

PPAR- γ Gene Expression Is Elevated in Skeletal Muscle of Obese and Type II Diabetic Subjects

Kyong Soo Park, Theodore P. Ciaraldi, Leslie Abrams-Carter, Sunder Mudaliar, Svetlana E. Nikoulina, and Robert R. Henry

The peroxisome proliferator activated receptor PPAR- γ has been identified as a nuclear receptor for thiazolidenediones, which are compounds with insulin-sensitizing properties in several tissues, including skeletal muscle. To determine whether this receptor is expressed and possibly involved in insulin action/resistance in skeletal muscle, PPAR- γ mRNA abundance and its regulation by insulin were quantified in muscle tissue and cultures from lean and obese nondiabetic and type II diabetic subjects using competitive reverse transcription-polymerase chain reaction (RT-PCR). In muscle biopsy specimens, PPAR- γ mRNA was elevated in obese nondiabetic and type II diabetic subjects (23.4 ± 4.2 and $28.0 \pm 5.69 \times 10^3$ copies/ μ g total RNA, respectively; both $P < 0.05$) compared with lean nondiabetic control subjects ($9.4 \pm 2.3 \times 10^3$ copies/ μ g total RNA). Significant positive correlations were present among skeletal muscle PPAR- γ mRNA levels, BMI ($r = 0.67$, $P < 0.01$), and fasting insulin concentration ($r = 0.76$, $P < 0.001$). PPAR- γ mRNA levels were also elevated in muscle cultures from type II diabetic subjects compared with lean nondiabetic control subjects (330.1 ± 52.9 vs. $192.1 \pm 27.0 \times 10^3$ copies/ μ g total RNA, $P < 0.05$). Insulin stimulation of muscle tissue (by hyperinsulinemic-euglycemic clamp for 3–4 h) or muscle cultures (30 nmol/l for 120 min) stimulated PPAR- γ mRNA expression up to fourfold (10.0 ± 2.7 to $41.3 \pm 7.4 \times 10^3$ copies/ μ g total RNA, $P < 0.05$, and 174.9 ± 56.9 to $268.2 \pm 78.6 \times 10^3$ copies/ μ g total RNA, $P < 0.05$, respectively). In summary, PPAR- γ mRNA expression in human skeletal muscle is acutely regulated by insulin and is increased in both obese nondiabetic and type II diabetic subjects in direct relation to BMI and fasting insulinemia. We conclude that abnormalities of PPAR- γ may be involved in skeletal muscle insulin resistance of obesity and type II diabetes. *Diabetes* 46:1230–1234, 1997

Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptors comprising three subtypes designated PPAR- α , PPAR- γ , and PPAR- δ (also termed PPAR- β , FAAR, or NUC-1) (1–4). Although the exact functions of the PPARs are unknown, evidence to date indicates that these receptors may be important regulators of lipid homeostasis, adipocyte differentiation, and insulin action (3–5). Interest in the PPARs, and in particular PPAR- γ , has been kindled by recent demonstrations that several thiazolidenediones, fatty acids, and prostaglandin derivatives can activate PPARs (3,4). In rodents, thiazolidenediones have been shown to reduce *ob* gene expression through activation of PPAR- γ (6). Equally provocative is evidence that thiazolidenedione derivatives improve muscle insulin resistance and that one of these agents with proven antidiabetic actions, Troglitazone, acts as an insulin sensitizer and binds to PPAR- γ (5,7). Recent studies also indicate that there is a close relationship between the potency of various thiazolidenediones to stimulate PPAR- γ and their antidiabetic action (8). Since skeletal muscle is the major site of impaired peripheral insulin action in obesity and type II diabetes, such observations raise the possibility that these receptors may be involved in modulating insulin action in human muscle tissue.

