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Determination of PPAR γ Activity in Adipose Tissue and Spleen

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Abstract

Peroxisome proliferator-activated receptor-gamma (PPAR γ) is a nuclear transcription factor that regulates many genes and is involved in extensive biological functions. Accurately determining PPAR γ activity in various tissues is important to understanding mechanisms of human physiology and pathophysiology. Thus, we evaluated a PPAR γ DNA binding immunoassay using nuclear extracts of spleen and adipose tissue from rats treated with rosiglitazone (20 mg/kg in food, 7 days, n=6) or vehicle (n=6) and compared results to mRNA expression of PPAR γ target genes, a well-established method to investigate PPAR γ activity. In adipose tissue, the PPAR γ immunoassay showed that rosiglitazone did not change PPAR γ binding, but qPCR analysis showed that expression of two PPAR γ target genes, CD36 and liver X receptor- α , were significantly increased. In spleen, the PPAR γ immunoassay showed that rosiglitazone decreased PPAR γ binding, but qPCR analysis showed no significant change. The different results obtained between PPAR γ binding immunoassay and target gene expression suggest that PPAR γ immunoassays may not be suitable when used with fresh homogenates of spleen and adipose tissue. Validation of the assay with each individual tissue is recommended.

Keywords

Immunoassay; peroxisome proliferator-activated receptor-gamma; rosiglitazone

INTRODUCTION

Peroxisome proliferator-activated receptor-gamma (PPAR γ) is a nuclear transcription factor that regulates the expression of many genes. PPAR γ is involved in large varieties of biological functions, including lipid and glucose metabolisms, anti-inflammatory effects, and has extensive cardiovascular effects.^[1–5] Synthetic activators of PPAR γ , the thiazolidinediones (TZD), have been used for treatment of type II diabetes since the 1990s.^[6] Determination of PPAR γ activity and expression in various tissues, particularly adipose tissue and spleen, where PPAR γ is highly expressed,^[7, 8] is common in biomedical research and thus important to understanding the mechanisms of physiological processes and disease pathologies.

There are several techniques available to measure PPAR γ in tissue. The more common approaches involve antibody-based methods, including immunohistochemistry, immunofluorescence and Western blotting. These methods can be combined in order to measure PPAR γ protein levels and also provide localization of protein expression within a

tissue. The disadvantages, however, are that specificity of the antibody may limit accuracy and that activity of the transcription factor cannot be determined. Other methods, such as measurement of PPAR γ target gene expression using PCR or a PPAR response element (PPRE) luciferase assay, offer measurement of PPAR γ transcriptional activity. A relatively new DNA binding immunoassay is available (Cayman Chemicals, Ann Arbor, MI, USA) for the measurement of PPAR γ DNA binding activity to PPRE, intended to replace the cumbersome radioactive electrophoretic mobility shift assay. This immunoassay measures the amount of free PPAR γ in nuclear extracts that bind to PPRE pre-coated on the bottom of the plate. The PPAR γ binding activity to PPRE in this immunoassay was tested in adipocyte cell line 3T3-L1 by the manufacturer. Using cell lysates of 3T3-L1, the immunoassay detected PPAR γ binding in as little as 1 μ g total protein. However, the use of this immunoassay has been limited in animal tissue^[9, 10] and has not been validated to measure PPAR γ activity. Thus, the aim of this study was to validate the result from this assay using nuclear extracts from homogenates of two types of tissue, spleen and adipose, where PPAR γ is highly expressed.^[7, 8] Results from this assay were compared to mRNA expression of PPAR γ target genes, which has been used extensively for determination of PPAR γ transcriptional activity in several tissues.^[11, 12] In order to ensure increased PPAR γ activity, we harvested tissues from animals treated with a PPAR γ activator rosiglitazone or vehicle for 7 days and compared the results from these two methodologies.

MATERIALS AND METHODS

Animals and Treatment Groups

All procedures were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Female virgin Sprague Dawley rats (250–300 g, 14–16 weeks, Charles River, Canada) were used for all experiments and housed in the University of Vermont Animal Care Facility. Animals were randomly selected and grouped as rosiglitazone (20 mg/day, in food, n=6), or vehicle treatment (n=6) for 7 days.

