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Insulin regulates the expression of adiponectin and adiponectin receptors in mature porcine adipocytes

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Abstract

Adiponectin is an adipocyte-derived hormone that can improve insulin-sensitivity. Its functions in regulating glucose utilization and fatty acid metabolism in mammals are mediated by two subtypes of adiponectin receptors (AdipoR1 and AdipoR2). This study was conducted to determine the effect of insulin on the expression of adiponectin and its receptors. The expression of both AdipoR mRNAs was increased in the liver and s. c. adipose tissue of fasted pigs compared with fed pigs. The data also showed that the expression of either AdipoR1 and AdipoR2 mRNA in muscle and visceral adipose tissue was not different between the fasted and fed conditions. We demonstrated that in the presence of 10 nM insulin, addition of 1 uM of insulin or rosiglitazone [a peroxisome proliferator - activated receptor γ (PPARγ) agonist] had no effect on the expression of adiponectin and AdipoR genes in differentiated porcine adipocytes. However, the addition of 1 μM insulin plus 1 μM rosiglitazone significantly increased the AdipoR2 mRNA in well-differentiated porcine adipocytes. Using the phosphatidylinositol 3 - kinase inhibitor (PI3K inhibitor, LY294002), we found that insulin inhibited the expression of AdipoR2 through the PI3K pathway and this inhibition can be blocked by addition of rosiglitazone. When porcine adipocytes were cultured without insulin, supplementation with 10 nM insulin inhibited the expression of AdipoR2 and this inhibition effect can also be blocked by addition of rosiglitazone. Therefore, these data suggest that a PPARγ agonist increases expression of AdipoR2 and that insulin inhibits the expression of AdipoR2 through the PI3K pathway.

Introduction

Adiponectin is an adipocyte-produced protein hormone circulating in the blood (1-4). Administration of adiponectin to mice decreases plasma glucose, free fatty acids, and triglycerides, but increases muscle fatty acid oxidation and induces weight loss (5). The function of adiponectin is carried out through the activation of AMP-activated protein kinase (4). Adiponectin-deficient mice are mildly insulin resistant and glucose intolerant when fed a standard diet (6, 7). Furthermore, decreased circulating adiponectin concentrations are associated with insulin resistance, obesity, and type Ⅱ diabetes (8, 9). Therefore, adiponectin may modify the function of insulin in mammals.

Thiazolidinediones (a class of type Ⅱ diabetes drugs), ligands for peroxisome proliferator-activated receptor γ (PPARγ) increase adiponectin expression and plasma adiponectin concentration in rodents (8, 10). Thiazolidinediones also increase the expression of adiponectin in type Ⅱ diabetes mellitus and obese patients (11, 12). Dual activation of PPARα and γ increases serum adiponectin concentration in adipose tissue of obese diabetic KKAy mice (13). Furthermore, there is a functional PPAR-response element (PPRE) in the promoter region of the adiponectin gene (14). Thus, adiponectin should be regulated by PPARγ and its ligands.

Yamauchi *et al*. (15) first cloned the cDNA encoding adiponectin receptors 1 (AdipoR1) and 2 of human and mouse. These two adiponectin receptors contain 7 transmembrane domains, but they are structurally and functionally distinct from G-protein-coupled receptors (15). Both AdipoR1 and AdipoR2 can mediate the function of adiponectin and the expression of these two receptors is regulated by PPARγ ligands or a combination of ligands for PPARα and PPARγ in obese patients and mice(11-13). In order to clarify the regulation of gene expression of adiponectin receptors in pigs, we have cloned the full length cDNA from porcine AdipoR genes

(16); and in this study, we determined the insulin regulation of AdipoR1 and AdipoR2 expression in adipocytes. We also investigated the interaction of a PPARγ ligand and insulin on the expression of adiponectin and AdipoR genes in porcine adipocytes.

Materials and Methods

Fasting and feeding animals. The animal protocol was approved by the Experimental Animal Care and Use Committee at National Taiwan University. Four male and four female crossbred pigs (*Sus domesticus*; sows were predominantly Landrace-Yorkshire crossbreds mated to a Duroc boar) were weaned at 28 d of age and fed a commercial diet (a corn, soy-based diet containing 18% crude protein and 4% fat) ad libitum and raised to 60 d of age for the experiment. In order to determine the effect of nutritional conditions on the expression of adiponectin and its receptors, fed pigs were euthanized by electrical stunning coupled with exsanguination at 10:00 h after feeding at 8:00 h, whereas the fasted group was killed after a 24 h fast. Liver, longissimus muscle, visceral adipose tissue of the belly and dorsal subcutaneous (s.c.) adipose tissue were dissected and frozen in liquid N_2 and stored at -70℃ for RNA extraction. The average body weight of pigs for both treatments was 20.4 $\pm$ 0.66 kg when euthanized. Total RNA was extracted for detecting AdipoR1, AdipoR2, and β-actin gene expression by Northern analysis

Isolation of porcine stromal vascular (S/V) cells. Porcine adipose tissue samples were digested and S/V cells were isolated and cultured as previously described (17, 18). In brief, adipose tissue from 9 d old crossbred pigs was removed from the dorsal s.c. depot in the neck, shoulder, and back regions. The slices of adipose tissue were digested with collagenase (Sigma C6885; Sigma, St Louis, MO, USA) in sterile Krebs Ringer bicarbonate buffer at 37℃ for 90 min. The S/V cell fraction was isolated by centrifugation at 800 x g for 10 min and the pellet was

93 washed three times by resuspension with DMEM/F12 medium (Sigma D8900)
94 supplemented with $NaHCO_3$, 100 U penicillin/mL, 100 mg streptomycin/mL, 1.5
95 μg/mL amphotericin B and 10% fetal bovine serum. Before the last washing step,
96 the S/V cell fraction was treated with red blood cell lysing buffer (155 mM NH_4Cl,
97 5.7 mM K_2HPO_4, 0.1 mM EDTA at pH 7.3) to remove red blood cells which may
98 reduce adhesion of S/V cells. The washed S/V cells were resuspended in
99 DMEM/F12 containing 10% fetal bovine serum and plated at a concentration of 5 x
100 10^4 cells/cm^2. The S/V cells were then cultured at 37℃ in air containing 5% CO_2 for
101 48 h to let the cells fully attach to the dish.
102 *Cell culture and differentiation of porcine adipocytes*. After 48 h of initial
103 incubation for proliferation (defined as day 0), the medium was removed and replaced
104 by serum-free, hormone-supplemented differentiation medium (DMEM/F12
105 containing $NaHCO_3$, 25 mM glucose, 1 μM bovine insulin, 10 μg transferrin/mL, 2
106 mM L-glutamine, 33 μM biotin, 17 μM pantothenate, 100 nM dexamethasone, 1 nM
107 triiodothyronine, 100 U penicillin/mL, 100 mg streptomycin/mL, 1.5 μg/mL
108 amphotericin B and 1 μM rosiglitazone) for 3 d to induce adipogenesis. The
109 medium was then changed to differentiation medium without rosiglitazone. The
110 medium was replaced every 3 d. After 9 d, up to 90-95% of the attached cells were
111 differentiated to cells with visible lipid droplets. For studying the expression of
112 genes during porcine adipocyte differentiation, porcine S/V cells were induced to
113 differentiation and on the indicated days (0, 3, 6, 9), total RNA was extracted for
114 detecting adiponectin, AdipoR1, AdipoR2, and β-actin mRNA by Northern analysis.
115 The results are the means of 3 independent experiments with S/V cells isolated from 3
116 different pigs.
117 *Effect of rosiglitazone and insulin on AdipoRs in porcine adipocytes.* To study
118 the effect of insulin on the expression of adiponectin and AdipoRs, differentiated

adipocytes were washed with phosphate-buffered saline and then cultured in low glucose DMEM/F12 (DMEM, Sigma D5523 : nutrient mixture F12, Sigma N6760 = 1 : 1, with a final glucose concentration of 7.78 mM) with 10 nM insulin for 6 hrs, and then 1 μM insulin or 1 μM rosiglitazone or insulin + rosiglitazone were added for 2 , 12, or 24 hrs. Total RNA was extracted to detect adiponectin, AdipoR1, AdipoR2, and β-actin mRNA by Northern analysis. The results are the means of 4 independent experiments using cells isolated from 4 different pigs.

The involvement insulin signal pathways on regulation expression of AdipoRs in porcine adipocytes. Porcine S/V cells were differentiated for 9 days and then differentiated adipocytes were cultured in low glucose (7.78 mM) DMEM/F12 with 10 nM insulin for 6 h. After 6 h, 1 μM insulin〔concentration was reported to be effective, (19)〕 + 1 μM rosiglitazone (20) were added to some cells and incubation was continued for 24 h. Also at 6 h, the mitogen-activated protein kinase (MAPK) inhibitor, PD98059 at 25 μM (21) or the phosphatidylinositol 3 - kinase (PI3K) inhibitor, LY294002 at 10 μM (19) were added along with 1 μM insulin + 1 μM rosiglitazone; incubation was continued for 24 h. Total RNA was extracted for determining AdipoR1, AdipoR2, and β-actin gene expression by Northern analysis. The results are the means of 4 independent experiments using cells isolated from 4 different pigs.

Insulin, rosiglitazone, and PI3K inhibitor treatments in porcine adipocytes. Porcine S/V cells were differentiated for 9 days and then differentiated adipocytes were cultured in low glucose (7.78 mM) DMEM/F12 without 10 nM insulin for 6 hrs, then 10 nM insulin, 1 μM insulin, 1 μM rosiglitazone, or 10 μM LY294002 were added to study the effect of low insulin, high insulin concentrations and insulin sensitizer on the expression of AdopoRs. Total RNA was extracted for determining adiponectin, AdipoR2, and β-actin gene expression by Northern analysis. The

145 results are the means of 4 independent experiments using cells isolated from 4
146 different pigs.

147 *Northern analysis*. Total RNA was extracted by the guanidinium - phenol -
148 chloroform extraction method (22). The integrity of RNA was determined by
149 examination of the 18S and 28S ribosomal RNA bands after electrophoresis. The
150 RNA was quantified by spectrophotometry at 260 nm and stored at -70℃. Total
151 RNA (10 μg of each sample) was electrophoresed and transferred to nylon membranes
152 for Northern analysis following the procedure described by Liu *et al* (23). The
153 porcine adiponectin, AdipoR1, AdipoR2 and β-actin probe sequences were previously
154 described (16, 18) and labeled by P^{32}dC with PCR amplification. Hybridization
155 blotting images were quantified using a Typhon 9200 phosphorimage scanner and
156 ImageQuant TL v2005 software (GE). The densitometric value for an individual
157 transcript in a sample lane was normalized to the densitometric value for the β-actin
158 mRNA in the same lane.

159 *Statistical analysis*. Data were presented as mean ± S.E.M. Statistical
160 analysis were using an ANOVA procedure to determine the major effects of insulin,
161 rosiglitazone, and insulin signaling pathway blockers. Duncan's new multiple-range
162 test was used to evaluate differences among means (SAS Inst., Inc., Cary, NC). A
163 significant difference was indicated at $P \leq 0.05$.

164

165 ## *Results and Discussion*

166 *Fasting increased AdipoR genes expression in s.c. adipose tissue and liver*. In
167 porcine s.c. adipose tissue and liver, the AdipoR1 and AdipoR2 mRNAs increased
168 ($P<0.05$) after a 24 h fast, but there was no effect of fasting on the expression of
169 either receptor in the visceral adipose tissue and muscle (Fig. 1A and 1B). The data
170 suggest differential regulation of AdipoR1 and AdipoR2 by nutritional status in

171 various tissues. Previously, we indicated that the expression of AdipoR2, but
172 AdipoR1 and adiponectin was increased in the s.c. adipose tissue after an 8 h fast
173 (16). In rodents, similar to the current study in pigs, both AdipoR1 and AdipoR2
174 gene expression are up-regulated after 48 h fasting in the liver (24). The reason
175 that fasting increased the expression of AdipoR genes in mouse muscle (24), but not
176 in pig muscle is not known. We speculate that the 48 h fast in the mouse is more
177 extreme than the 24 h fast in the pig; this difference may also contribute to the
178 observed species difference. Others have showed that serum adiponectin remained
179 stable concentrations during 72 h of fasting in normal- and over-weight humans (25).
180 Thus, hormonal regulation of the feeding / fasting status on adiponectin function
181 may mainly act through regulating the expression of AdipoRs. In summary, hepatic
182 and s.c. adipose tissue AdipoR genes, but not visceral adipose tissue and muscle
183 AdipoR genes, were responsive to the feeding / fasting status in pigs.