The tissue specificity, relative abundance, and regulation of expression of these receptors in human tissues need to be defined to address the physiological functions of PPARs. In rodents, all PPAR subtypes have been identified in muscle tissue, with PPAR- δ being the most abundantly expressed (3). Accurate evaluation of PPARs in samples of human muscle can be problematic owing to the concomitant presence of adipose tissue, which predominantly expresses PPAR- γ (3,9). Additionally, interpretation can be confounded by in vivo homeostatic regulatory mechanisms, which can rapidly modify or counteract metabolic manipulations (9). These limitations can be largely overcome by the use of a human skeletal muscle culture model, which is devoid of other cell types and has been shown to metabolically reflect the in vivo situation (10). By comparing events in tissue to those in culture, we have been able to accurately compare muscle PPAR expression in different study populations and their response to insulin stimulation. Our results indicate that PPAR- γ in human skeletal muscle is acutely regulated by insulin and that its expression is increased in the hyperinsulinemic insulin-resistant states of obesity and type II diabetes.

From the Department of Medicine, University of California at San Diego, La Jolla; and Veterans Affairs Medical Center, San Diego, California.

Address correspondence and reprint requests to Robert R. Henry, MD, VA Medical Center, San Diego (V111G), 3350 La Jolla Village Dr., San Diego, CA 92161. E-mail: rhenry@vapop.ucsd.edu.

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aP2, adipocyte lipid binding protein; PPAR, peroxisome proliferation activated receptor; RT-PCR, reverse transcription-polymerase chain reaction.

TABLE 1
Clinical characteristics of subjects

	Lean ND	Obese ND	Type II diabetes
Muscle tissue			
<i>n</i>	6	5	5
Age (years)	40 ± 1	43 ± 3	50 ± 3*
Body mass index (kg/m ²)	25.0 ± 2.0	35.3 ± 2.8*	31.9 ± 1.7*
Fasting insulin (pmol/l)	24 ± 6	78 ± 24*	72 ± 4*
Fasting glucose (mmol/l)	5.0 ± 0.6	5.5 ± 0.2*†	14.1 ± 1.3*
Diabetic treatment	—	—	4 OHA, 1 INS
Muscle culture			
<i>n</i>	8	—	8
Age (years)	39 ± 4	—	47 ± 5
BMI (kg/m ²)	27.0 ± 1.0	—	33.0 ± 2.0*
Fasting insulin (pmol/l)	36 ± 6	—	174 ± 54*
Fasting glucose (mmol/l)	5.1 ± 0.2	—	7.0 ± 0.4*
Diabetic treatment	—	—	4 OHA, 1 INS, 3 DIET

Data are *n* or means ± SD. ND, nondiabetic subjects; OHA, oral hypoglycemic agents; INS, insulin. **P* < 0.05 compared with lean ND subjects; †*P* < 0.05 compared with obese diabetic subjects.

RESEARCH DESIGN AND METHODS

Materials. All supplies and reagents were obtained as described in detail previously (10).

Human subjects. Characteristics of the study groups are summarized in Table 1. For the studies in muscle tissue, five subjects with type II diabetes and five obese and six lean nondiabetic subjects had a needle biopsy performed in the overnight fasted state. Five of the lean nondiabetic subjects also had needle muscle biopsies performed after a 3- to 4-h hyperinsulinemic-euglycemic clamp (see below). For muscle culture studies, eight subjects with type II diabetes and eight nondiabetic subjects also had a muscle biopsy performed. Glucose tolerance was determined in all nondiabetic subjects after a 75-g oral glucose tolerance test (11). None of the nondiabetic subjects had a family history of type II diabetes or were on any medications known to influence glucose metabolism. The experimental protocol was approved by the Committee on Human Investigation of the University of California at San Diego. Informed written consent was obtained from all subjects after explanation of the protocol.

Glucose clamp technique. Five nonobese nondiabetic control subjects underwent a 3- to 4-h hyperinsulinemic (360–1,800 pmol · m⁻² · min⁻¹) euglycemic (5.0–5.5 mmol/l) clamp as described in detail previously (12). A needle biopsy of vastus lateralis was performed before and after the clamp study.

