



Role of peroxisome proliferator-activated receptor alpha in the expression of hepatic fatty acid oxidation-related genes in chickens

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2 hepatic fatty acid oxidation-related genes in chickens

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11 Running title: ROLE OF PPAR α IN CHICKENS

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Abstract

Liver is the most important target organ for investigation of lipid metabolism in domestic fowls. However, little is known about the regulatory mechanism of fatty acid oxidation in chicken liver. In mammals, proliferator-activated receptor alpha (PPAR α), a transcription factor, plays an essential role in the regulation of hepatic fatty acid oxidation. The aim of the present study was to investigate the regulatory mechanisms of PPAR α -induced gene expression involved in hepatic fatty acid oxidation in chickens *in vivo* and *in vitro*. WY14643, a PPAR α agonist, significantly increased the mRNA levels of carnitine palmitoyltransferase 1a (CPT1a) and acyl-coenzyme A oxidase (ACO), but not long-, middle-, and short-chain acyl-coenzyme A dehydrogenase (LCAD, MCAD, and SCAD, respectively), hydroxyacyl-coenzyme A dehydrogenase (HAD), and PPAR α itself in chicken hepatoma cells. In contrast, WY14643 significantly increased the mRNA levels of CPT1a, ACO, MCAD, SCAD, HAD, and PPAR α in human hepatoma cells. The mRNA levels of CPT1a and ACO in the liver were significantly increased by 6 h of fasting in chickens, whereas the mRNA levels of LCAD, MCAD, SCAD, and HAD were unchanged. These results suggest that, unlike in mammals, CPT1a and ACO might play an important role in PPAR α -induced fatty acid oxidation in the liver of chickens.

Keywords: chickens, fasting, liver, PPAR

INTRODUCTION

Broiler chickens have been intensively selected over many generations with specific emphasis on increasing body weight. This increased body weight has been accompanied by unintended changes such as increased body fat (Havenstein *et al.* 2003). In view of the obesity epidemic, chickens that provide several times the fat energy compared with protein seem illogical (Wang *et al.* 2010). In addition, excessive fat accumulation not only increases inedible products such as visceral adipose tissue but also results in metabolic diseases, which are a serious problem for the poultry industry (Julian 2005). Therefore, the regulatory mechanisms of lipid metabolism in chickens have been a focus of research by poultry nutritionists.

Liver plays a primary role in lipid metabolism in birds. In addition, fatty liver is a serious problem in many types of domestic fowl (Julian 2005). Therefore, liver is the most important target for investigation of lipid metabolism in domestic fowls. Peroxisome proliferator-activated receptors (PPARs) are in the nuclear receptor family of ligand-activated transcription factors. In particular, PPAR α plays an essential role in the metabolic adaptation of the liver to fasting situations by inducing gene expression of the rate-limiting enzymes for mitochondrial and peroxisomal fatty acid oxidation, such as carnitine palmitoyltransferase 1a (CPT1a), hydroxyacyl-coenzyme A dehydrogenase (HAD), and acyl-coenzyme A oxidase (ACO) in mammals (Schoonjans *et al.* 1996; Mandard *et al.* 2004). Fatty acid oxidation-related genes such as long-, middle-, and short-chain acyl-coenzyme A dehydrogenase

(LCAD, MCAD, and SCAD, respectively), HAD, and PPAR α itself are also target genes of PPAR α (Schoonjans *et al.* 1996; Kersten *et al.* 1999; Mandard *et al.* 2004). Therefore, PPAR α plays important roles in fatty acid oxidation in mammals.

The PPAR α agonist fenofibrate is used as a medicine for non-alcoholic fatty liver disease in humans (Kostapanos *et al.* 2013). Chicken liver expresses PPAR α , which has high homology with human PPAR α (Diot & Douaire 1999). There is evidence that PPAR α agonists have complex effects on hepatic lipid metabolism in chickens. For example, the addition of PPAR α agonist clofibrate did not influence the mRNA level of the PPAR α in the liver in laying hens, although the mRNA levels of CPT1a and ACO were increased (König *et al.* 2007a). PPAR α agonists GW7647 and perfluorooctane sulfonate significantly increased mRNA levels of ACO, but not PPAR α and CPT1a, in the liver from chicken embryos (Strömqvist *et al.* 2012). It is therefore likely that the target genes of PPAR α in chickens might differ from those in mammals.