184 *Expression of adiponectin and AdipoR genes during porcine adipocyte*
185 *differentiation*. Expression of both adiponectin and AdipoR2 increased during
186 adipocyte differentiation, whereas expression of AdipoR1 increased during the initial
187 3 d with no further increase during the latter stages of differentiation (Fig. 2). The
188 data suggest that either AdipoR2 is more sensitive to the hormones present in the
189 adipocyte cell culture medium than AdipoR1 or the AdipoR2 is a more important
190 receptor mediating adiponectin function in the differentiated adipocyte. A recent
191 study indicates that adiponectin increases PPARγ2 expression in porcine adipocytes
192 (26), suggesting that adiponectin may involve in regulating the PPARγ2 function
193 during adipogenesis. Furthermore, overexpression of adiponectin not only
194 promotes adipogenesis by prolonging and enhancing the key adipogenic
195 transcription factors, ie., PPARγ, CCAAT/enhancer binding protein α (C/EBPα), and
196 adipocyte determination and differentiation factor 1/sterol-regulatory element

197 binding protein 1c (ADD1/SREBP1c), but also stimulates glucose uptake through
198 increasing glucose transporter 4 gene expression in 3T3-L1 adipocytes (27).
199 Therefore, a greater expression of the adiponectin gene in the latter stages of porcine
200 adipocyte differentiation may function to enhance or maintain adipocyte morphology
201 through the regulation of PPARγ and other adiponectin target genes.

202 Fu *et al*. (27) found that the expression of both AdipoR genes were
203 down-regulated in adiponectin-overexpressing adipocytes, suggesting that
204 adiponectin may act in an autocrine or paracrine fashion to regulate the function of
205 its receptors in adipose tissue. A similar study also found that adiponectin
206 downregulated its own production and the expression of its AdipoR2 receptor in
207 transgenic mice (28). The receptor down-regulation may decrease the adiponectin
208 responses to slow the adipogenic progression; however, perhaps receptor
209 down-regulation only occurs when adiponectin concentration reaches extreme levels,
210 as in the overexpressing cells and mice.

211 *Effects of insulin and rosiglitazone on expression of adiponectin and AdipoRs*.
212 Treatment with a high concentration of insulin for 2 to 24 hr did not change the
213 expression of adiponectin or the AdipoRs (Fig. 3). In contrast, several studies
214 indicate that insulin inhibits the expression of adiponectin and AdipoR genes in
215 adipocytes of humans and rodents (13, 19, 29). We cannot exclude the possibility
216 that the 10 nM insulin concentration used in the pre-incubation medium was great
217 enough to suppress the expression of adiponectin and AdipoR genes so that the
218 higher insulin concentration (1 μM) used to test for insulin effects had no additional
219 effect.

220 The PPARγ agonist, rosiglitazone alone did not increase the expression of
221 adiponectin or the AdipoRs (Fig. 3). However, the combination of a high insulin
222 concentration (1 μM) plus rosiglitazone (1 μM) increased expression of adiponectin

223 and AdipoR2 in porcine adipocytes. In 3T3-L1 adipocytes cultured with DMEM +
224 10% FBS, rosiglitazone does not affect the expression of adiponectin, even though
225 there is a PPAR response element (PPRE) in the adiponectin promoter region (30).
226 Rosiglitazone does increase plasma adiponectin concentration in humans and mice *in*
227 *vivo* (30, 31). The authors suggest that an adequate insulin concentration may be
228 necessary to observe the rosiglitazone-stimulated increase in the expression of the
229 adiponectin gene (30). Recent studies also showed that AdipoR2, but not AdipoR1,
230 was increased by rosiglitazone treatment in mouse primary adipocytes, and HepG2
231 hepatocytes cultured with FBS (13, 20).

232 In order to clarify the relationship between insulin and the PPARγ agonist, we
233 used insulin signal pathway inhibitors to block phosphatidylinositol 3-kinase (PI3K)
234 or mitogen-activated protein kinase (MAPK). Expression of AdipoR2, but not
235 AdipoR1 mRNA was increased in the presence of 1 μM insulin + 1 μM rosiglitazone
236 (Fig. 4), confirming data in Fig. 3B, C. Addition of the MAPK inhibitor, PD98059
237 had no effect on expression of AdipoR1 or AdipR2 mRNA. However, the AdipoR2
238 mRNA was increased when the PI3K signal pathway was inhibited by addition of
239 LY294002, suggesting that insulin may decrease the expression of AdipoR2 through
240 the PI3K pathway. Tsuchida *et al*. (24) recently demonstrated that insulin
241 suppressed the expression of AdipoRs via the PI3K/Foxo1-dependent pathway in
242 rodent hepatocytes and myocytes. Although we did not observe that insulin alone
243 decreased AdipoR2 mRNA expression, the increase in AdipoR2 mRNA when the
244 PI3K pathway was inhibited by addition of LY294002, suggests that insulin has at
245 least a partial suppressive effect via the PI3K pathway. In addition, studies also
246 showed evidence of a direct insulin-induced inhibition of AMPK activity through
247 PI3K/Akt pathway in the heart (32), which is a possible mechanism by which the
248 expression of AdipoRs are inhibited.

In order to exclude the possibility that 10 nM insulin, used in most experiments to maintain cell viability, suppressed adiponectin or AdipoRs gene expression, we designed another experiment to clarify the interaction of insulin and a PPARγ agonist on adiponectin and AdipoRs gene expression. After treating with insulin free medium for 6 h, addition of 10 nM insulin reduced both adiponectin and AdipoR2 (Fig. 5A) mRNAs, indicating a minute amount of insulin suppresses the expression of adiponectin and AdipoR2 genes. In pigs, the normal concentration of insulin is maintained at 106 ± 12.5 pM and increased to 850 ± 137.5 pM 30 mins post-prandial (33). Hence, supplementation with 10 nM or 1 μM insulin in porcine adipocyte culture is much higher than concentrations *in vivo*. In mammals, the concentration of feeding / fasting insulin could not affect the abundance of adiponectin (16, 25). Insulin may regulate the ratio of high molecular weight adiponectin (active form) or the expression of AdipoRs to modify the adiponectin function. This high insulin concentration is similar to the conditions in type II diabetes or other hyperinsulinemia-induced metabolism syndromes.

Adiponectin overexpressed mice have been shown to be able to improve insulin resistance (34), whereas adiponectin deficient mice were shown to be mildly insulin resistant and glucose intolerant when fed a standard diet, and severe insulin resistant triggered by high fat diet (6, 7). Mice lacking adiponectin show decreased hepatic insulin sensitivity and reduced responsiveness to PPARγ agonists (35). In addition, several SNPs on adiponectin and AdipoR genes and other environmental factors such as obesity appears to be hypoadiponectinemic and associated with type II diabetes (36). Hence, regulation of adiponectin function may have therapeutic potential for treating type II diabetes mellitus and obesity.

Because insulin decreased the expression of adiponectin and AdipoR2, we cultured well-differentiated adipocytes in medium without insulin for 6 h. Addition

of the PPARγ agonist, rosiglitazone to these cells for 24 h significantly increased adiponectin mRNA expression, but not AdipoR2 mRNA (Fig. 5B). It should be noted that in the presence of 10 nM insulin, rosiglitazone had no effect on the expression of AdipoR2 (Fig. 3C). This result confirmed data from our first experiment and was similar to that reported in differentiated human myotubes (37), indicating that the PPARγ agonist may improve insulin sensitivity by increasing the serum adiponectin level, but had no effect on its receptors. Recently research also showed that administrating TZD for type 2 diabetes mellitus to improve insulin sensitivity and increase adiponectin levels did not affect the expression of AdipoR1 and AdipoR2 in muscle and adipose tissue (38). Although rosiglitazone may require the presence of insulin to increase the expression of adiponectin and AdipoR2 genes (Fig. 3), the effect of rosiglitazone might be in reversing the inhibitory effect of insulin. It was interesting that LY294002 promoted the expression of both adiponectin and AdipoR2 mRNAs more strongly than insulin alone, rosiglitazone alone or rosiglitazone plus insulin (Fig. 5). Hence, these findings suggest that insulin suppresses the expression of adiponectin and AdipoR2 mRNAs through PI3K pathway. When PI3K pathway blocked, both rosiglitazone and insulin could increase the expression of adiponectin and AdipoR2 mRNAs through a yet to be determined mechanism.

In conclusion, the expression of the adiponectin and AdipoR2 mRNAs is more strongly inhibited by insulin and stimulated by the PPARγ agonist, rosiglitazone than the AdipoR1 mRNA. The PPARγ agonist effects depend on the presence of insulin, perhaps act in a permissive manner. The insulin effect mimicking the type II diabetes was mediated through the PI3K pathway as indicated by use of the PI3K inhibitor, LY294002. Therefore, understanding of interaction between insulin, PPARγ and expression of adiponectin and its receptors will provide mechanisms that

may lead to control of adipose fat deposition and to the treatment of type II diabetes.

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Fig 1. The effect of fasting on AdipoR1 and AdipoR2 gene expression in pigs. Samples of liver, longissimus muscle, visceral adipose tissue (VAT) and subcutaneous adipose tissue (AT) were taken 2 hrs after feeding (Fed) or 24 hrs after feeding (Fasted). The total RNA from each tissue of each pig (10 μg) was electrophoresed and transferred to a nylon membrane. The membranes were hybridized with cDNA probes for adiponectin receptor 1 (AdipoR1), adiponectin receptor 2 (AdipoR2), and β-actin. The mRNA abundance was determined by phosphorimage technology and the densitometeric value for each gene was normalized to β-actin. The data represent the means of 4 crossbred pigs (2 males and 2 females) per treatment. Data were analyzed with ANOVA and Duncan's new multiple range test was used to evaluate the differences from the fed AT value (set to 100). Each bar represents the mean ± S.E.M.; *, $P \leq 0.05$; **, $P \leq 0.01$.

Fig 2. Differentiation-dependent adiponectin, and AdipoR genes expression in porcine adipocytes. Porcine S/V cells were differentiated and on the indicated days (0, 3, 6, 9), total RNA was extracted for detecting adiponectin, AdipoR1, AdipoR2, and β-actin gene expression by Northern analysis. The results are the means of 3 independent experiments with S/V cells isolated from 3 different pigs. The average of AdipoR1 day 0 data was set to 100 and other data were expressed as relative abundance to this value. Each point represents the mean ± S.E.M. and mRNA values were normalized to β-actin. Means without a common letter differ significantly, $P \leq 0.05$.