Adipose tissue biopsy. Adipose tissue was obtained by open biopsy of the lower abdominal wall after an overnight fast, as previously described (13).

Muscle biopsy procedure and culture. Percutaneous biopsies of vastus lateralis were performed as described previously (12), and muscle tissue was either immediately snap-frozen in liquid N₂ (12) or processed for culture (10). Studies were performed on cultures after 4 days of fusion to mature myotubes (10).

Isolation and extraction of RNA. Total cellular RNA was extracted and purified from adipose and muscle biopsy tissue and cultured muscle cells using Trizol reagent (Gibco BRL, Gaithersburg, MD), according to manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR) of PPAR-γ. A competitive RT-PCR assay was developed for quantitation of PPAR-γ mRNA (14). The internal standard was designed to use the same primers as the target, but to yield a different sized PCR product (302 vs. 250 bp). A synthetic gene, which was used to produce cRNA for the internal control, was constructed by amplification of a heterologous DNA fragment using a pair of composite primers. The composite primers contain the primer sequences for PPAR-γ contiguous to the sequences that anneal to a heterologous DNA fragment and a *EcoRI* or *BamHI* restriction site. This fragment was digested with *EcoRI* and *BamHI* and subcloned into a pGEM 3Z vector (Promega, Madison, WI). The resulting pKSP-2 plasmid was linearized by *BamHI* and used as a template for transcription by T7 polymerase, according to the transcription protocol of Promega. The cRNA was purified with phenol/chloroform extractions and ammonium acetate-ethanol precipitation to remove unincorporated nucleotides. The cRNA was dissolved in water treated with diethylpyrocarbonate, quantitated by spectrophotometry, and stored at -70°C. Also, 0.25 μg of total RNA and serial dilutions (2.5 – 600 × 10⁹ copies) of cRNA were

reverse-transcribed using 200 U Moloney murine leukemia virus reverse transcriptase (Gibco) with 200 ng of random hexamers in 20 μl reaction volume for 1 h at 37°C, then heated to 70°C for 15 min.

PCR amplification was performed using 4 μl of the RT reaction in PCR buffer (50 mmol/l KCl, 2 mmol/l MgCl₂, 20 mmol/l Tris-HCl, pH 8.3), 200 μmol/l dNTP, 25 pmol forward and reverse primers, and 1 U AmpliTaq Gold (Perkin-Elmer/Cetus, Norwalk, CT) in a final volume of 50 μl. The upstream primer was GTT CAT GCT TGT GAA GGA TGC (nt 545–565), and the downstream primer was ACT CTG GAT TCA GCT GGT CG (nt 775–794) (15). Cycling parameters were 94°C for 1 min, 55°C 1 min, and 72°C for 1 min and 30 s for 30–35 cycles after the initial denaturation at 95°C for 12 min. At the end of the last cycle, samples were incubated for 7 min at 72°C. Of the amplification products, 10 μl were separated on a 3% agarose gel, stained with ethidium bromide, photographed, and quantitated using NIH Image (image software available from the National Institutes of Health). Quantitation of the RT-PCR method was established by performing standard curves using at least four different concentrations of cRNA. A linear relationship was found for cRNA vs. log (target/cRNA ratio) in basal state (*r* = 0.99) and following hyperinsulinemic

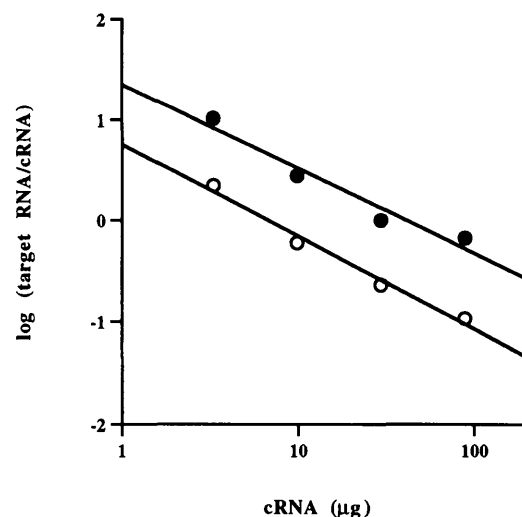


FIG. 1. Validation of RT-PCR for PPAR-γ. Representative standard curve performed on RNA isolated from skeletal muscle biopsy of lean nondiabetic subject before (○) and after (●) a hyperinsulinemic-euglycemic clamp. In the basal condition, *r* = 0.99 and following the clamp, *r* = 0.98.