Fasting triggers a complex array of adaptive metabolic responses. Therefore, the effects of fasting on the expression of genes involved in the metabolic adaptations to feeding in the liver have been extensively studied in chickens. We previously showed that 4 hours of fasting significantly increased the mRNA levels of PPAR α , CPT1a, and ACO in the liver in broiler chickens (Saneyasu *et al.* 2013). However, the mRNA level of PPAR α was significantly increased after 2 hours of fasting, whereas the mRNA levels of CPT1a and ACO were unchanged (Saneyasu *et al.* 2013). In addition, we found that the mRNA

levels of LCAD, MCAD, SCAD, and HAD were unchanged by both 2 and 4 hours of fasting in chickens (unpublished data).

In the present study, we focused on the different responses of fatty acid oxidation-related genes between humans and chickens. The results show the first direct evidence that target genes of PPAR α might substantially differ between humans and chickens.

MATERIALS AND METHODS

Cell culture

Vanden Heuvel *et al.* (2003) demonstrated the direct effect of WY14643 on gene expression in a human hepatoma cell line (HepG2). They chose the short treatment time (6 h) and relatively high concentration of WY14643 (50 μ M) in order to focus on early and predominantly transcriptional events. In fact, 24 h incubation of rat hepatoma cells with WY 14643 significantly increased not only PPAR α target genes but also cholesterol metabolism-related genes (König *et al.* 2007b). According to these findings, we confirmed the effect of 6 h incubation with WY14643 on hepatoma cell lines. Also, since there is no report showing the effect of WY14643 on the expression of PPAR α target genes in chicken hepatocytes, a wider range of concentration (0-100 μ M) of WY14643 was used in a chicken hepatoma cell line (LMH) experiment.

LMH was purchased from ATCC (VA, USA; ATCC # CRL-2117). Cells were grown on 6-well culture dishes in Waymouth's MB 752/1 containing 10%

Fetal Clone III (HyClone, UT, USA), 100 IU/ml penicillin, and 100 µg/ml streptomycin under a controlled atmosphere (95% air and 5% CO₂). LMH cells were cultured prior reaching confluence and then incubated with the medium supplemented with either WY14643 (25, 50 or 100 µM) or vehicle (DMSO, 0.1% v/v) for 6 h. After removing the cell culture medium, cells were washed twice with cold PBS, and total RNA was extracted as described in the Real-time PCR analysis section.

HepG2 was purchased from ATCC (VA, USA; ATCC # HB-8065). Cells were grown on 6-well culture dishes in DMEM containing 10% Fetal Clone III (HyClone, UT, USA), 100 IU/ml penicillin, and 100 µg/ml streptomycin under a controlled atmosphere (95% air and 5% CO₂). HepG2 cells were prior reaching confluence and then incubated with the medium supplemented with either 0 or 50 µM WY14643 for 6 h. After removing the cell culture medium, cells were washed twice with cold PBS, and total RNA was extracted as described in the Real-time PCR analysis section.

Animals and diet

Day-old male chicks of a Ross 308 broiler (*Gallus gallus domesticus*) were purchased from a local hatchery (Ishii Co., Ltd., Tokushima, Japan). They were maintained in an electrically heated brooder at 32°C ± 2°C with an automatically controlled 12-h light:dark cycle (18:00-6:00 h). They were given free access to water and a commercial chick starter diet (Nippon Formula Feed Mfg. Co., Ltd., Kanagawa, Japan). This study was approved by the Institutional

Animal Care and Use Committee and carried out according to the Kobe University Animal Experimentation Regulation.

