Retinoid X Receptor Expression in Skeletal Muscle of Nondiabetic, Obese and Type 2 Diabetic Individuals

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Retinoid X receptor (RXR) is a nuclear receptor that functions as an obligate heterodimeric partner of peroxisome proliferator-activator receptor (PPAR). Studies have shown that the α isoform of RXR and PPARγ act synergistically to regulate gene expression and insulin action. The aim of the current study was to compare the expression and regulation of RXR in the primary insulin-sensitive tissue, skeletal muscle, of various degrees of insulin-resistant states including obese type 2 diabetic (T2D), obese nondiabetic (OND), and lean nondiabetic (LND) subjects. Insulin action/resistance was determined by a 3-hour hyperinsulinemic, euglycemic (5.0 to 5.5 mmol/L) clamp. Percutaneous biopsy of the vastus lateralis muscle was performed before and after the clamp. RXRα mRNA was measured using a quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay, while protein was determined by Western blotting. All 3 isoforms of RXR, α, β, and γ, were present in skeletal muscle. Protein expression of RXR isoforms did not differ between groups; RXR α mRNA was also similar between groups. Neither RXR α mRNA, RXR -β nor -γ protein displayed significant relationships with any of the clinical or laboratory parameters measured, including insulin sensitivity. RXR α exhibited a negative correlation with free fatty acids levels (r, -.42, $P < .05$). There was also no relationship between RXR α and PPARγ protein levels. RXR α mRNA was unaltered following insulin infusion. We conclude that RXR isoform (α, β, γ) expression is not tightly controlled by insulin, insulin resistance or type 2 diabetes. Instead, RXR isoforms are likely constitutive proteins or controlled by other factors.

MEMBERS OF THE NUCLEAR receptor superfamily are involved in regulation of a broad array of genes.[1] The vitamin D/thyroid/retinoid nuclear receptor family, also named the class II nuclear receptor family, comprises a large group of receptors, which preferentially bind to their hormone response elements in vitro as heterodimers.[2] In the majority of situations, the heterodimeric partner is the retinoid X receptor (RXR). Ligand binding to the RXR partner can either augment[3,4] or impair[5] gene transcription under control of the heterodimer. RXR is crucial for the function of so many other nuclear receptors that it has been characterized as a "master regulator."[2] Three different isoforms of RXR, α, β, and γ, the products of different genes, have been described.[6]

RXR is particularly important in the function of the peroxisome proliferator-activated receptors (PPARs). For example, PPARγ is transcriptionally inactive unless part of a heterodimer with RXR.[7] The PPARs have been identified as crucial for the control of a number of genes involved in glucose and lipid metabolism.[7,8] The PPARs have also been a topic of considerable interest due, in part, to the observation that the thiazolidinedione antidiabetic drugs are selective agonists for PPARγ,[9] with a close relationship between the ability to activate transcription in PPARγ reporter systems and potency to lower blood glucose in insulin-resistant animals.[10,11] Given the obligate role of RXR in PPARγ function, it seems possible that activation of RXR could also influence metabolism. Indeed, selective RXR agonists, besides acting synergistically with thiazolidinediones to augment transcriptional activity in PPARγ/RXRα reporter systems,[12,13] also display blood glucose lowering activity in diabetic animals.[12,14,15] Thus, RXR agonists may have potential utility as antidiabetic agents.

Skeletal muscle is the major site of impaired glucose metabolism and insulin resistance in type 2 diabetic subjects.[16] We have reported that, in an in vitro system, thiazolidinediones can act directly on skeletal muscle to improve glucose metabolism in type 2 diabetic subjects.[17] Studies in diabetic animal models also show that skeletal muscle metabolism is improved with thiazolidinedione treatment.[18-21] Both lines of evidence indicate that skeletal muscle is a potential target for thiazolidinedione action. In support of this idea, we have recently reported that PPARγ protein is abundant in skeletal muscle.[22] Interestingly, the relationship between skeletal muscle PPARγ protein expression and insulin-stimulated glucose disposal rate, a measure of whole body insulin sensitivity, was different between nondiabetic and type 2 diabetic subjects,[22] suggesting potential impairments in PPARγ function in diabetes. As RXR is essential for PPARγ function, it could also contribute to the behavior specific to the diabetic subjects. The aim of the current study was to determine which isoforms of RXR are present in skeletal muscle and evaluate the expression of RXRs to establish if differences in RXR isoform expression in diabetic subjects could contribute to alterations in glucose and lipid metabolism.