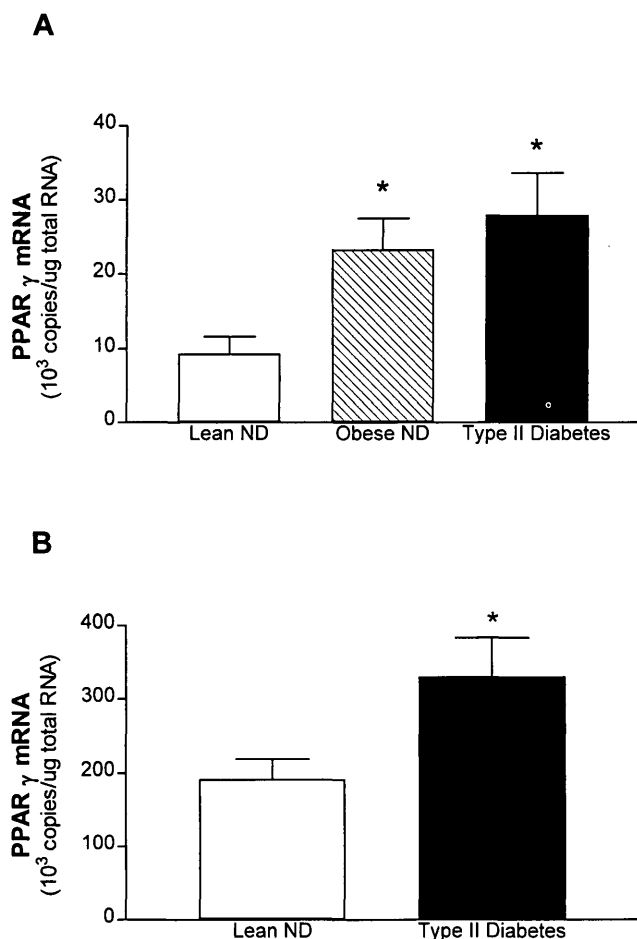


FIG. 2. PPAR- γ gene expression in human skeletal muscle tissue and culture. **A:** PPAR- γ mRNA levels in skeletal muscle tissue of lean and obese nondiabetic (ND) subjects and obese patients with type II diabetes. **B:** PPAR mRNA levels in skeletal muscle cultures of lean nondiabetic (ND) subjects and obese patients with type II diabetes. * $P < 0.05$ compared to lean nondiabetic subjects.

clamps ($r = 0.98$) (Fig. 1). Intra-assay variation was 8.9%, and interassay variation was 15.4% for RT-PCR.

Northern blotting of PPAR- δ and adipocyte lipid binding protein (aP2). Of total RNA, 10 μ g was electrophoresed through a denaturing formaldehyde 1% agarose gel and transferred to a NYTRAN membrane (Schleicher and Schuell, Keene, NH). Hybridization with cDNA [α -³²P]dCTP]-labeled probes was carried out at 68°C, according to manufacturer's instructions. To remove nonspecific binding, membranes were washed twice at room temperature, first with 2 \times saturated sodium citrate (SSC), 0.1% SDS buffer and then once with 0.2 \times SSC, 0.1% SDS buffer, followed by washing at 60°C with 0.1 \times SSC, 0.1% SDS buffer.

Statistical analysis. Statistical significance was evaluated by analysis of variance (ANOVA) or Student's *t* test using StatView II (Abacus Concepts, Berkeley, CA). All data are expressed as the mean \pm SE. Correlation coefficients were calculated using the method of least squares. Significance was accepted at the $P < 0.05$ level.