Sampling and preparation

A total of 24 thirteen-day-old male broiler chicks were weighed and allocated based on body weight to six cages (1,725 mm × 425 mm × 320 mm, six birds in each group). Twelve chicks were fasted for 0 or 6 hours prior to euthanasia by decapitation. The remaining twelve chicks were fasted for 12 hours and refed for 0 or 6 hours prior to euthanasia by decapitation. The liver was excised, weighed, and frozen immediately using liquid nitrogen for real-time PCR analysis.

Real-time PCR analysis

Total RNA was extracted from the liver and hepatoma cells using Sepazol-RNA I (Nacalai Tesque, Inc., Kyoto, Japan). First-strand cDNA was synthesized from 2 µg of DNase I (Ambion Inc., Austin, Texas, USA)-treated total RNA using a ReverTra Ace[®] qPCR RT Kit (TOYOBO CO. LTD., Osaka, Japan) with random primers. Complementary DNAs of chicken PPAR α , CPT1a, ACO, and LCAD were amplified with the primers as described previously (Motoki *et al.* 2012; Saneyasu *et al.* 2013). Complementary DNAs of chicken MCAD (GenBank accession no. BM426980), SCAD (GenBank accession no. NM_001031246), and HAD (GenBank accession no. XM_418403) were amplified with the primers as follows: MCAD sense, 5'-GCG GAA GGG CGA

155 TGA GT-3'; MCAD antisense, 5'-TCC GTT GGT GAT CCA CAT CTT-3';
 156 SCAD sense, 5'-ATA TCC TCG GGC AGA TTG GA-3'; SCAD antisense, 5'-
 157 TGC CAC CAT TCA GCA TTC C-3'; HAD sense, 5'-CAA GCA ACA CTT CAT
 158 CCT TGC A-3'; HAD antisense, 5'-GTC CTG CCT GGT GGT TGA GT-3'.
 159 Complementary DNAs of chicken lipogenic genes such as sterol regulatory
 160 element-binding protein 1 (SREBP1), acetyl-coenzyme A carboxylase α ,
 161 (ACC α), and fatty acid synthase (FAS) were also amplified with the primers as
 162 described previously (Saneyasu *et al.* 2013). As an internal standard,
 163 complementary DNA of chicken ribosomal protein S17 (RPS17) was also
 164 amplified with the primers as described previously (Ibuki *et al.* 2013).
 165 Complementary DNAs of human genes, such as CPT1a (GenBank accession no.
 166 NM_001031847), ACO (GenBank accession no. NM_001185039), LCAD
 167 (GenBank accession no. NM_001608), MCAD (GenBank accession no.
 168 NM_000016), SCAD (GenBank accession no. NM_000017), HAD (GenBank
 169 accession no. NM_001184705), PPAR α (GenBank accession no.
 170 NM_001001928), and RPS17 (GenBank accession no. NM_001021), were
 171 amplified with the primers as follows: CPT1a sense, 5'-TGG TGG GCG TGA
 172 TGA CAA C-3'; CPT1a antisense, 5'-GAG TCC GAT TGA TTT TTG CAA TT-
 173 3'; ACO sense, 5'-TCT TCA CTT GGG CAT GTT CCT-3'; ACO antisense, 5'-
 174 TTC CAG GCG GGC ATG A-3'; LCAD sense, 5'-AGC TTA TGT GGA TGC
 175 CAG AGT TC-3'; LCAD antisense, 5'-TCT TGC AAT CAG CTC CTT CAT
 176 TAT-3'; MCAD sense, 5'-GCT GGT GCT GTT GGA TTA GCA-3'; MCAD
 177 antisense, 5'-CCT TTC CAG GGC ATA CTT GGT-3'; SCAD sense, 5'-TTT

178 GCC AGC ACG GAC AGA-3'; SCAD antisense, 5'-GGA CCA GGA AGG CAC
179 TGA TG-3' ; HAD sense, 5'-GAT TCG CTG GCC TCC ATT T-3'; HAD
180 antisense, 5'-TTT AAT GAC CTC CAC AAG TTT CAT G-3'; PPAR α sense, 5'-
181 AAC ATC CAA GAG ATT TCG CAA TC-3'; PPAR α antisense, 5'-CCG TAA
182 AGC CAA AGC TTC CA-3' ; RPS17 sense, 5'-CGC CAT TAT CCC CTG CAA-
183 3'; RPS17 antisense, 5'-CAG ATG CGT GAC ATA ACC TGC TA-3'.
184 THUNDERBIRDTM SYBR[®] qPCR Mix was purchased from TOYOBO CO.
185 LTD. (Osaka, Japan), and mRNA expression was quantified in duplicate using
186 the Applied Biosystems 7300 Real-Time PCR system according to the supplier's
187 recommendations.