Fig 3. The effect of rosiglitazone and insulin on adiponectin, and AdipoR genes expression in porcine adipocytes. Porcine S/V cells were differentiated for 9 days, as indicated in Fig 2. The 9 days differentiated adipocytes were then cultured in low

476 glucose (7.78 mM) DMEM/F12 with 10 nM insulin for 6 hrs, and then 1 μM insulin
477 or 1 μM rosiglitazone (Rosi) or insulin + Rosi were added for 2 , 12or 24 hrs. Total
478 RNA was extracted to detect adiponectin (panel A), AdipoR1 (panel B), AdipoR2
479 (panel C), and β-actin gene expression by Northern analysis, as indicated in Fig 2.
480 The results are the means of 4 independent experiments using cells isolated from 4
481 different pigs. The average of the 2 h control medium data was set to 100 and other
482 data were expressed on relative abundance to the 2 h data. Each point represents the
483 mean ± S.E.M.; *, $P \leq 0.05$.
484
485 Fig 4. The effect of an insulin signal pathway inhibitor on rosiglitazone-mediated
486 induction of expression of AdipoR genes in porcine adipocytes. Porcine S/V cells
487 were differentiated for 9 days and then differentiated adipocytes were cultured in low
488 glucose (7.78 mM) DMEM/F12 with 10 nM insulin for 6 h. Control cells were
489 incubated in this medium for an additional 24 h. After 6 h, 1 μM insulin (I) plus 1
490 μM rosiglitazone (R) were added to some cells and incubation was continued for 24 h.
491 Also at 6 h, the MAPK inhibitor, PD98059 at 25 μM (PD) or the PI3K inhibitor,
492 LY294002 at 10 μM (LY) were added along with I + R; incubation was continued for
493 24 h. Total RNA was extracted for detecting AdipoR1, AdipoR2, and β-actin gene
494 expression by Northern analysis as indicated in Fig 2. The results are the means of 4
495 independent experiments using cells isolated from 4 different pigs and the average of
496 the 24 h control medium data was set to 100 with other data expressed as relative
497 abundance to the control value. Each bar represents the mean ± S.E.M. and mRNA
498 values were normalized to β-actin. Means without a common letter differ
499 significantly, $P \leq 0.05$.
500
501 Fig 5. The effects of insulin and rosiglitazone on the expression of adiponectin and

AdipoR genes in porcine adipocytes. Porcine S/V cells were differentiated for 9 days and then differentiated adipocytes were cultured in low glucose (7.78 mM) DMEM/F12 without 10 nM insulin for 6 hrs, then 10 nM insulin (10 nM I), 1 μM insulin (I), 1 μM rosiglitazone (Rosi), or 10 μM LY294002 (LY) were added to separate plates for 24 hrs. Total RNA was extracted for detecting adiponectin, AdipoR2, and β-actin gene expression by Northern analysis as indicated in Fig 2. The results are the means of 4 independent experiments using cells isolated from 4 different pigs and the average of 24 hrs control medium data was set to 100 with other data expressed as relative abundance to the control value. Each bar represents the mean ± S.E.M. and mRNA values were normalized to β-actin. Means without a common letter differ significantly, $P \leq 0.05$.

513 Table 1. List of the Northern analysis probes

Genes[1]	Primers[2]	Source of primers	Size, bp
Adiponectin (AY589691)	S 5‘-GCTCAGGATGCTGTTGTTGG-3‘ A 5‘-TGGTGGAGGCTCTGAGTTGG-3‘	Pig	768
AdipoR1 (AY578142)	S 5‘-AGGTACCAGCCAGATGTCTT-3‘ A 5‘-CTCTTCCTCTCACTTCAGCA-3‘	Human	1220
AdipoR2 (AY606803)	S 5‘-AAAGGCTTGGGTATCCCATG-3‘ A 5‘-CTCCTCTGGTACTGGCATCA-3‘	Human	1391
β-actin (AF054837)	S 5'-GTGGGCCGCTCTAGGCACCA-3' A 5'-CGGTTGGCCTTAGGGTTCAGGGGGG-3'	Mouse	245

514

515 [1] GenBank accession number is indicated parenthetically.

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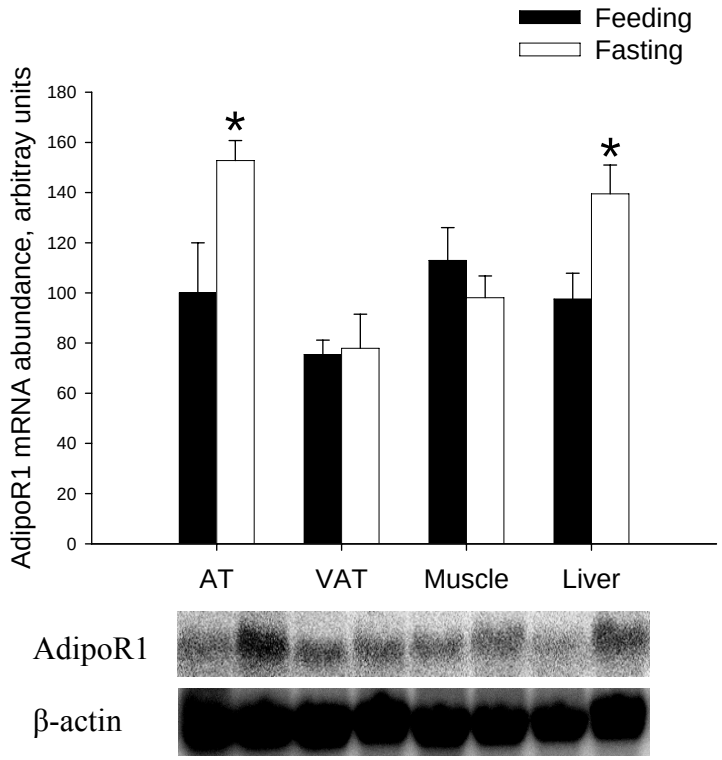
517 [2] S: sense; A: antisense.

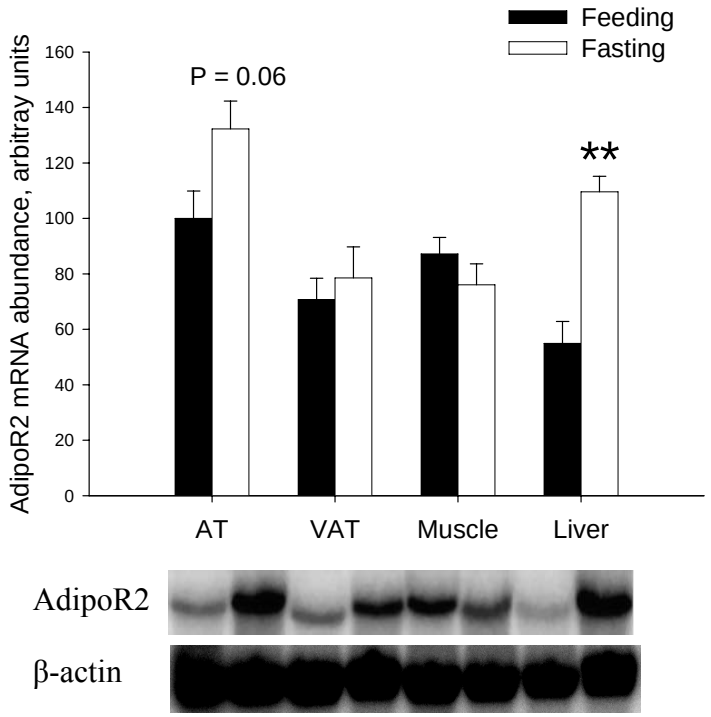
518

519

A


Feeding
Fasting
AdipoR1 mRNA abundance, arbitray units
180
160
140
120
100
80
60
40
20
0
*
*
AT
VAT
Muscle
Liver
AdipoR1
β-actin

B


Feeding
Fasting
AdipoR2 mRNA abundance, arbitray units
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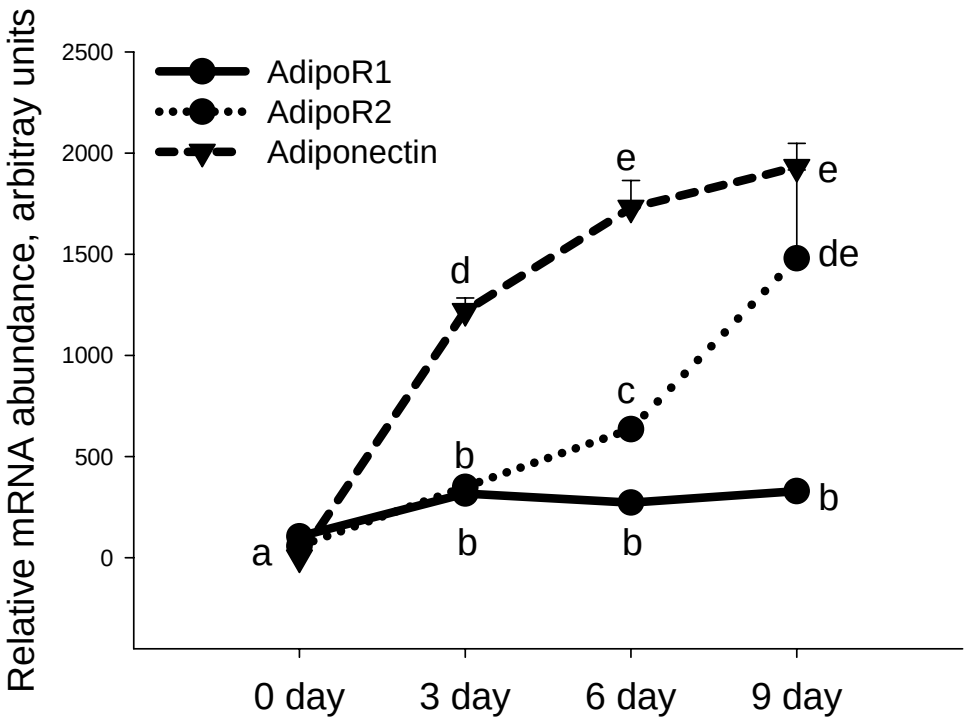
140
120
100
80
60
40
20
0
P = 0.06
**
AT
VAT
Muscle
Liver
AdipoR2
β-actin

Fig 1.


Relative mRNA abundance, arbitray units
2500
2000
1500
1000
500
0
AdipoR1
AdipoR2
Adiponectin
a
b
b
b
c
d
e
e
de
0 day
3 day
6 day
9 day

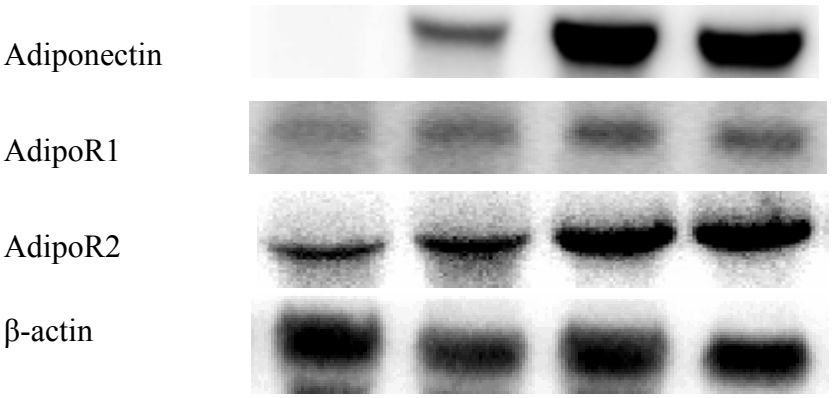
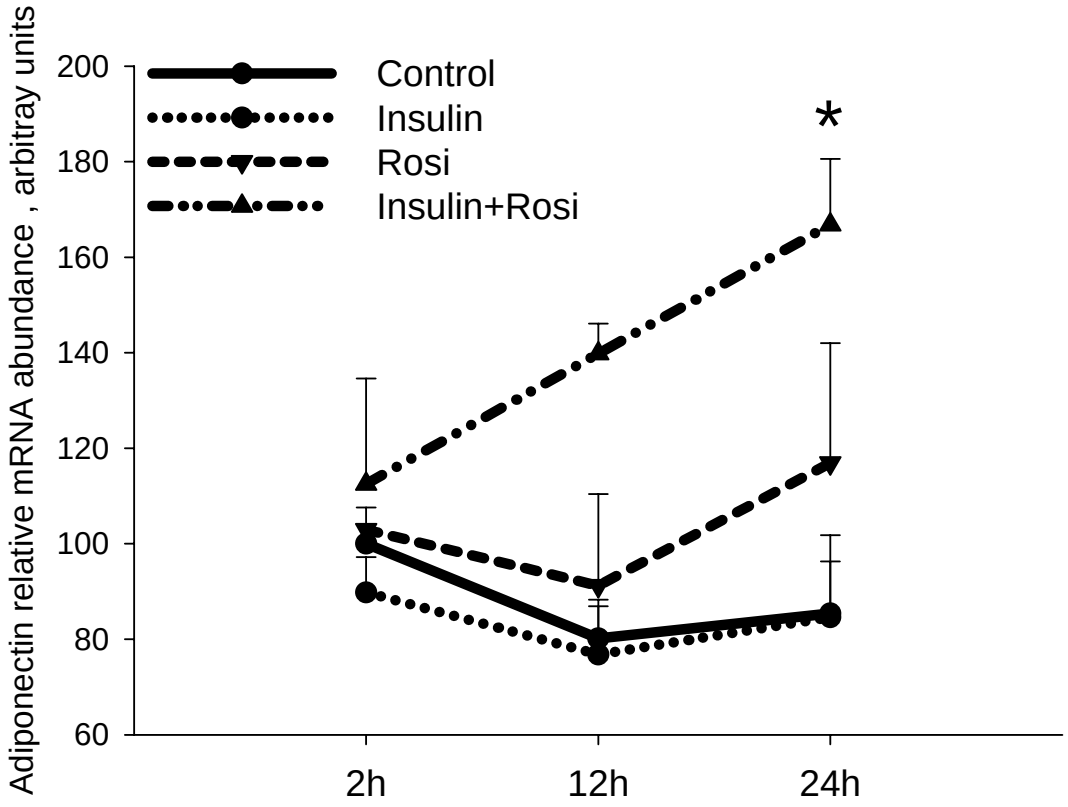

Adiponectin
AdipoR1
AdipoR2
β-actin

Fig 2.