RESULTS

PPAR mRNA abundance in muscle tissue and cultures. As shown in Fig. 2A, PPAR- γ mRNA expression was significantly greater in muscle tissue from both obese nondiabetic and obese type II diabetic subjects than lean nondiabetic subjects (23.4 ± 4.2 and 28.0 ± 5.69 vs. $9.4 \pm 2.3 \times 10^3$ copies/ μ g total RNA, respectively; both $P < 0.05$). PPAR- δ mRNA levels, however, were similar among the three

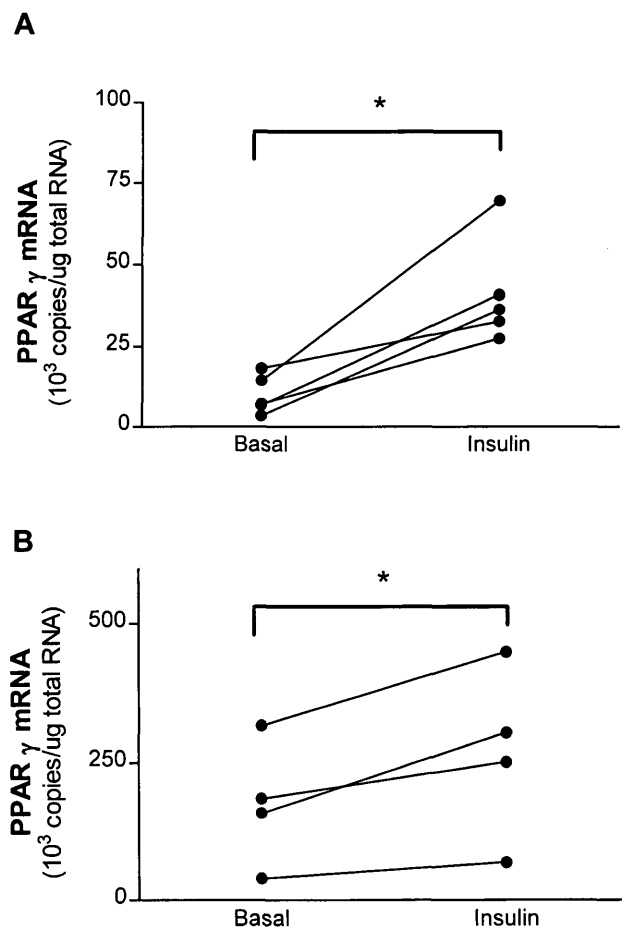


FIG. 3. Effect of insulin on PPAR- γ gene expression in human skeletal muscle tissue and culture. **A:** PPAR- γ mRNA levels in human skeletal muscle tissue of lean nondiabetic (ND) subjects before and following 3–4 h of a hyperinsulinemic (360 – 1800 pmol \cdot m⁻² \cdot min⁻¹) euglycemic (5.0 – 5.5 mmol/l) clamp. **B:** PPAR- γ mRNA levels in human skeletal muscle cultures of lean nondiabetic (ND) subjects before and after 30 nmol/l insulin for 120 min.

groups (data not shown, NS). For purposes of comparison, PPAR- γ mRNA levels measured in abdominal adipose tissue were $950 \pm 84 \times 10^3$ copies/ μ g total RNA. Thus, expression of PPAR- γ in muscle tissue is ~ 1 to 5% of that in adipose tissue. Since PPAR- γ is abundantly expressed in adipose tissue, mRNA expression of aP2, an adipocyte-specific marker, was measured to determine whether elevated PPAR- γ levels in obese nondiabetic and diabetic muscle might reflect an increased adipose content of muscle biopsy samples. Also, aP2 mRNA abundance was very low in muscle tissue but significantly greater in both obese groups (data not shown), suggesting that increased adipose content of muscle biopsy samples might contribute to the differences in PPAR- γ levels.