188

189 **Data analysis**

190 Data from an *in vitro* experiment using LMH were analyzed by one-way
191 ANOVA followed by the Tukey-Kramer test. Other data were analyzed by
192 Student's *t*-test. All statistics was performed using a commercial software
193 package (StatView version 5, SAS Institute, Cary, North Carolina, USA, 1998).

194

195 **RESULTS**

196

197 We first examined the effects of WY14643 on fatty acid oxidation-
198 related gene expression in chicken and human hepatoma cells. The mRNA
199 levels of CPT1a, ACO, MCAD, SCAD, HAD, and PPAR α were significantly
200 increased by WY14643 in HepG2 cells (Fig. 1). On the other hand, WY14643

significantly increased the mRNA levels of CPT1a and ACO, but not LCAD, MCAD, SCAD, HAD, and PPAR α in LMH cells (Table 1). We also analyzed the effects of WY14643 on lipogenic gene expression in chicken hepatoma cells because PPAR α influenced the expression of lipogenic genes in mammals (Yoshikawa *et al.* 2003; König *et al.* 2009; Fernández-Alvarez *et al.* 2011). However, WY14643 did not influence the mRNA levels of SREBP1, FAS, and ACC α in LMH cells (Table 1).

We next examined the effects of fasting and refeeding on the mRNA levels of fatty acid oxidation-related genes in the liver in chickens. The mRNA levels of CPT1a, ACO, and PPAR α were significantly increased by fasting, whereas the mRNA levels of LCAD, MCAD, SCAD, and HAD were not influenced by fasting (Fig. 2A). The mRNA levels of these enzymes were not changed by refeeding, whereas the mRNA levels of CPT1a, ACO, and PPAR α were significantly decreased (Fig. 2B).

DISCUSSION

LCAD is a mitochondrial fatty acid oxidation enzyme whose expression in humans is low or absent in organs known to utilize fatty acids for energy, such as heart, muscle, and liver. (He *et al.* 2007; Chegary *et al.* 2009; Maher *et al.* 2010). In the present study, we analyzed the LCAD mRNA level in HepG2 cells, but complementary DNA of LCAD cannot be amplified by real-time PCR. Therefore, we showed the effects of fasting and WY14643 on LCAD mRNA levels in chicken hepatoma cells (Table 1) and liver (Fig. 2), but not in human

224 hepatoma cells (Fig. 1).

225 The PPAR α agonist WY14643 significantly increased the mRNA levels
226 of CPT1a and ACO in chicken hepatoma cells (Table 1). The addition of the
227 PPAR α agonist clofibrate in the diet significantly increased the mRNA levels of
228 CPT1a and ACO in the liver of laying hens (König *et al.* 2007a). *In ovo*
229 injection of PPAR α agonists GW7647 and perfluorooctane sulfonate
230 significantly increased the ACO mRNA level in the liver of chicken embryos
231 (Strömqvist *et al.* 2012). These findings and our results suggest that CPT1a and
232 ACO are target genes of PPAR α in chickens.