558 **A**



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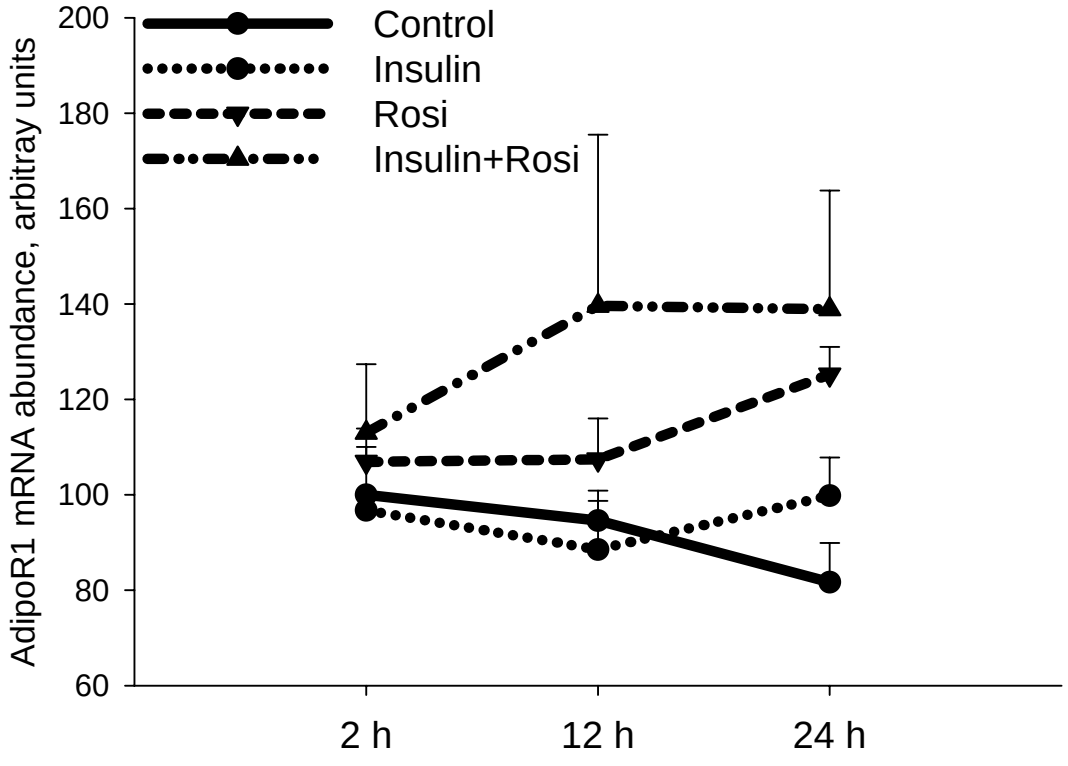
561 **B**



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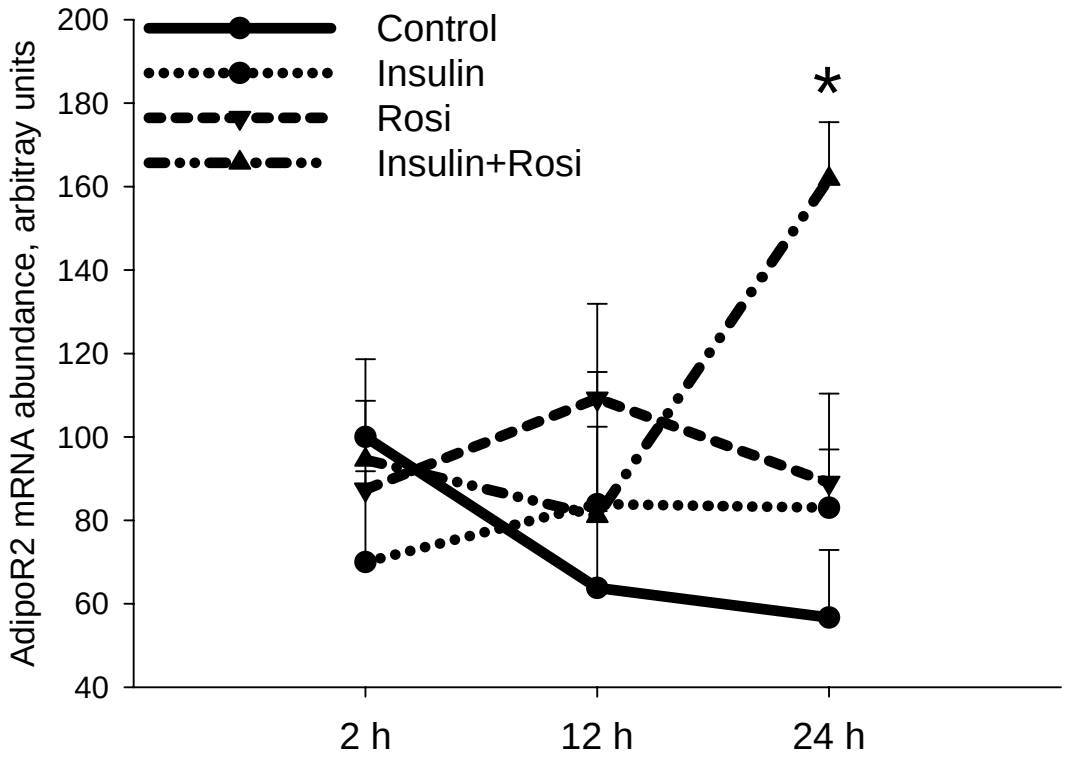
563 **C**



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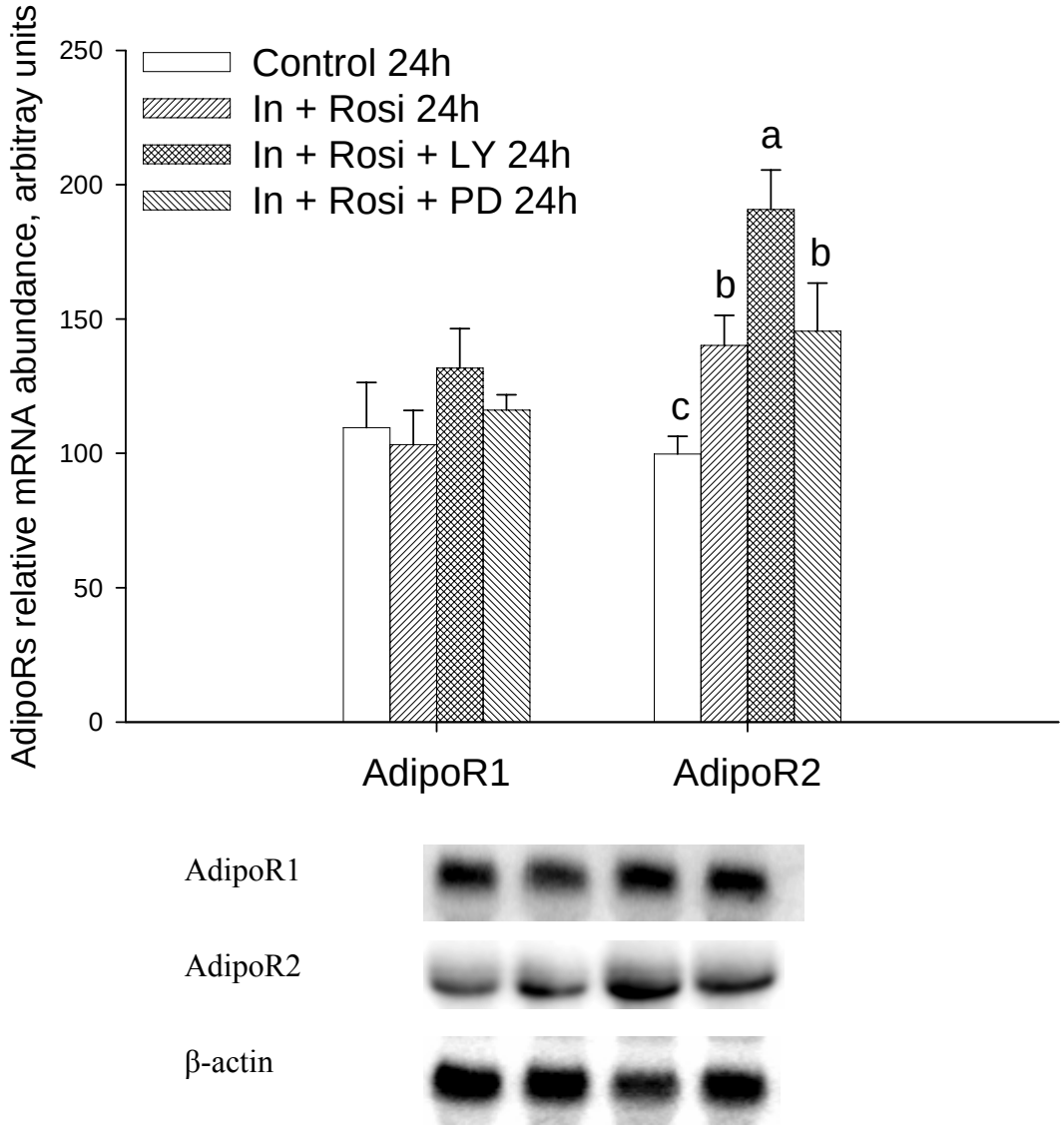
565 Fig 3.



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571 Fig 4.

