up-regulation of *UCP2* gene expression in adipose tissues (5, 19, 29, 30). However,

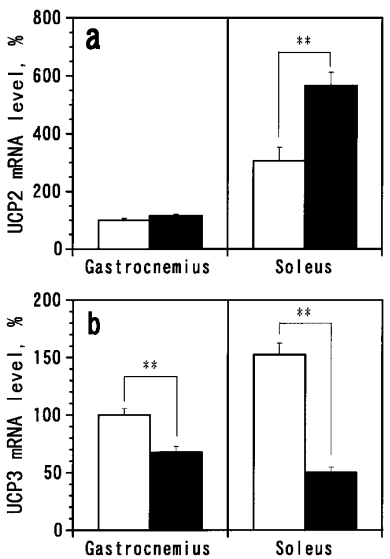

FIG. 1. Effect of pioglitazone on UCP2 and UCP3 mRNA levels in skeletal muscles of KK mice. Total RNA was prepared from gastrocnemius and soleus muscles treated with pioglitazone (■) or saline (□) as described in Materials and Methods. Uncoupling protein (UCP2 and UCP3) mRNA levels were determined by a real time quantitative RT-PCR method. Relative mRNA levels (means ± S.E.) are represented as gastrocnemius in control group as 100 %. Statistical significance vs. control: **: $p<0.001$.

little was known about the mechanisms of TZD on energy expenditure in skeletal muscles, which are pivotal tissues in terms of glucose metabolism. The thermoregulatory mitochondrial uncoupling protein UCP2 mRNA levels in soleus muscles increase dramatically, but mRNA levels do not significantly increase in gastrocnemius (Fig. 1a). This interesting result may indicate the differential control of *UCP2* and *UCP3* among these muscle types, gastrocnemius and soleus like adipose tissue (31). These results suggest that pioglitazone treatment stimulates energy expenditure partly through upregulation of *UCP2* gene expression in soleus-type muscles, which contains relatively abundant myoglobin, oxidative enzymes, and lipids compared to gastrocnemius type muscles (21). In skeletal muscles, PPAR γ 1 expression is relatively high compared to PPAR γ 2 (32), suggesting the PPAR γ 1 expression plays a key role in determining tissue sensitivity to insulin (33). Skeletal muscle *UCP2* gene upregulation caused by pioglitazone treatment may therefore have acted through PPAR γ 1 directly or indirectly like *UCP1* and *UCP2* gene expressions in adipose tissue (34). However, this study can not rule out the possibility that other factors such as cytokines may also be involved, directly or indirectly (35). *In vitro* experiments using rat skeletal muscle myoblast L6 cells, indicated that TZD stimulated UCP2 mRNA expression via PPAR γ (19). Furthermore, the improvement of hyperglycemia in KK mice may not be due to stimulation of *UCP2* gene expression in skeletal muscle by TZD treatment, since UCP2 mRNA level in soleus-type muscle does not significantly correlate with blood glucose level in KK mice.

In contrast, expression of muscle-specific UCP3 mRNA showed a marked decrease in both gastrocnemius and soleus tissues in KK mice of the pioglitazone-treatment group (Fig. 1b). This *in vivo* effect of pioglitazone also raises questions of direct vs. indirect effects through PPAR γ, or effects due to confounding factors. However, the strong positive correlation between UCP3 mRNA levels in both type muscles and blood glucose levels does not seem to be a simple result of secondary response to the hypoglycemic effect of pioglitazone, since no other positive correlations were observed for these factors between normoglycemic C57/BL mice and hyperglycemic KK mice ($r=0.0.471$, $p=0.089$ in gastrocnemius; $r=0.193$, $p=0.509$ in soleus)[1]. This UCP3 decrease may result from physiologic responses in skeletal muscles to effects complicated by acting through other tissues such as adipocytes, i.e. down-regulation of leptin (35), decrease of serum free fatty acid level as observed (36) or exercise training (37). Further experiments are required to clarify the mechanism of this phenomenon.

In summary, the PPAR γ ligand pioglitazone showed *in vivo* stimulation of UCP2 mRNA expression in skeletal muscles, especially soleus muscles. In contrast, UCP3 mRNA level decreased by this ligand, indicating differential *in vivo* gene regulation. *In vivo* data suggest that the insulin sensitizing effect of TZD in skeletal muscles may be due to increased energy expenditure through *UCP2* gene expression, but not *UCP3*, although further evidence on protein expression and fucntion is needed.

ACKNOWLEDGMENTS

We thank Dr. Kunihiro Niigata for preparing pioglitazone hydrochloride. We thank Drs. Yoshitaka Ueda, Masahide Goto and Satomi Nishijima for their technical assistance and helpful discussions. We are grateful to Mr. Steven E. Johnson for reviewing this manuscript.

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