233 Mitochondrial fatty acid oxidation is the main mechanism providing
234 energy from fatty acids in cells. Long-chain fatty acids are imported into the
235 mitochondrial matrix after the transfer of acyl groups from coenzyme A to
236 carnitine by CPT1a (Bartlett & Eaton 2004). After the transport of fatty acids
237 from the cytosol to mitochondria, they are oxidized by the β -oxidation pathway,
238 in which HAD is considered as the rate-limiting enzyme in mammals (McGarry
239 *et al.* 1989; Eaton 2002). Very long-chain fatty acids were oxidized in
240 peroxisomes, and ACO, the rate-limiting enzyme of peroxisomal fatty acid
241 oxidation, is transcriptionally regulated by PPAR α in mammals (Mandard *et al.*
242 2004). CPT1a, ACO, HAD, PPAR α , and gene for other β -oxidation-related
243 enzymes including LCAD, MCAD, and SCAD are known to be the target genes
244 of PPAR α in mammals (Schoonjans *et al.* 1996; Kersten *et al.* 1999; Mandard
245 *et al.* 2004). Therefore, we examined the effects of WY14643 on the mRNA
246 levels of these genes in chicken hepatoma cells. However, WY14643 did not

influence the mRNA levels of LCAD, MCAD, SCAD, HAD, and PPAR α in chicken hepatoma cells (Table 1), although the mRNA levels of CPT1a and ACO were significantly increased. On the other hand, WY14643 significantly increased the mRNA levels of CPT1a, ACO, MCAD, SCAD, HAD, and PPAR α in human hepatoma cells (Fig. 1). PPAR α agonists did not increase the PPAR α mRNA level in laying hens and chicken embryos (König *et al.* 2007a; Strömqvist *et al.* 2012). These findings and our results suggest that the target genes of PPAR α differ between chickens and humans.

In the present study, the mRNA levels of LCAD, MCAD, SCAD, and HAD were unchanged by fasting (Fig. 2A) and refeeding (Fig. 2B) in chickens, although the mRNA levels of CPT1a and ACO were significantly changed. CPT1a and ACO function as rate-limiting enzymes for mitochondrial and peroxisomal fatty acid oxidation, respectively, in mammals (Mandard *et al.* 2004). Our findings suggest that the transcriptional change of CPT1a and ACO plays an important role in hepatic fatty acid oxidation in chickens.

We previously showed that the mRNA levels of CPT1a and ACO were not increased by 2 hours of fasting in chickens (Saneyasu *et al.* 2013), although the PPAR α mRNA level was increased. In the present study, the PPAR α agonist WY14643 significantly increased the mRNA levels of CPT1a and ACO, but not PPAR α , in chicken hepatoma cells (Table 1), suggesting that PPAR α itself is not the target gene of PPAR α . All our findings clearly demonstrate that the regulatory mechanisms of fasting-induced gene expression differ between PPAR α and its target genes CPT1a and ACO.

Fatty acid is known to be one of the natural ligands of PPAR α (Bocos *et al.* 1995). During fasting, fatty acids are released from white adipose tissue and travel to the liver, where they bind and activate PPAR α , which results in stimulation of the fatty acid oxidative pathway in the liver (Mandard *et al.* 2004). In fact, a significant increase in the mRNA level of PPAR α at 8 hours of fasting occurred after the significant increase of plasma NEFA concentration at 4 hours of fasting in rats (Palou *et al.* 2008). However, we recently found that the PPAR α mRNA level was significantly increased after 2 hours of fasting in chickens, although the plasma non-esterified fatty acid (NEFA) concentration was not significantly changed (Saneyasu *et al.* 2013). In the present study, WY14643, an artificial ligand of PPAR α , did not influence the mRNA level of PPAR α in chicken hepatoma cells (Table 1), unlike in human hepatoma cells (Fig. 1). These findings suggest that the fasting-induced PPAR α gene expression in the liver does not depend on elevation of the plasma NEFA concentration in chickens.

The transcription factor SREBP1 plays important roles in regulation of the expression of lipogenic genes including ACC α and FAS in the liver in both mammals and chickens (Wang *et al.* 2009; Shimano 2009). However, several differences between animal species have been demonstrated previously in terms of the regulation of lipogenic gene expression by PPAR α . For example, WY14643 increased the mRNA levels of SREBP1 and FAS in rat hepatoma cells (König *et al.* 2009). The overexpression of PPAR α in HEK293 cells has been shown to inhibit mouse SREBP1 promoter activity (Yoshikawa *et al.*

2003). In humans, PPAR α seems to be an activator of the SREBP1 promoter (Fernández-Alvarez *et al.* 2011). In the present study, however, we showed that WY14643 did not influence the mRNA levels of SREBP1, ACC α , and FAS in chicken hepatoma cells (Table 1). Our findings provide new insight into the species-specific mechanism underlying the regulation of hepatic lipogenesis.