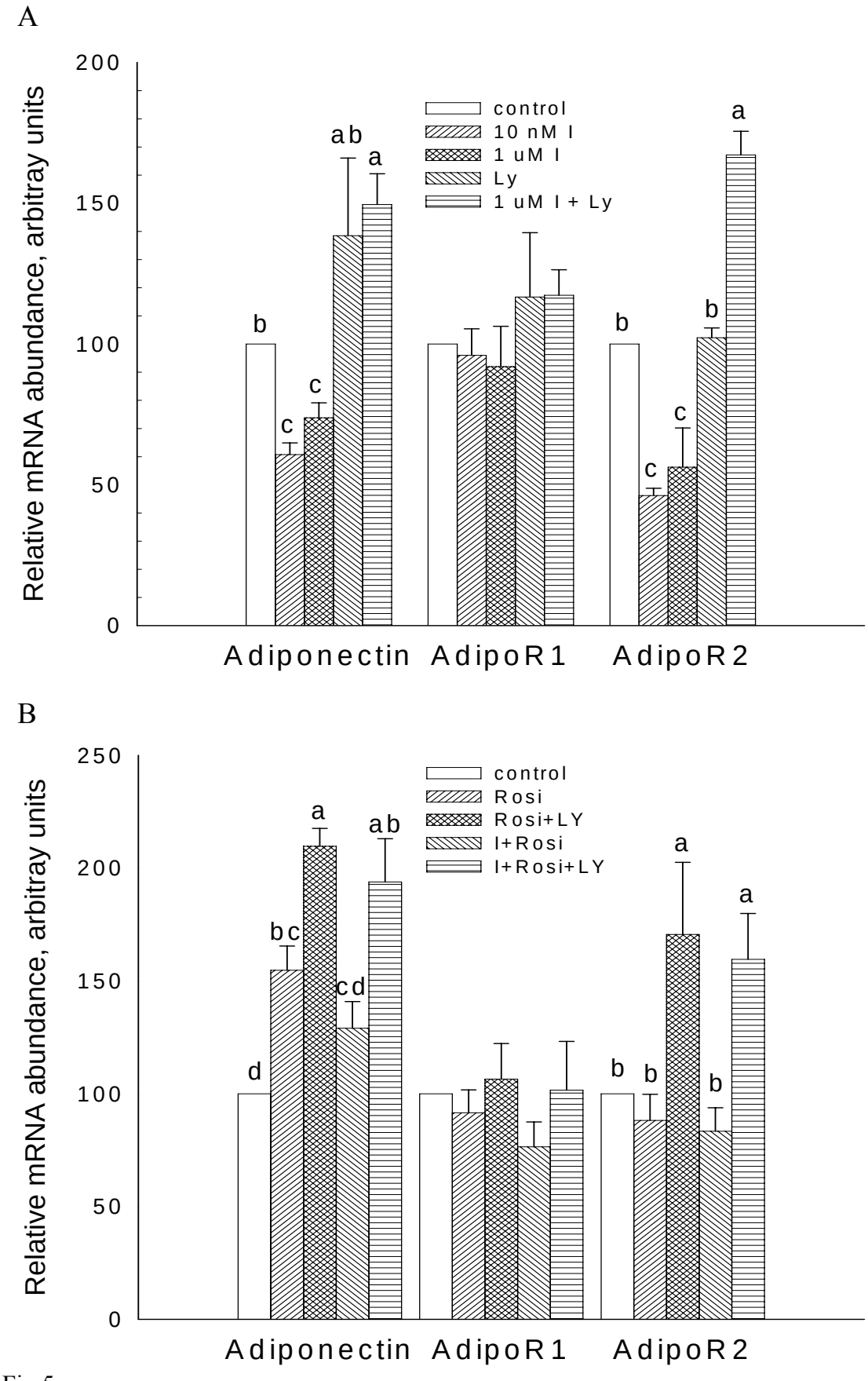
A
Relative mRNA abundance, arbitray units
200
150
100
50
0
control
10 nM I
1 uM I
Ly
1 uM I + Ly
b
c
c
ab
a
b
c
c
b
a
Adiponectin
AdipoR1
AdipoR2
B
Relative mRNA abundance, arbitray units
250
200
150
100
50
0
control
Rosi
Rosi+LY
I+Rosi
I+Rosi+LY
d
bc
a
cd
ab
b
b
a
b
a
Adiponectin
AdipoR1
AdipoR2

Fig 5.

行政院國家科學委員會補助國內專家學者出席國際學術會議報告

96 年 5 月 14 日

附件三

報告人姓名	丁詩同	服務機構及職稱	國立台灣大學動物科學技術學系教授
時間 會議 地點	95 年 4 月 26 日至 5 月 3 日 美國華盛頓首府	本會核定 補助文號	94-2313-B-002-024
會議名稱	(中文)實驗生物學聯合會和美國農部區域討論會 (英文)Experimental Biology 2007 and USDA NCC0097		
發表論文題目	(中文)豬 PPAR d 在脂肪細胞分化的功能 (英文) The functionality of porcine peroxisomal proliferator activated receptor delta in adipocyte differentiation		

報告內容應包括下列各項：

一、　參加會議經過

April 26 Flew to Washington DC, USA. Stay in Red Roof Inn, 500 H Street NW, Washington DC 20001.
April 27 Attended the meeting for USDA regional meeting NCC0097, Presented a talk on two topics: The function of PPARg and PPARd in pigs and The expression of genes in porcine adipose tissue under the treatment of porcine serum amyloid protein A. The fee for the meeting is 70 US dollars. Went to the dinner function with the scientists with common interests in adipocyte biology. The dinner cost for 67 US dollars.

April 28. Attended the Keynote speech by Two scientist both named Tony on the discovery of tyrosine kinases and its function in regulating physiological process and gene expression in ASBMB Biochemistry session. Attended the poster section on lipid metabolism. Attended the evening session on enzyme expression regulation.

April 29. Attended the Keynote speech on Phosphoinisitide molecules and the genes involved in making the enzymes. What we learn from yeast model. Also attend Lipid metabolism. Went to Symposium on animal model for human nutrition 1 delivered by DH Baker and 2 delivered by Spurlock on adipocyte models. Also went to a biochemistry teaching for cultivating future strong biochemist. The speakers were too good. But picked up some points, including bring research into classroom, set the expectation high, recognize the students, enthusiasm in research is contagous, and interactive teaching to enhance student learning. We also posted our poster. The full length of the report is attached at the end of the report.

April 30. Attended the ASBMB Merck Award for the talk on PEPC Kinase by R.W. Hanson. I also attended a section on role of nuclear receptors in metabolic syndrome in the morning. Went to poster section for genetics and metabolic approaches to obesity and proteionics: proteomics and bioinformatics. Went to Symposium on lipid metabolism and transport and also lipid signaling track.

May 1. I attended the Nutrition section and mostly the poster and two keynote speeches. Two areas are very important, one was on companion animal nutrition and the other was on aging. There were lots of research on the feed limitation on aging and well-being of animals. Two specific experiments on monkey long term restriction on feeding improve the health condition and longevity.

May 2. Attended the FASEB Excellence in Science Award before we took off to Columbus, Ohio for an international exchange program discussion.

二、 與會心得

This year I started to get the feeling of how well we have done researchwise. We have demonstrated a very specific pathway of regulating nutrition metabolism and such a finding is world-class. We need to collect the most recent progress of the research in order to know where we are and what to do to compete with international scientists. This meeting always has a lot of activities going on. Lots of science to learn, but too little time available. I have also got to interact with lots of scientist in my field of research. That help me develop the sense of where we are and our competitiveness.

三、 考察參觀活動(無是項活動者省略)

四、 建議

The areas of nutritional science research are evolving very fast during the past few years. This Meeting collects a broad spectrum of research progress which is important for the researchers in Taiwan. I would suggest that we should encourage researchers to go and joint this meeting to get updated research progress report and to improve our research quality.

五、 攜回資料名稱及內容

Experimental Biology 2007, Conference information and scientific program in a CD format. All the station reports from the USDA regional meeting NCR-97.

六、 其他

The full article of our presentation.

Ectopic expression of porcine peroxisome-proliferator-activated receptor delta regulates adipogenesis in myoblasts

Y. H.Yu[1] and S. T. Ding[1]
Department of Animal Science and Technology, National Taiwan University, 50, Lane 155, Kee-Long Rd. Sec. 3, Taipei 106, Taiwan [1]

ABSTRACT

It is well known that peroxisome-proliferator-activated receptor γ (PPAR γ) plays a critical role in regulating adipogenesis. In rodents, PPAR δ is expressed before PPAR γ during adipocyte differentiation. Thus, the interaction between PPAR δ and PPAR γ during adipogenesis needs to be elucidated. The current experiment was designed to study the interaction of porcine PPAR δ and PPAR γ in mouse myoblast cells. Inhibition of myogenesis was observed in myoblasts expressing porcine PPAR δ, similar to myoblast expressing PPAR γ. Treatment of myoblasts expressing PPAR δ with ligands for both PPAR δ and γ enhanced lipogenesis to a greater extent than treatment with a PPAR γ ligand alone. The ability to transdifferentiate myoblasts into adipocytes was decreased in myoblasts co-expressing PPAR δ with either wild-type or mutated PPAR γ (serine 112 was mutated to alanine) compared to myoblasts expressing either type of PPAR δ alone. Adipose transdifferentiation in myoblasts co-expressing PPAR δ and mutated PPAR γ was greater than in myoblasts co-expressing PPAR δ and wild-type PPAR γ. Our results suggest that PPAR δ has two different roles in regulating adipogenesis, ie., suppression of myogenesis to enhance transdifferentiation of myoblasts into adipocytes and interaction with PPAR γ to modify adipogenesis. Therefore, PPAR δ may have a significant role in adipogenesis.

Key Words: Adipocyte differentiation, Peroxisome proliferator-activated receptor δ, Peroxisome proliferator-activated receptor γ.

表 Y04

INTRODUCTION

In rodent, peroxisome-proliferator-activated receptor δ (PPAR δ) is wildly expressed in several tissues, including adipose tissue, intestine, skeletal muscle, lung and heart. The expression of PPAR δ in proliferating preadipocytes is undetectable and increases gradually

INTRODUCTION

In rodent, peroxisome-proliferator-activated receptor δ (PPAR δ) is wildly expressed in several tissues, including adipose tissue, intestine, skeletal muscle, lung and heart. The expression of PPAR δ in proliferating preadipocytes is undetectable and increases gradually during adipocyte differentiation (Amri *et al.,* 1995). Preadipocyte overexpressing PPAR δ with long chain fatty acids promotes adipogenesis (Bastie *et al.,* 2000). Ectopic expression of PPAR δ in fibroblasts with long chain fatty acids alone do not induce adipogenesis but stimulation in the presence of PPAR γ ligand (Bastie *et al.,* 1999). Therefore, PPAR δ seems to have a facilitating role in adipogenesis.

The information of porcine PPAR δ is still poorly understood, especially in functional study. In previous studies, we have demonstrated that ectopic expression of porcine PPAR γ induces adipogenesis in myoblasts (Yu *et al.,* 2006). The expression of PPAR δ is earlier than PPAR γ during adipocyte differentiation in rodent adipocytes (Amri *et al.,* 1995). We hypothesize that a relationship between PPAR δ and PPAR γ in regulating adipocyte differentiation. In this study, we created C2C12 myoblasts expressing porcine PPAR δ, or co-expressing PPAR δ with either wild-type or mutated PPAR γ (serine 112 was mutated to alanine). Transfected myoblasts with porcine PPAR δ stimulated adipogenesis after addition of both PPAR δ and PPAR γ ligands, whereas a decreased lipid accumulation was observed in myoblasts co-expressing PPARs compared with expressing PPAR γ alone.

MATERIALS AND METHODS

Stably transformed cells with PPAR δ or PPAR γ and induction of myoblast transdifferentiation

The porcine PPAR δ cDNA was cloned from porcine adipose tissue. The PCR products were cloned into a mammalian expression vector and transfected into C2C12 myoblasts by lipofection. To establish expression of both porcine PPAR δ and PPAR γ cell models, C2C12 myoblasts containing either wild-type PPAR γ or mutated PPAR γ were also transfected with porcine PPAR δ. Myoblasts stably expressing PPAR δ were established by puromycin selection. After drug selection, the cells were cultured without selection medium and allowed to propagate to 80% confluence in DMEM with 10% FBS. Confluent cells were then cultured in adipogenic differentiation medium [DMEM containing 10% fetal bovine serum, 1μM dexamethasone, and 5μg/mL insulin] and with or without 1 μM rosiglitazone, a PPAR γ ligand and 1 μM L165041, a PPAR δ ligand. After 10 days of culture, total RNA was purified to determine gene expression.

Northern blot and statistical analysis

The RNA was separated by electrophoresis and blotted to nylon membranes. The membrane was prehybridized at 42 ℃ and then hybridized with isotope labeled complementary DNA probes. Hybridization results were quantified by phosphor-image analysis. The densitometric value for an individual transcript in a sample lane was normalized to the densitometric value for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in the same lane. The treatment effects were analyzed using an ANOVA procedure to determine the main effects of the form of PPAR δ and PPAR γ in presence or absence of its ligands. Duncan's new multiple range test was

used to evaluate differences among means (SAS Inst. Inc., Cary, NC). A significant difference indicates that P value is not greater than 0.05.