To further explore these findings, PPAR- γ mRNA levels were measured in muscle cultures derived from eight lean nondiabetic and eight type II diabetic subjects. In agreement with the results in muscle tissue samples, PPAR- γ mRNA levels (Fig. 2B) were also significantly greater in muscle cultures from diabetic compared with nondiabetic subjects (330.1 ± 52.9 vs. $192.1 \pm 27.0 \times 10^3$ copies/ μ g total RNA,

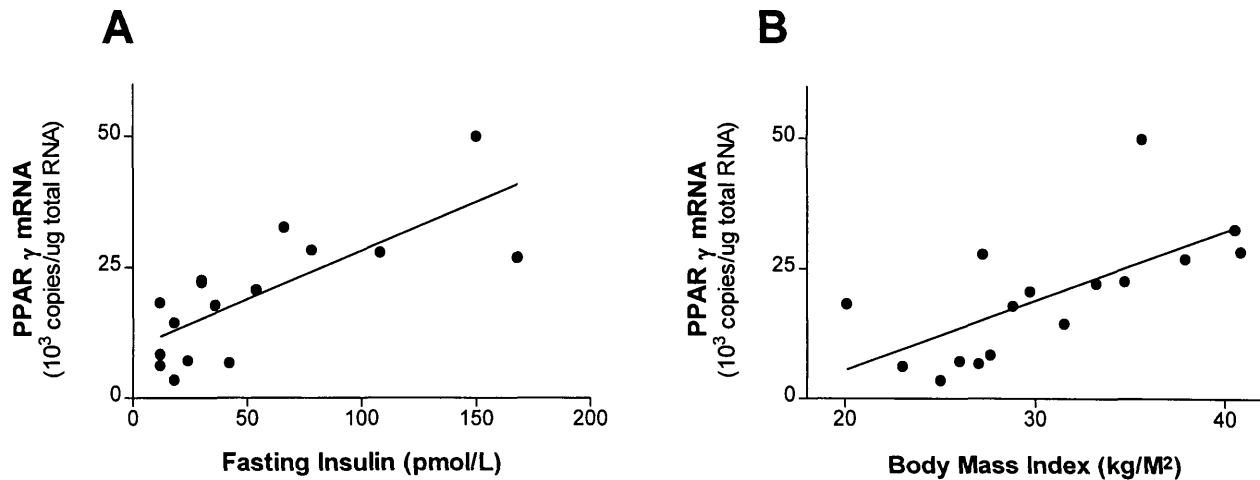


FIG. 4. Relationship between PPAR- γ mRNA levels in human skeletal muscle tissue, fasting insulin levels (A) and BMI (B) in lean and obese nondiabetic and obese type II diabetic subjects

respectively; $P < 0.05$). PPAR- γ mRNA expression in muscle cell cultures was 30–40% of that in adipose tissue, and aP2 mRNA signals were not detected in muscle cultures from either group.

PPAR gene regulation in muscle tissue and cultures. To determine whether muscle PPAR expression might be regulated by insulin, mRNA levels were measured before and after 3–4 h of acute insulin administration in muscle samples from five lean nondiabetic subjects. As shown in Fig. 3A, PPAR- γ mRNA levels were increased in all subjects by insulin (from 10.0 ± 2.7 to $41.3 \pm 7.4 \times 10^3$ copies/ μ g total RNA; $P < 0.05$). Similar to the *in vivo* situation, PPAR- γ mRNA levels in nondiabetic muscle cultures ($n = 4$) also increased significantly (from 174.9 ± 56.9 to $268.2 \pm 78.6 \times 10^3$ copies/ μ g total RNA, $P < 0.05$) after treatment with 30 nmol/l insulin for 120 min (Fig. 3B). Consistent with an insulin effect on PPAR- γ transcription, strong correlations were found to exist between muscle tissue PPAR- γ mRNA levels and fasting insulin levels ($r = 0.76$, $P < 0.001$, Fig. 4A) as well as BMI ($r = 0.67$, $P < 0.01$) (Fig. 4B).