In summary, the PPAR α agonist WY14643 significantly increased the mRNA levels of PPAR α , MCAD, SCAD, and HAD in human hepatoma cells, but not in chicken hepatoma cells. Fasting significantly increased the mRNA levels of PPAR α , CPT1a, and ACO, but not MCAD, SCAD, and HAD, in chicken liver. These results suggest that the mechanisms underlying the transcription of fatty acid oxidation-related genes in the liver might differ between humans and chickens.

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FIGURE LEGENDS

Figure 1 Effects of WY14643 on the mRNA levels of fatty acid oxidation-related genes in human hepatoma cells. Data are the means \pm S.E.M. of four wells in each group. Data were analyzed by Student's *t*-test. *, $p < 0.05$; **, $p <$

431 0.01

432

433 Figure 2 Effects of fasting (A) or refeeding (B) on the mRNA levels of fatty
434 acid oxidation-related genes in the liver in chickens. Data are the means \pm
435 S.E.M. of six chicks in each group. Data were analyzed by Student's *t*-test. *, *p*
436 < 0.05 ; **, *p* < 0.01

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ニワトリにおける肝臓脂肪酸酸化関連遺伝子の発現におけるペルオキシ
ソーム増殖活性化受容体 α の役割

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肝臓は、家禽の脂質代謝研究における最も重要な標的臓器となっ
ている。しかしながら、ニワトリの肝臓における脂肪酸酸化調節機構に
ついては殆どわかっていない。哺乳動物においては、転写因子であるペ
ルオキシソーム増殖活性化受容体 (PPAR) α が肝臓脂肪酸酸化において
極めて重要な役割を果たす。本研究では、ニワトリ肝臓におけるPPAR α
誘導性の脂肪酸酸化関連遺伝子の発現調節機構を *in vivo* および *in vitro*
条件下で調べることを目的とした。PPAR α アゴニストであるWY14643
は、ニワトリ肝癌細胞においては、カルニチンパルミトイルトランスフ
ェラーゼ1a (CPT1a) およびアシルCoAオキシダーゼ (ACO) のmRNA量
を有意に増加させたが、長鎖、中鎖、短鎖アシルCoAデヒドロゲナーゼ
(LCAD、MCADおよびSCAD)、ヒドロキシアシルCoAデヒドロゲナー
ゼ (HAD) およびPPAR α のmRNA量には影響しなかった。一方、ヒト肝
癌細胞においては、WY14643は、CPT1a、ACO、MCAD、SCAD、HADお
よびPPAR α のmRNA量を有意に増加させた。ニワトリ肝臓におけるCPT1a
およびACOのmRNA量は6時間の絶食により有意に増加したが、LCAD、

462 MCAD、SCADおよびHADのmRNA量は変化しなかった。これらの結果か
463 ら、哺乳動物とは異なり、ニワトリ肝臓におけるPPAR α 誘導性の脂肪酸
464 酸化においては、CPT1aおよびACOが重要な役割を果たしている可能性
465 が示された。

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467 キーワード：ニワトリ、絶食、肝臓、PPAR

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Table 1 Effects of WY14643 on the mRNA levels of fatty acid metabolism-related genes in chicken hepatoma cells