SUBJECTS AND METHODS

Subjects

Twelve patients with type 2 diabetes (T2D), 11 obese nondiabetic (OND), and 11 lean nondiabetic (LND) subjects participated in the study. Glucose tolerance was determined in all subjects after a 75 g oral glucose tolerance test. The clinical characteristics of the 3 groups are shown in Table 1. Of the diabetic subjects, 7 were treated with oral antidiabetic agents, 4 with insulin, and 1 with diet only. Diabetic

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patients had their medication withheld on the morning of biopsy. None of the nondiabetic subjects 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, San Diego. Informed written consent was obtained from all subjects after explanation of the protocol. Insulin action was determined by the 3-hour hyperinsulinemic (300 mU/m^2/min) euglycemic (5.0 to 5.5 mmol/L) clamp procedure as described in detail previously.[23] The glucose disposal rate (GDR) was determined during the last 30 minutes of the insulin infusion.

Materials

Bovine serum albumin (BSA, fraction V) was purchased from Boehringer Mannheim (Indianapolis, IN). Reagents for electrophoresis were purchased from Bio-Rad (Richmond, CA). Polyclonal antisera against the α, β, and γ isoforms of RXR were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antirabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase was purchased from Amersham (Arlington Heights, IL). Super Signal chemiluminescence substrate was from Pierce Chemical Co, Rockford, II. Unless indicated, all other reagents were purchased from Sigma Chemical (St Louis, MO).

Muscle Biopsy Procedure

Percutaneous biopsies of vastus lateralis muscle were obtained before and after the clamp procedure as described previously,[23] and muscle tissue was immediately snap-frozen in liquid N_2. Total cellular RNA was extracted and purified from the muscle biopsy tissue using Trizol reagent (Gibco BRL, Gaithersburg, MD) according to manufacturer's instructions.

Quantitation of RXRα mRNA by Reverse Transcriptase-Polymerase Chain Reaction

A competitive reverse transcriptase-polymerase chain reaction (RT-PCR) assay was developed for quantitation of RXRα mRNA. The internal standards were designed to use the same primers as the target gene, but to yield different sized PCR products (338 bp for standard *v* 389 bp for RXRα). A synthetic gene, which was used to produce cRNA for the internal standard, was constructed by amplification of a heterologous DNA fragment using a pair of composite primers. The composite primers contain the primer sequences for the target gene, RXRα, contiguous to the sequences that anneal to a heterologous DNA fragment. After amplification of this DNA with these primers, the resulting PCR product was cloned to a plasmid with the use of Topo TA cloning kit (Invitrogen, Carlsbad, CA). The plasmid was linearized by BamHI and used as a template for in vitro transcription by T7 polymerase, according to the manufacturer's transcription protocol (Gibco BRL). The resulting cRNA was purified by phenol/chloroform extraction and ammonium acetate-ethanol precipitation to remove unincorporated nucleotides. The cRNA was dissolved in water treated with diethypyrocarbonate, quantitated by spectrophotometry, and stored at −70°C. The new synthethic DNA was sequenced with a 373 automated DNA sequencer (ABI, Perkin Elmer, Boston, MA). The resulting sequence was, as expected, 338 bp and had in its extreme the sequence of the RXRα primers. The RXRα target was also subcloned into the same plasmid and sequenced. The PCR product had the expected sequence for RXRα.

A total of 0.75 mg of total RNA and serial dilutions (100 to 8,000 × 10^3 copies) of each cRNA construct were reverse transcribed using 200 U Moloney murine leukemia virus reverse transcriptase (Gibco) with 200 ng of random hexamers in 20 mL reaction volume for 1 hour at 37°C, then heated to 70°C for 15 minutes.