RESULTS AND DISCUSSION

The presence or absence of rosiglitazone and L165041 in adipogenic medium had no effect on myogenesis (Figure 1B, C and D). The myotube formation was inhibited when myoblast expressing PPAR δ compared with transfection of empty vector cells (Figure 1A vs. E). Similar results were observed in our previous studies, myoblasts containing PPAR γ had an ability to interfere in myocyte differentiation (Yu *et al.,* 2006). After exposure of rosiglitazone to the adipogenic differentiation medium for 10 days, lipid-droplets were visualized in myoblasts expressing PPAR δ but absence in addition L165041 in adipogenic differentiation medium (Figure 1). It was well known that ligands for PPARs can activate more than one receptor isoform, hence adipocyte differentiation was increased in medium containing rosiglitazone even if absence of L165041 (Figure 1F). The maximum of lipid accumulation was observed in addition of both PPAR ligands (Figure 1H). This result suggests indirectly that PPAR γ with its ligand has crucial potential in modulating adipocyte differentiation. In loss of function study, it has been demonstrated that lipid accumulation and adipogenic marker genes are decreased in PPAR δ -null adipocytes (Matsusue *et al.,* 2004). In our results, myoblats expressing PPAR δ with PPAR γ and PPAR δ ligands enhanced adipogenesis. It implies that PPAR δ appeared to accelerate adipogenesis. The downstream gene of PPAR γ, adipocyte fatty acid binding protein (aP2) mRNA was highly expressed in myoblasts containing PPAR δ in the presence of rosiglitazone. However, addition of PPAR δ and PPAR γ ligands in adipogenic medium had a greater stimulation of aP2 expression compared with presence of single PPAR ligand. For lipoprotein lipase (LPL) mRNA, it was also increased in the same condition. It has been known that expression of aP2 and LPL are regulated by PPAR γ. Deducing from our results, we hypothesize that high aP2 and LPL transcripts were attributed to PPAR γ function and ectopic PPAR δ modulated PPAR γ expression by binding its peroxisome proliferator response element. A late myogenic marker gene, myogenin was decreased in cells transfected with PPAR δ and both of ligands had no effect on myogenesis (Figure 3). Similar results were observed in another myogenic marker gene, myogenic regulatory factor 4 (MRF4). The suppression of myogenic marker genes in myoblasts expressing PPAR δ was consistent with ectopic expression of PPAR γ in myocytes. These results demonstrated that adipogenesis related transcription factors have the capability of impairing myogenesis. Furthermore, mRNA for aP2 and LPL were expressed at a low level in myoblasts containing either wild-type PPAR γ or mutated PPAR γ and PPAR δ compared with expressing either wild-type PPAR γ or mutated PPAR γ alone (Figure 4). This phenomenon was also found in preadipocyte expressing PPAR δ and PPAR γ. The over-expression of PPAR δ indeed can suppress PPAR γ-mediated adipogenesis (Shi *et al.,* 2002). However, reduction of myogenic genes expression was enhanced in C2C12 myoblasts containing both PPARs (Figure 5). Thus, PPAR δ and PPAR appeared to have a synergic effect in the inhibition of myogenesis.

CONCULSION

In the current study, we demonstrated that PPAR δ has the ability to promote transdifferentiation of myoblasts into adipocytes and interact with PPAR γ to modify adipogenesis. Therefore, PPAR δ may have a significant role in adipogenesis.

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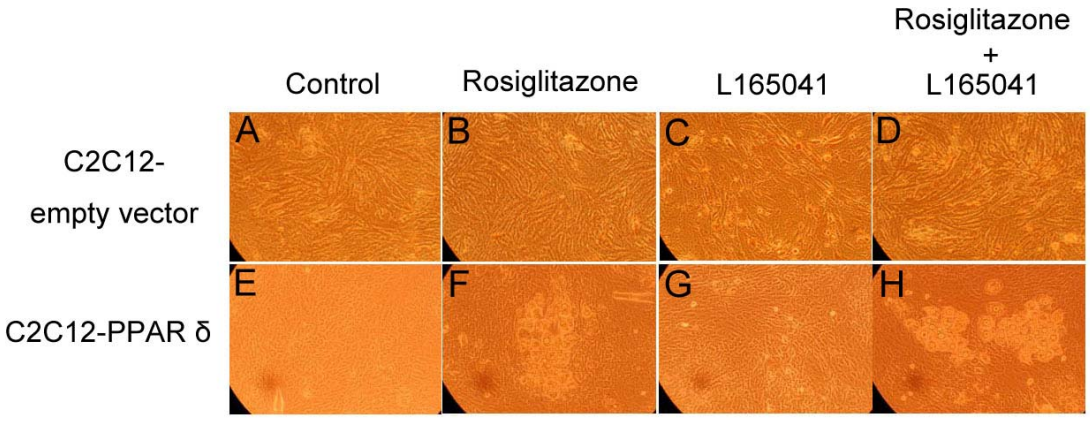

Figure 1. Ligand-induced morphological alterations and accumulation of lipid droplets. Microscographs of C2C12 myocyte with empty vector (A-D), C2C12 expressing porcine peroxisome proliferator-activated receptor δ (PPAR δ; E-H) are shown. Cells were maintained in adipogenic medium (Dulbecco's modified Eagle medium/dexamethasone/insulin/10% fetal bovine serum) ± 1 μM rosiglitazone (B and F), 1 μM L165041 (C and G) and both of ligands (D and H) to

d 10 postconfluence. Magnification was 60.

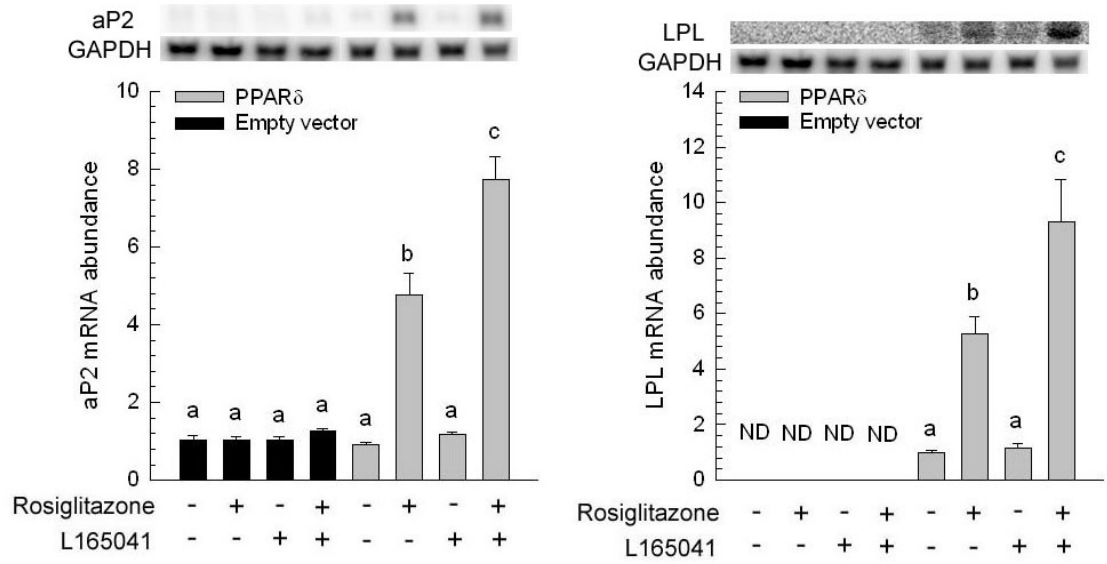

Figure 2. Expression of adipogenic marker genes. After confluence, C2C12 myocytes with empty vector (Empty vector) and C2C12 expressing porcine peroxisome proliferator-activated receptor δ (PPAR δ) were cultured for 10 d. The expression of adipocyte-specific genes [adipocyte fatty acid-binding protein (aP2) and lipoprotein lipase (LPL)] was determined and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The bars indicate the means ± SE for cells from 3 independent replicates (n = 3). ND = not detected. [a–c] Means without a common letter differ, $P < 0.05$.

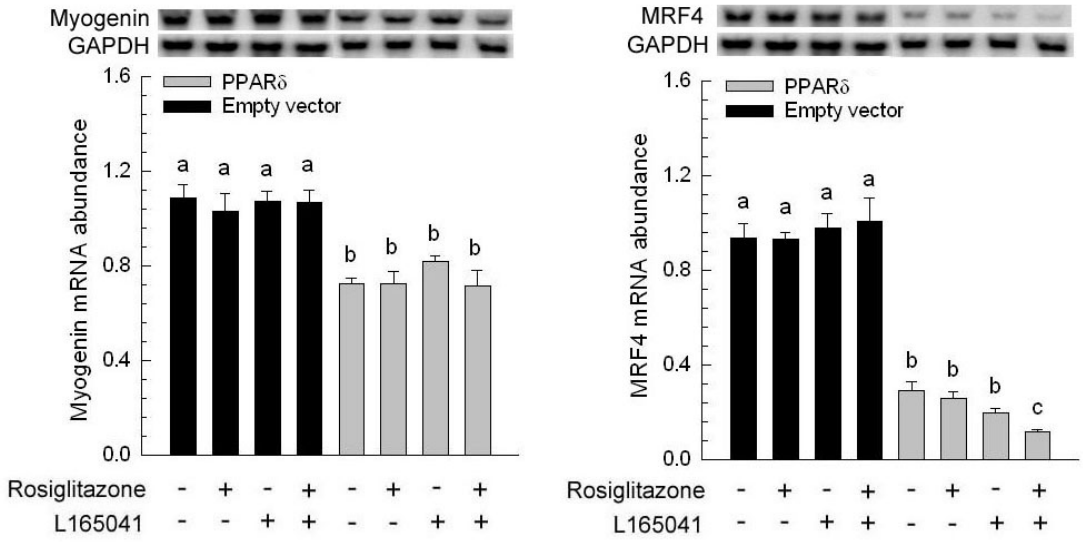

Figure 3. Expression of myogenic marker genes. After confluence, C2C12 myocyte with empty vector (Empty vector) and C2C12 expressing porcine peroxisome proliferator-activated receptor δ (PPAR δ) were cultured for 10 d. The expression of myogenic genes [Myogenin and myogenic regulatory factor-4 (MRF4)] was determined and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The bars indicate the means ± SE for cells from 3 independent replicates

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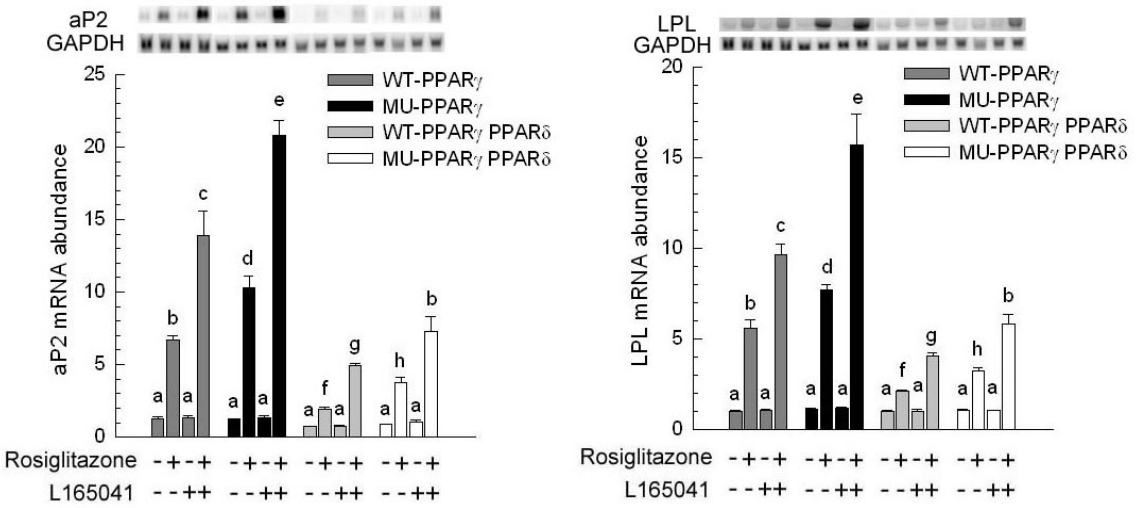