DISCUSSION

PPARs are nuclear receptors that act to modulate gene transcription and are believed to be involved in regulation of lipid metabolism, adipocyte differentiation, and insulin action (1–6). A possible role for PPARs in regulating insulin action has been strengthened by the recent demonstration that the insulin-sensitizing thiazolidenedione class of compounds are synthetic ligands for PPAR- γ (7) and appear to exert antidiabetic actions in direct relation to their PPAR- γ stimulating potency (8). In human muscle tissue, the presence and abundance of the PPAR- γ as well as its regulation and physiological functions remain to be defined. Because skeletal muscle is the major peripheral target tissue for insulin and is associated with insulin resistance in obesity and type II diabetes, the abundance and regulation by insulin of PPAR- γ mRNA was determined in human muscle from lean and insulin-resistant obese nondiabetic and type II diabetic subjects. These studies demonstrate that PPAR- γ is regulated by insulin and abnormally elevated in the insulin-resistant states of obesity and type II diabetes.

In a recent study, PPAR- γ mRNA was detected in rodent skeletal muscle (9). In adipose tissue from these same animals, PPAR- γ mRNA expression was shown to be regulated by dietary manipulation and insulin treatment (9). The current study extends these observations by confirming that the message for this receptor is present in human skeletal muscle and is acutely regulated by insulin administration. To date, there is no direct evidence that PPAR- γ is involved in insulin action. However, an association between insulin action and PPAR- γ is further supported by the strong correlation of PPAR- γ mRNA levels in muscle biopsy tissue with fasting insulin levels and BMI. Such relationships may be explained by the well-recognized association of obesity with insulin resistance and compensatory hyperinsulinemia (16). By this scenario, muscle tissue from obese individuals with or without diabetes is insulin resistant and has elevated PPAR- γ mRNA levels. The crucial issue raised by these findings is whether the elevated PPAR- γ mRNA levels result from an intrinsic abnormality or develop subsequent to acquired insulin resistance in muscle. The fact that muscle PPAR- γ mRNA levels increase after acute insulin administration implies that elevations in obese nondiabetic and type II diabetics are the result of insulin resistance and hyperinsulinemia.

Measurement of PPAR- γ mRNA levels in muscle biopsy specimens can be confounded by the concomitant presence of adipose tissue, in which PPAR- γ mRNA is abundantly expressed. To overcome this limitation, PPAR- γ mRNA levels were measured in human skeletal muscle cultures. However, the issue arises as to whether these cells are, in fact, all of muscle origin. If some adipocyte precursors also proliferate during culture, then the PPAR- γ mRNA abundance and the stimulation by insulin could reflect measurements made in cells of adipose origin. Several lines of evidence suggest that this was not the case. First, the adipocyte-specific marker, aP2 mRNA, was not detectable in muscle cultures. Second, on histological examination, >90% of the cells were multinucleated and, therefore, of muscle origin. Finally, there was no apparent difference in muscle morphology of cultures from nondiabetic and type II diabetic subjects. Therefore, it seems likely that PPAR- γ mRNA measured in cultures are a true reflection of changes occurring in muscle. The explanation for

increased PPAR- γ mRNA expression in muscle cultures from subjects with type II diabetes is uncertain but cannot be explained by differences in media substrates (insulin, glucose, triglycerides, etc.) during growth and differentiation.

Several other potential limitations of these results need to be emphasized. Although mRNA levels were elevated in muscle of obese nondiabetic and type II diabetic subjects, such changes may not reflect levels of protein or bioactivity. Additionally, PPAR- γ mRNA exists in two isoforms, 1 and 2, that differ only in the 30 NH₂-terminal amino acids and may have different biological effects. It remains to be determined whether one isoform normally predominates in human muscle, if either or both isoforms are elevated in insulin-resistant muscle, and which isoform is stimulated by insulin. Differences in PPAR- γ mRNA abundance of more than tenfold also exist between muscle tissue and culture that are not readily explicable. These issues will need to be addressed in future studies to more fully elucidate the implications of PPAR- γ expression in skeletal muscle.