PCR amplification was performed in a Delta System IITM thermocycler (Ericomp, San Diego, CA) using 5 mL of the RT reaction in PCR buffer (50 mmol/L KCl, 1 mmol/L MgCl2, 20 mmol/L Tris-HCl, pH 8.3), 200 mmol/L deoxynucleotide 5′-triphosphate (dNTPs), 25 pmol forward and reverse primers, and 1 U AmpliTaq Gold (Perkin-Elmer/Cetus, Norwalk, CT) in a final volume of 50 mL. The upstream primer was CCG GGC ATG AGT TAG TCG (nt 53–71) and the downstream primer was GTG GGC GGG GAC CTT GAG (nt 424–442). Samples were incubated at 95°C for 12 minutes for initial denaturation. Cycling parameters were: 94°C for 1 minute, 63°C for 1 minute, and 72°C for 1 minute 30 seconds for 5 cycles; 94°C for 1 minute, 58 C for 1 minute, and 72°C for 1 minute 30 seconds for 24 cycles; then 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute 30 seconds for 1 cycle. At the end of the last cycle, samples were incubated for 7 minutes at 72°C. A total of 18 mL of the amplification products was separated on a 3% agarose gel, stained with ethidium bromide, photographed with a DC40 camera and quantitated using Kodak Digital Science 1D Image Analysis software (Kodak, Rochester, NY). The ratio of each target product/cRNA standard was plotted against the number of copies of cRNA added to yield the equivalence point between cRNA and target mRNA (Fig 1). The *r* value of the standard curves was between .95 to 1.00, with interassay and intrassay variations of 22.5% and 13.6%, respectively.

Analysis of Protein Expression

Tissue protein homogenization and Western blot analysis were performed as detailed previously.[24,25] In brief, tissue homogenates were solubilized in Laemmli's buffer and proteins size fractionated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and electrophoretically transferred to a nitrocellulose membrane (Protran, Schleicher & Schuell, Keene, NH). Equal amounts of total protein were loaded. Total protein content was determined by a dye-binding method.[26] All blots contained an extract of 3T3 cells as an internal standard. The results were normalized to this standard to correct for blot to blot variation. RXR proteins were identified using polyclonal antisera specific for the individual RXR isoforms (Santa

Table 1. Clinical Characteristics of Study Subjects

	Diabetic (n = 12)	Obese Nondiabetic (n = 11)	Lean Nondiabetic (n = 11)
Age (yr)	47.4 ± 1.7	43.3 ± 2.5	43.3 ± 3.5
BMI (kg/m^2)	35.3 ± 2.7*	31.4 ± 4.8*	23.1 ± 0.5
Fasting glucose (nmol/L)	9.5 ± 0.9*†	5.1 ± 0.1	4.9 ± 0.1
Fasting insulin (pmol/L)	154.0 ± 27.8*†	67.3 ± 12.2*	24.7 ± 6.1
HbA1c (%)	8.7 ± 0.8*†	5.3 ± 0.1	5.4 ± 0.1
Fasting FFA (mmol/L)	0.93 ± 0.10*†	0.64 ± 0.10	0.57 ± 0.10
GDR (mg/kg/min)	5.9 ± 0.6*†	8.9 ± 0.5*	12.9 ± 0.8

Abbreviations: BMI, body mass index; HbA1c, hemoglobin A1c; FFA, free fatty acids; GDR, glucose disposal rate.

* $P < .05$ *v* lean nondiabetic.

† $P < .05$ *v* obese nondiabetic.