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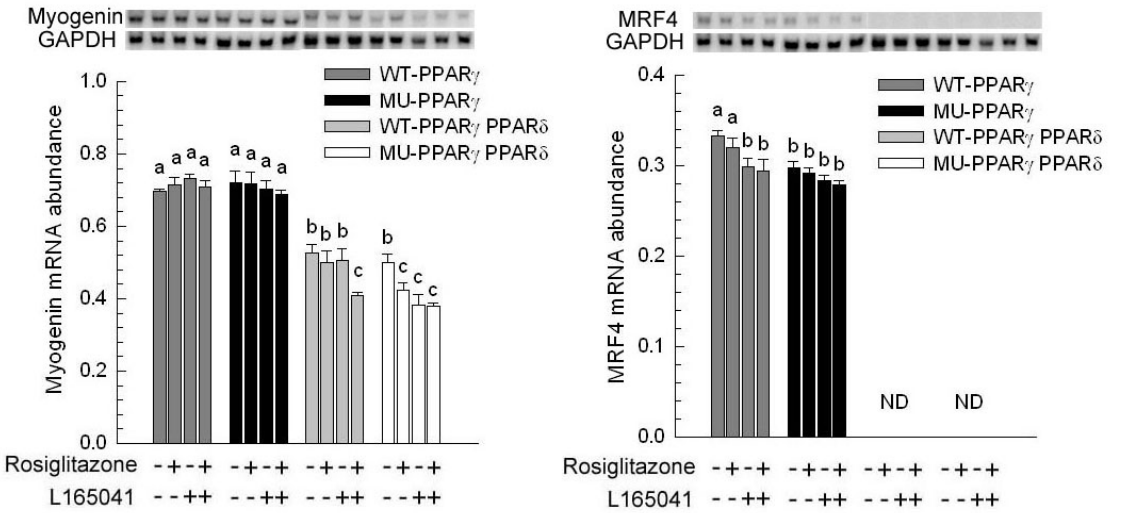

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表 Y04

行政院國家科學委員會補助國內專家學者出席國際學術會議報告

96 年 5 月 14 日

附件三

報告人姓名	丁詩同	服務機構及職稱	國立台灣大學動物科學技術學系教授
時間 會議 地點	95 年 4 月 26 日至 5 月 3 日 美國華盛頓首府	本會核定 補助文號	94-2313-B-002-024
會議名稱	(中文)實驗生物學聯合會和美國農部區域討論會 (英文)Experimental Biology 2007 and USDA NCC0097		
發表論文題目	(中文)豬 PPAR d 在脂肪細胞分化的功能 (英文) The functionality of porcine peroxisomal proliferator activated receptor delta in adipocyte differentiation		

報告內容應包括下列各項：

一、　參加會議經過

April 26 Flew to Washington DC, USA. Stay in Red Roof Inn, 500 H Street NW, Washington DC 20001.
April 27 Attended the meeting for USDA regional meeting NCC0097, Presented a talk on two topics: The function of PPARg and PPARd in pigs and The expression of genes in porcine adipose tissue under the treatment of porcine serum amyloid protein A. The fee for the meeting is 70 US dollars. Went to the dinner function with the scientists with common interests in adipocyte biology. The dinner cost for 67 US dollars.

April 28. Attended the Keynote speech by Two scientist both named Tony on the discovery of tyrosine kinases and its function in regulating physiological process and gene expression in ASBMB Biochemistry session. Attended the poster section on lipid metabolism. Attended the evening session on enzyme expression regulation.

April 29. Attended the Keynote speech on Phosphoinisitide molecules and the genes involved in making the enzymes. What we learn from yeast model. Also attend Lipid metabolism. Went to Symposium on animal model for human nutrition 1 delivered by DH Baker and 2 delivered by Spurlock on adipocyte models. Also went to a biochemistry teaching for cultivating future strong biochemist. The speakers were too good. But picked up some points, including bring research into classroom, set the expectation high, recognize the students, enthusiasm in research is contagous, and interactive teaching to enhance student learning. We also posted our poster. The full length of the report is attached at the end of the report.

April 30. Attended the ASBMB Merck Award for the talk on PEPC Kinase by R.W. Hanson. I also attended a section on role of nuclear receptors in metabolic syndrome in the morning. Went to poster section for genetics and metabolic approaches to obesity and proteionics: proteomics and bioinformatics. Went to Symposium on lipid metabolism and transport and also lipid signaling track.

May 1. I attended the Nutrition section and mostly the poster and two keynote speeches. Two areas are very important, one was on companion animal nutrition and the other was on aging. There were lots of research on the feed limitation on aging and well-being of animals. Two specific experiments on monkey long term restriction on feeding improve the health condition and longevity.

May 2. Attended the FASEB Excellence in Science Award before we took off to Columbus, Ohio for an international exchange program discussion.

二、 與會心得

This year I started to get the feeling of how well we have done researchwise. We have demonstrated a very specific pathway of regulating nutrition metabolism and such a finding is world-class. We need to collect the most recent progress of the research in order to know where we are and what to do to compete with international scientists. This meeting always has a lot of activities going on. Lots of science to learn, but too little time available. I have also got to interact with lots of scientist in my field of research. That help me develop the sense of where we are and our competitiveness.

三、 考察參觀活動(無是項活動者省略)

四、 建議

The areas of nutritional science research are evolving very fast during the past few years. This Meeting collects a broad spectrum of research progress which is important for the researchers in Taiwan. I would suggest that we should encourage researchers to go and joint this meeting to get updated research progress report and to improve our research quality.

五、 攜回資料名稱及內容

Experimental Biology 2007, Conference information and scientific program in a CD format. All the station reports from the USDA regional meeting NCR-97.

六、 其他

The full article of our presentation.

Ectopic expression of porcine peroxisome-proliferator-activated receptor delta regulates adipogenesis in myoblasts

Y. H.Yu[1] and S. T. Ding[1]
Department of Animal Science and Technology, National Taiwan University, 50, Lane 155, Kee-Long Rd. Sec. 3, Taipei 106, Taiwan [1]

ABSTRACT

It is well known that peroxisome-proliferator-activated receptor γ (PPAR γ) plays a critical role in regulating adipogenesis. In rodents, PPAR δ is expressed before PPAR γ during adipocyte differentiation. Thus, the interaction between PPAR δ and PPAR γ during adipogenesis needs to be elucidated. The current experiment was designed to study the interaction of porcine PPAR δ and PPAR γ in mouse myoblast cells. Inhibition of myogenesis was observed in myoblasts expressing porcine PPAR δ, similar to myoblast expressing PPAR γ. Treatment of myoblasts expressing PPAR δ with ligands for both PPAR δ and γ enhanced lipogenesis to a greater extent than treatment with a PPAR γ ligand alone. The ability to transdifferentiate myoblasts into adipocytes was decreased in myoblasts co-expressing PPAR δ with either wild-type or mutated PPAR γ (serine 112 was mutated to alanine) compared to myoblasts expressing either type of PPAR δ alone. Adipose transdifferentiation in myoblasts co-expressing PPAR δ and mutated PPAR γ was greater than in myoblasts co-expressing PPAR δ and wild-type PPAR γ. Our results suggest that PPAR δ has two different roles in regulating adipogenesis, ie., suppression of myogenesis to enhance transdifferentiation of myoblasts into adipocytes and interaction with PPAR γ to modify adipogenesis. Therefore, PPAR δ may have a significant role in adipogenesis.

Key Words: Adipocyte differentiation, Peroxisome proliferator-activated receptor δ, Peroxisome proliferator-activated receptor γ.

表 Y04

INTRODUCTION

In rodent, peroxisome-proliferator-activated receptor δ (PPAR δ) is wildly expressed in several tissues, including adipose tissue, intestine, skeletal muscle, lung and heart. The expression of PPAR δ in proliferating preadipocytes is undetectable and increases gradually

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In rodent, peroxisome-proliferator-activated receptor δ (PPAR δ) is wildly expressed in several tissues, including adipose tissue, intestine, skeletal muscle, lung and heart. The expression of PPAR δ in proliferating preadipocytes is undetectable and increases gradually during adipocyte differentiation (Amri *et al.,* 1995). Preadipocyte overexpressing PPAR δ with long chain fatty acids promotes adipogenesis (Bastie *et al.,* 2000). Ectopic expression of PPAR δ in fibroblasts with long chain fatty acids alone do not induce adipogenesis but stimulation in the presence of PPAR γ ligand (Bastie *et al.,* 1999). Therefore, PPAR δ seems to have a facilitating role in adipogenesis.

The information of porcine PPAR δ is still poorly understood, especially in functional study. In previous studies, we have demonstrated that ectopic expression of porcine PPAR γ induces adipogenesis in myoblasts (Yu *et al.,* 2006). The expression of PPAR δ is earlier than PPAR γ during adipocyte differentiation in rodent adipocytes (Amri *et al.,* 1995). We hypothesize that a relationship between PPAR δ and PPAR γ in regulating adipocyte differentiation. In this study, we created C2C12 myoblasts expressing porcine PPAR δ, or co-expressing PPAR δ with either wild-type or mutated PPAR γ (serine 112 was mutated to alanine). Transfected myoblasts with porcine PPAR δ stimulated adipogenesis after addition of both PPAR δ and PPAR γ ligands, whereas a decreased lipid accumulation was observed in myoblasts co-expressing PPARs compared with expressing PPAR γ alone.

MATERIALS AND METHODS

Stably transformed cells with PPAR δ or PPAR γ and induction of myoblast transdifferentiation

The porcine PPAR δ cDNA was cloned from porcine adipose tissue. The PCR products were cloned into a mammalian expression vector and transfected into C2C12 myoblasts by lipofection. To establish expression of both porcine PPAR δ and PPAR γ cell models, C2C12 myoblasts containing either wild-type PPAR γ or mutated PPAR γ were also transfected with porcine PPAR δ. Myoblasts stably expressing PPAR δ were established by puromycin selection. After drug selection, the cells were cultured without selection medium and allowed to propagate to 80% confluence in DMEM with 10% FBS. Confluent cells were then cultured in adipogenic differentiation medium [DMEM containing 10% fetal bovine serum, 1μM dexamethasone, and 5μg/mL insulin] and with or without 1 μM rosiglitazone, a PPAR γ ligand and 1 μM L165041, a PPAR δ ligand. After 10 days of culture, total RNA was purified to determine gene expression.

Northern blot and statistical analysis

The RNA was separated by electrophoresis and blotted to nylon membranes. The membrane was prehybridized at 42 ℃ and then hybridized with isotope labeled complementary DNA probes. Hybridization results were quantified by phosphor-image analysis. The densitometric value for an individual transcript in a sample lane was normalized to the densitometric value for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in the same lane. The treatment effects were analyzed using an ANOVA procedure to determine the main effects of the form of PPAR δ and PPAR γ in presence or absence of its ligands. Duncan's new multiple range test was

used to evaluate differences among means (SAS Inst. Inc., Cary, NC). A significant difference indicates that P value is not greater than 0.05.