In summary, PPAR- γ mRNA is expressed in skeletal muscle. PPAR- γ is acutely regulated by insulin and increased in both obese nondiabetic and type II diabetic subjects in direct relation to BMI and fasting insulinemia. These results suggest that PPAR- γ may play a role in skeletal muscle insulin resistance of obesity and type II diabetes.

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REFERENCES

- Keller H, Wahli W: Peroxisome proliferator-activated receptors: a link between endocrinology and nutrition? *Trends Endocrinol Metab* 4:291-296, 1993
- Schoonjans K, Staels B, Auwerx J: Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. *J Lipid Res* 37:907-925, 1996
- Schoonjans K, Staels B, Auwerx J: The peroxisome proliferator activated receptors (PPARs) and their effects on lipid metabolism and adipocyte differentiation. *Biochim Biophys Acta* 1302:93-109, 1996
- Spiegelman BM, Flier JS: Adipogenesis and obesity: rounding out the big picture. *Cell* 87:377-389, 1996
- Saltiel AR, Olefsky JM: Thiazolidinediones in the treatment of insulin resistance and type II diabetes. *Diabetes* 45:1661-1669, 1996
- DeVos P, Lefebvre A-M, Miller SG, Guerre-Millo M, Wong K, Saladin R, Hamann LG, Staels B, Briggs MR, Auwerx J: Thiazolidinediones repress *ob* gene expression in rodents via activation of peroxisome proliferator-activated receptor γ . *J Clin Invest* 98:1004-1009, 1996
- Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, Kliewer SA: An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPAR γ). *J Biol Chem* 270:12953-12956, 1995
- Willson TM, Cobb JE, Cowan DJ, Wiethel RW, Correa ID, Prakash SR, Beck KD, Moore LB, Kliewer SA, Lehmann JM: The structure-activity relationship between peroxisome proliferator-activated receptor gamma agonism and the antihyperglycemic activity of thiazolidinediones. *J Med Chem* 39:665-668, 1996
- Vidal-Puig A, Jimenez-Linan M, Lowell BB, Hamann A, Hu E, Spiegelman B, Flier JS, Moller DE: Regulation of PPAR γ gene expression by nutrition and obesity in rodents. *J Clin Invest* 97:2553-2561, 1996
- Henry RR, Abrams L, Nikoulina S, Ciaraldi TP: Insulin action and glucose metabolism in nondiabetic control and NIDDM subjects: comparison using human skeletal muscle cell cultures. *Diabetes* 44:936-946, 1995
- National Diabetes Data Group: Classification and diagnosis of diabetes mellitus and other categories of glucose tolerance. *Diabetes* 28:1039-1057, 1979
- Thorburn AW, Gumbiner B, Bulacan F, Brechtel G, Henry RR: Multiple defects of muscle glycogen synthase activity contribute to reduced glycogen synthesis in non-insulin dependent diabetes mellitus. *J Clin Invest* 87:489-495, 1991
- Freidenberg GR, Henry RR, Klein HH, Reichart DR, Olefsky JR: Decreased kinase activity of insulin receptors from adipocytes of non-insulin-dependent diabetic subjects. *J Clin Invest* 79:240-250, 1987
- Wang AM, Doyle MV, Mark DF: Quantitation of mRNA by the polymerase chain reaction. *Proc Soc Natl Acad Sci* 86:9717-9721, 1989
- Greene ME, Blumberg B, McBride OW, Yi HF, Kronquist K, Kwan K, Hsieh L, Greene G, Nimer SD: Isolation of the human peroxisome proliferator activated receptor gamma cDNA expression in hematopoietic cells and chromosomal mapping. *Gene Expr* 4:281-299, 1995
- DeFronzo RA, Ferrannini E: Insulin resistance: a multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. *Diabetes Care* 14:173-194, 1991