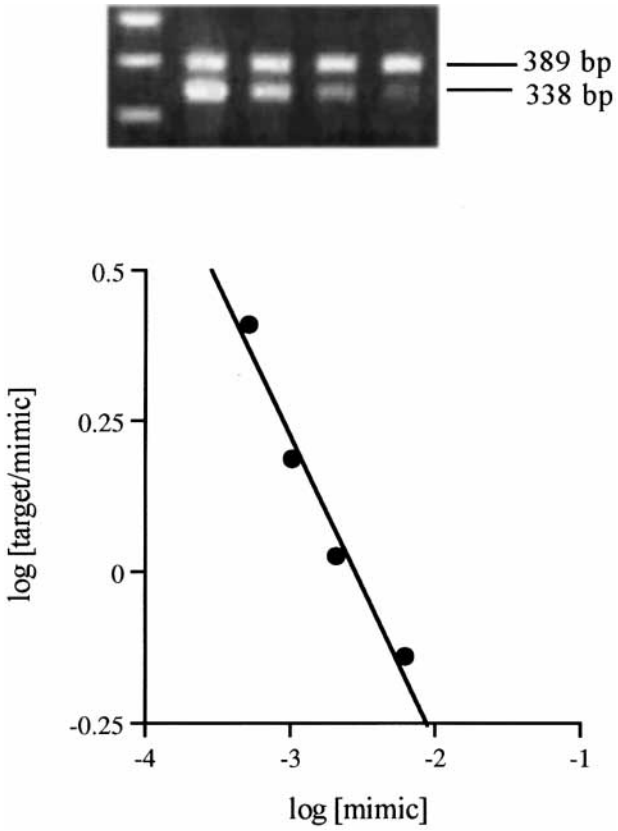

Fig 1. Validation for RT-PCR for RXRα. (A) Ethidium bromide stain of representative standard competition curve performed on RNA isolated from skeletal muscle biopsy of a lean nondiabetic subject. (B) Standard curve transformation of data, $r = .984$.

Cruz Biotechnology), and the secondary antibody was antirabbit IgG conjugated with horseradish peroxidase. Proteins were visualized with SuperSignal and exposed to high performance chemiluminescence film (Hyperfilm TM ECLTM; Amersham Life Science, Piscataway, NJ). Analysis of PPARγ protein expression in human skeletal muscle has recently been described.[22,24]

Statistical Analysis

Statistical significance was evaluated using Student's *t* test for dependent and for independent samples and Pearson's analysis of correlation (Prism; Graphpad, San Diego, CA). Significance was accepted at the $P < .05$ level. Results are presented as mean ± standard error.

RESULTS

The clinical characteristics of the study subjects are shown in Table 1. The subjects were matched for age, while both obese groups (nondiabetic and type 2 diabetic) were matched for body mass index (BMI). The diabetic subjects were hyperglycemic and hyperlipidemic. As indicated by the GDR, the diabetic subjects were demonstrated to be insulin-resistant, while the obese, nondiabetic subjects were intermediate in insulin action compared with the lean nondiabetic subjects.

RXR occurs as 3 isoforms, α, β, and γ.[7] Protein for all 3 isoforms was readily detected in skeletal muscle of the 3 groups of subjects (Fig 2A). Due to differences in the affinities of the antibodies, it is not possible to draw conclusions about the relative expression of the isoforms. While there was considerable intragroup variability, there were no statistically significant differences between groups with regards to expression of any of the RXR isoforms (Fig 2B). RXRα mRNA expression was also comparable between groups in the baseline condition (Fig 3).

The possible relationship between RXR isoform expression and a variety of clinical parameters was investigated (Table 2). There were no statistically significant correlations between either RXRα mRNA and a wide array of factors including: BMI, fasting glucose, fasting insulin, fasting FFA or triglyceride levels, and HbAlc. RXRβ and RXRγ protein levels did not display significant relationships with any of the clinical or laboratory parameters measured. However, RXRα protein expression exhibited a negative correlation with free fatty acid levels ($r = -.42$, $P < .05$). There were no correlations between RXRα, -β, and -γ protein and skeletal muscle content of PPARγ protein measured in the same subjects. This lack of correlation was observed both for the entire