| Gene | WY14643 (μ M) | | | |
|---------------|------------------------------|-------------------------------|-------------------------------|------------------------------|
| | 0 | 25 | 50 | 100 |
| PPAR α | 0.97 \pm 0.01 | 1.06 \pm 0.05 | 0.99 \pm 0.12 | 0.76 \pm 0.07 |
| CPT1a | 0.66 \pm 0.05 ^b | 1.02 \pm 0.16 ^{ab} | 1.28 \pm 0.20 ^{ab} | 1.44 \pm 0.20 ^a |
| ACO | 1.38 \pm 0.16 ^b | 1.94 \pm 0.37 ^{ab} | 2.56 \pm 0.22 ^{ab} | 2.99 \pm 0.42 ^a |
| LCAD | 3.45 \pm 0.20 | 4.58 \pm 1.00 | 4.88 \pm 0.11 | 4.83 \pm 0.58 |
| MCAD | 0.38 \pm 0.20 | 0.43 \pm 0.10 | 0.49 \pm 0.12 | 0.61 \pm 0.15 |
| SCAD | 2.10 \pm 0.10 | 2.10 \pm 0.14 | 2.41 \pm 0.37 | 1.98 \pm 0.12 |
| HAD | 1.11 \pm 0.11 | 1.13 \pm 0.04 | 1.04 \pm 0.03 | 0.87 \pm 0.05 |
| SREBP1 | 0.67 \pm 0.05 | 0.76 \pm 0.10 | 0.97 \pm 0.20 | 0.90 \pm 0.11 |
| ACC α | 0.73 \pm 0.08 | 0.67 \pm 0.11 | 0.72 \pm 0.20 | 0.82 \pm 0.11 |
| FAS | 2.11 \pm 0.22 | 2.26 \pm 0.44 | 2.48 \pm 0.20 | 2.41 \pm 0.33 |

Data are the means \pm S.E.M. of four wells in each group. Data were analyzed by ANOVA followed by Tukey-Kramer test. Groups with different letters are significantly different ($p < 0.05$).

Figure 1

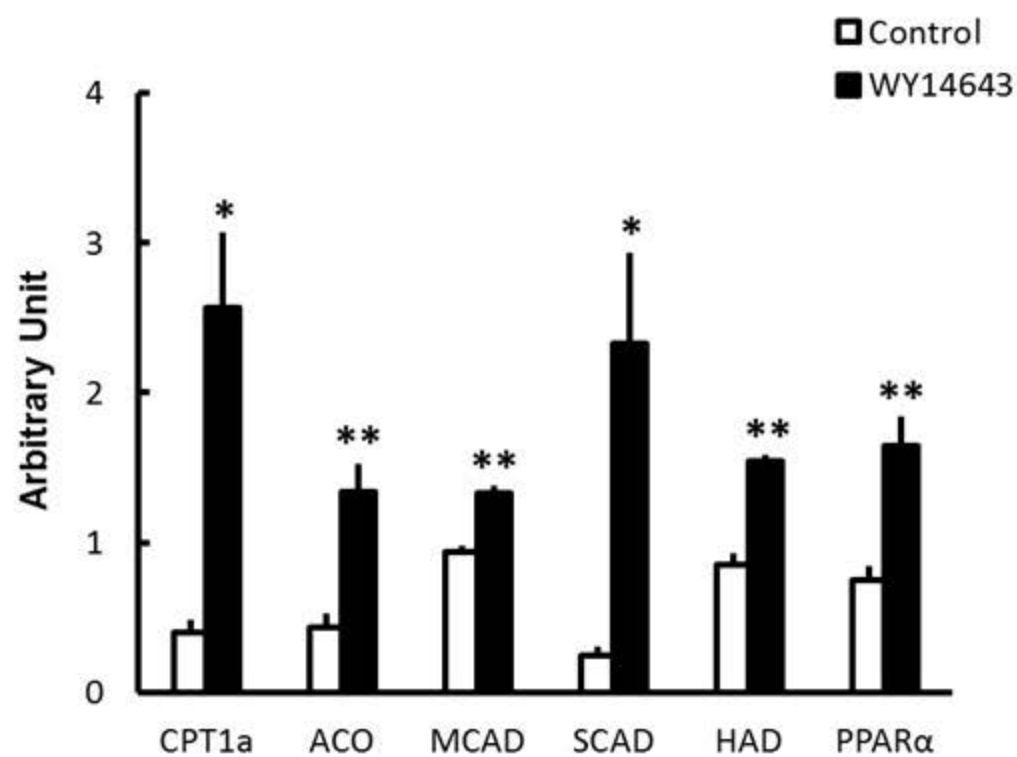


Figure 2