RESULTS AND DISCUSSION

The presence or absence of rosiglitazone and L165041 in adipogenic medium had no effect on myogenesis (Figure 1B, C and D). The myotube formation was inhibited when myoblast expressing PPAR δ compared with transfection of empty vector cells (Figure 1A vs. E). Similar results were observed in our previous studies, myoblasts containing PPAR γ had an ability to interfere in myocyte differentiation (Yu *et al.,* 2006). After exposure of rosiglitazone to the adipogenic differentiation medium for 10 days, lipid-droplets were visualized in myoblasts expressing PPAR δ but absence in addition L165041 in adipogenic differentiation medium (Figure 1). It was well known that ligands for PPARs can activate more than one receptor isoform, hence adipocyte differentiation was increased in medium containing rosiglitazone even if absence of L165041 (Figure 1F). The maximum of lipid accumulation was observed in addition of both PPAR ligands (Figure 1H). This result suggests indirectly that PPAR γ with its ligand has crucial potential in modulating adipocyte differentiation. In loss of function study, it has been demonstrated that lipid accumulation and adipogenic marker genes are decreased in PPAR δ -null adipocytes (Matsusue *et al.,* 2004). In our results, myoblats expressing PPAR δ with PPAR γ and PPAR δ ligands enhanced adipogenesis. It implies that PPAR δ appeared to accelerate adipogenesis. The downstream gene of PPAR γ, adipocyte fatty acid binding protein (aP2) mRNA was highly expressed in myoblasts containing PPAR δ in the presence of rosiglitazone. However, addition of PPAR δ and PPAR γ ligands in adipogenic medium had a greater stimulation of aP2 expression compared with presence of single PPAR ligand. For lipoprotein lipase (LPL) mRNA, it was also increased in the same condition. It has been known that expression of aP2 and LPL are regulated by PPAR γ. Deducing from our results, we hypothesize that high aP2 and LPL transcripts were attributed to PPAR γ function and ectopic PPAR δ modulated PPAR γ expression by binding its peroxisome proliferator response element. A late myogenic marker gene, myogenin was decreased in cells transfected with PPAR δ and both of ligands had no effect on myogenesis (Figure 3). Similar results were observed in another myogenic marker gene, myogenic regulatory factor 4 (MRF4). The suppression of myogenic marker genes in myoblasts expressing PPAR δ was consistent with ectopic expression of PPAR γ in myocytes. These results demonstrated that adipogenesis related transcription factors have the capability of impairing myogenesis. Furthermore, mRNA for aP2 and LPL were expressed at a low level in myoblasts containing either wild-type PPAR γ or mutated PPAR γ and PPAR δ compared with expressing either wild-type PPAR γ or mutated PPAR γ alone (Figure 4). This phenomenon was also found in preadipocyte expressing PPAR δ and PPAR γ. The over-expression of PPAR δ indeed can suppress PPAR γ-mediated adipogenesis (Shi *et al.,* 2002). However, reduction of myogenic genes expression was enhanced in C2C12 myoblasts containing both PPARs (Figure 5). Thus, PPAR δ and PPAR appeared to have a synergic effect in the inhibition of myogenesis.

CONCULSION

In the current study, we demonstrated that PPAR δ has the ability to promote transdifferentiation of myoblasts into adipocytes and interact with PPAR γ to modify adipogenesis. Therefore, PPAR δ may have a significant role in adipogenesis.

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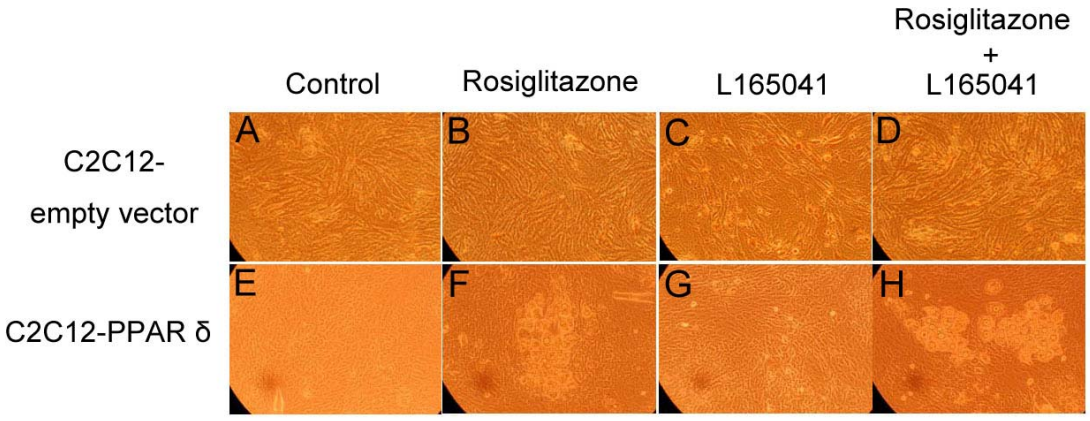
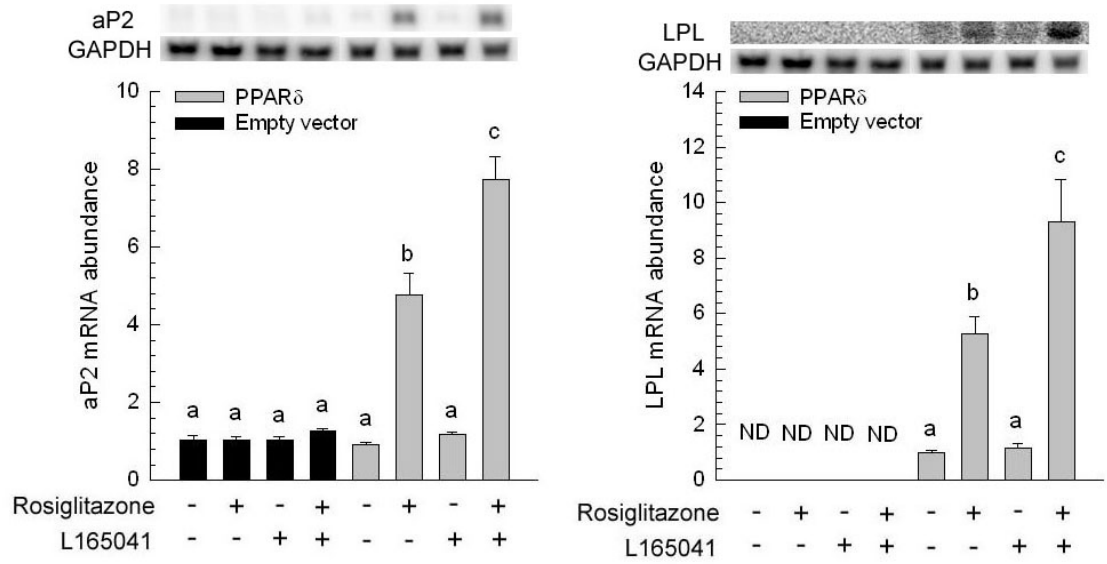


Figure 1. Ligand-induced morphological alterations and accumulation of lipid droplets. Microscographs of C2C12 myocyte with empty vector (A-D), C2C12 expressing porcine peroxisome proliferator-activated receptor δ (PPAR δ; E-H) are shown. Cells were maintained in adipogenic medium (Dulbecco's modified Eagle medium/dexamethasone/insulin/10% fetal bovine serum) ± 1 μM rosiglitazone (B and F), 1 μM L165041 (C and G) and both of ligands (D and H) to

d 10 postconfluence. Magnification was 60.



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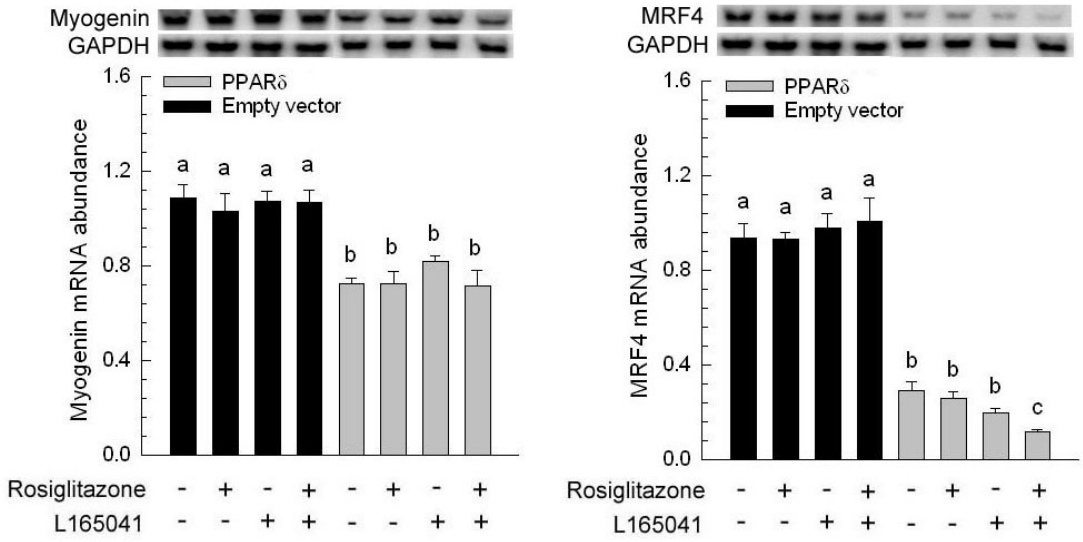

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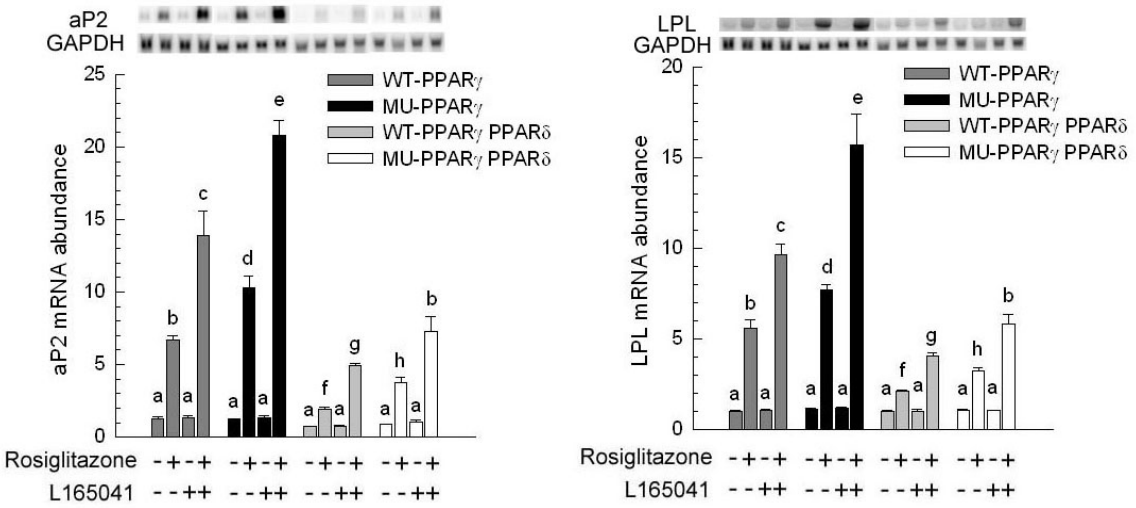

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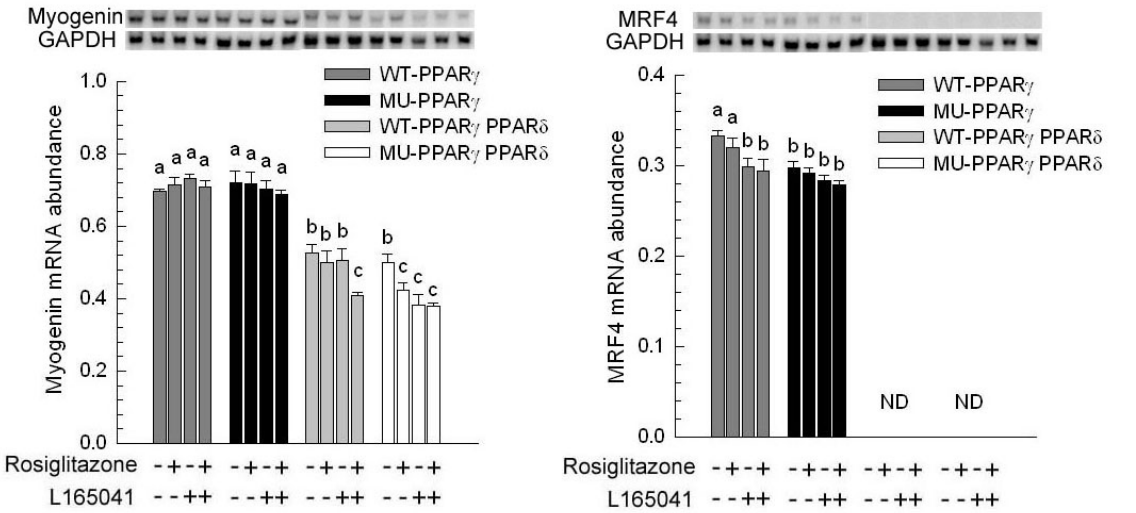

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