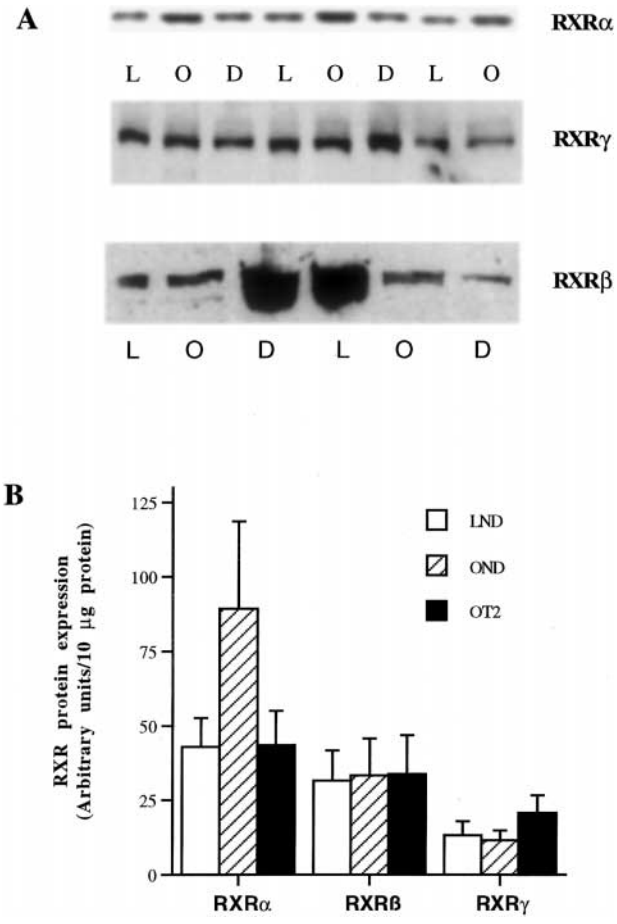

Fig 2. RXR isoform protein expression in skeletal muscle biopsies from lean nondiabetic (L or LND), obese nondiabetic (O or OND), and type 2 diabetic (D or OT2) subjects. (A) Representative Western blots, equal amounts of protein loaded in each lane. The blot for RXRα was stripped and reprobed for RXRγ; a separate membrane was probed for RXRβ. Starting from the left, the same subjects were analyzed on each membrane. (B) Quantitation of RXR protein expression. Results are average + SEM, n = 6 to 8.

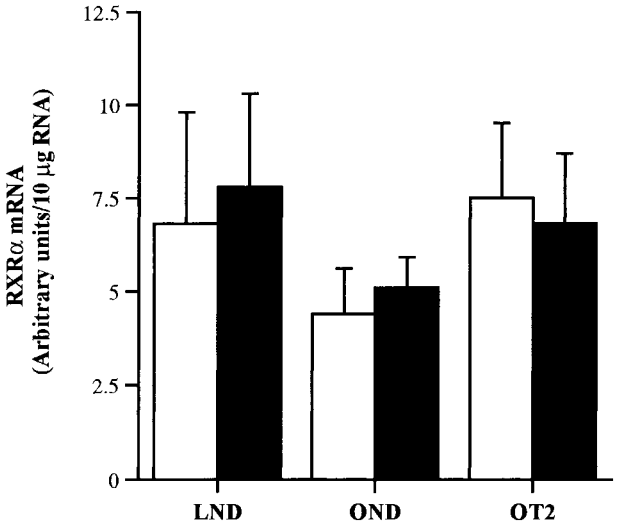

Fig 3. Quantitation of RXRα mRNA in skeletal muscle of LND, OND, and OT2 subjects. Tissue was obtained in the baseline state (open bars) and after hyperinsulinemic/euglycemic clamp procedure (solid bars). Results are average + SEM.

subject population combined or when each of the 3 groups was analyzed separately.

Acute insulin exposure can influence the expression of many genes, including PPARγ.[27] However, 3 hours of insulin infusion during the clamp procedure was without effect on RXRα mRNA expression in any of the subject groups (Fig 3). Protein content of RXRα was also unaltered (not shown). Postclamp levels of RXRα mRNA and protein were also not correlated with any of the parameters analyzed, including GDR.

DISCUSSION

The PPARs and RXRs have been shown to be especially important in the control of glucose and lipid metabolism.[8,28,29] There is extensive literature concerning the in vitro interactions between RXRα and PPARs,[3,12,30] including synergism for regulation of reporter gene transcription. Of greater physiologic relevance, synergism of RXR and PPAR agonists has also been demonstrated for the control of a number of genes involved in glucose and lipid metabolism including: acyl-CoA oxidase in liver cells;[3] GLUT4, the p85 subunit of phosphatidylinositol 3-kinase and uncoupling protein-2 (UCP-2) in human adipocytes;[31] and UCP-3 in L6 muscle cells.[32] Such responses might explain the demonstrated ability of RXR agonists to reduce elevated glucose and triglyceride levels in insulin-resistant ob/ob[14] and db/db mice[16] and to improve whole body insulin action. Considering this background, we decided to evaluate the expression of RXR in skeletal muscle of adult humans, including obese diabetic, obese nondiabetic, and lean control subjects.

All 3 isoforms of RXR, RXRα, RXRβ, and RXRγ, were identified in skeletal muscle by Western blotting, showing that multiple RXR are present in skeletal muscle, available for dimerization with other nuclear receptors. As a number of studies have shown the importance of RXRα for PPARγ function,[3,30] this isoform was chosen for additional study. To advance this goal, a competitive RT-PCR assay was developed and validated for quantitative evaluation of RXRα mRNA. The first observation to be made was that there are wide interpersonal variations in the RXRα mRNA content of skeletal muscle. Second, there were no differences in RXRα mRNA between any of the groups and no trends suggesting that differences might become apparent if additional subjects were to be studied. This result is in agreement with the data for RXRα protein expression. There were also no significant differences between groups for RXRβ and RXRγ protein expression. The skeletal muscle content of PPARγ protein, measured in many of the same subjects, also did not differ between groups.[22] However, when those subjects were grouped on the basis of insulin sensitivity, as determined by the hyperinsulinemic/euglycemic clamp, the most insulin-resistant subjects were found to have the greatest PPARγ protein content. No such relationship exists for RXR isoforms, at either the protein or mRNA level. There also appears to be no relationship between RXR isoform and PPARγ protein expression. In light of the fact that RXR partners with a number of nuclear receptors, it is not surprising that its expression is independent from that of PPARγ.

Little is known about the regulation of RXR expression. The lack of correlation of skeletal muscle RXRα, -β, or -γ protein or RXRα mRNA with metabolite (glucose, triglyceride or free fatty acid), or hormone (insulin) levels suggests that other factors are regulating their expression. Yet, in cultured liver cells free fatty acids, alone or in combination with dexamethasone, upregulated RXRα mRNA.[33] Insulin was able to block a portion of this increase,[34] indicating potential tissue specificity of RXRα control. In the same report, maximal effects of free fatty acids on RXRα mRNA were attained by 12 to 24 hours, and increases were detectable within 2 to 4 hours. Lipopolysaccharide treatment of rodents caused a similarly rapid (2 to 4 hours) decrease in the mRNA for RXR isoforms,[35] yet we found muscle RXRα mRNA expression to be unaltered after 3 hours of insulin infusion. This contrasts with the behavior of PPARγ in humans, in which the mRNA content of both skeletal muscle[27] and adipose tissue[36] are increased after acute (11/2 to 3-hour) exposure to insulin, further evidence of a lack of coupling between PPARγ and RXRα expression.

From this first evaluation of RXR expression in human skeletal muscle, we conclude that in this tissue, RXRs may be constitutively expressed proteins or controlled by factors other than glucose, insulin, and free fatty acids. Because RXR is the heterodimeric partner to multiple nuclear receptors that belong

Table 2. Correlation Analysis for RXRα mRNA Levels and RXRα, RXRβ, and RXRγ Protein Expression With Different Clinical Parameters

	RXRα mRNA	RXRα Protein	RXRβ Protein	RXRγ Protein
Age	−.13	−.03	.16	.16
BMI	−.08	−.16	.17	.17
Fasting glucose	.2	−.03	.18	.17
Fasting Insulin	.05	−.05	.02	.02
HbA1C (%)	.23	.10	−.01	−.01
Fasting FFA	−.23	−.42*	−.07	−.07
GDR	−.08	−.05	−.04	.17

NOTE. Data are shown as Pearsons *r* values.
* $P < .05$.

to the class II nuclear receptor family, it is not unexpected that a strict correlation with diabetes or parameters of insulin action could not be found. These data suggest that RXR expression is not involved in the pathogenesis of type 2 diabetes, but leaves open the question about whether RXR agonists could have clinical usefulness as insulin-sensitizing agents.

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