Purification of Tissue Nuclear Extract

As the majority of PPAR γ protein is localized in the nucleus, the first procedure was to isolate and purify nuclear protein from fresh tissues. Animals were anesthetized with isoflurane (3 % in oxygen), decapitated, and the adipose tissue around the mesenteric beds and spleen were quickly removed and placed in cold phosphate buffered saline (PBS). Nuclear extracts were obtained using a commercial Nuclear Extraction Kit (#10009277, Cayman Chemical, Ann Arbor, MI, USA), recommended by the manufacturer for the use with the PPAR γ immunoassay. All procedures were performed on ice (4 °C) unless stated otherwise. Briefly, adipose or spleen tissues were homogenized with a Dounce homogenizer in hypotonic buffer (3 ml) and incubated for 15 min. The homogenate was centrifuged in a pre-chilled tube at 300 $\times$ g for 10 min. To ascertain the cells were lysed, the cell pellet was gently resuspended in 500 μ l hypotonic buffer and incubated again for 15 min. The homogenate was then centrifuged at 14,000 $\times$ g for 30 s and supernatant discarded. The pellet was resuspended in 50 μ l extraction buffer (with protease and phosphatase inhibitors). The resulting aliquot was vortex for 15 s and incubated for 15 min on a shaking platform. The aliquot was then vortex for 30 s and incubated for another 15 min. After the nuclear extraction procedure, the aliquot was centrifuged at 14,000 $\times$ g for 10 min. The supernatant (nuclear extract) was collected, snap frozen with liquid nitrogen and stored –80 °C.

Determination of Nuclear Protein Content

Total protein of nuclear extracts of spleen and adipose tissue was determined by Protein Determination Kit based on the Bradford method (#704002, Cayman Chemical). Because

the recommended range of detection is 5.6 to 32 $\mu\text{g/ml}$ protein, we determined the proper dilution of the nuclear extracts by a trial run. The dilution factor was 500 for spleen and 200 for adipose tissue. We performed a protein assay to determine the amount of total protein in nuclear extracts. This result was used to standardize the amount of total protein in the nuclear extract that was added to the PPAR γ immunoassay (100, 200 or 300 μg). We then ran the protein assay again to compare this measured protein content to the calculated values.

Determination of PPAR γ Activity Using Immunoassay

To determine the effect of rosiglitazone on PPAR γ activity, PPAR γ binding to PPRE was determined by a PPAR γ Transcription Factor Assay Kit (#10006855, Cayman Chemical). PPRE was pre-coated at the bottom of each well by the manufacturer for capturing free PPAR γ in samples. The volume of nuclear extracts that was spiked in the assay was adjusted based on protein content of individual samples. Based on the results from the protein assay, nuclear protein was adjusted to 7.7, 38.5, and 231.0 μg for spleen and 9.1 and 45.5 μg for adipose tissue. We did not perform adipose samples at higher nuclear protein content because we did not have enough sample of this tissue. All sample volumes were adjusted to 100 μl with a Complete Transcription Factor Binding Assay Buffer. After incubation of samples overnight at 4 $^{\circ}\text{C}$, wells were washed 5 times with 200 μl of wash buffer, followed by adding 200 μl of PPAR γ primary antibody. Samples were then incubated at room temperature for 1 hr, wells were washed and 100 μl of goat anti-rabbit HRP conjugate was added. After incubation for 1 hr, wells were washed and 100 μl of developing solution was added. The plate was incubated at room temperature with gentle agitation protected from light. Absorbance at 655 nm was checked periodically for proper color development. When the positive control wells approaching over-development (about 45 min), 100 μl of stop solution was added and absorbance was read at 450 nm within 5 min.

Determination of PPAR γ Target Gene Expression

To determine the effect of rosiglitazone on PPAR γ transcriptional activity, expression of PPAR γ target gene fatty acid translocase (FAT) (also known as CD36) and liver X receptor- α (LXR α) was determined in adipose and spleen tissues. Expression of these PPAR γ target genes has been shown to be increased by rosiglitazone.^[12–14] All collected samples were stored in RNase inhibitor (1 unit/ μl , RiboLock, Fermentas, Glen Burnie, MD, USA) at -80°C . Standard techniques for real-time qPCR were performed by the Vermont Cancer Center DNA analysis facility at the University of Vermont, as described previously.^[15] Briefly, RNA was extracted from tissues and concentration and integrity was determined by a NanoDrop ND-1000 Spectrophotometer (Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Santa Clara, SA, USA), respectively. Samples with low RNA quality were excluded. cDNA was then made by a SuperScript III Kit (Invitrogen, Carlsbad, SA, USA). Real-time qPCR was set up as follows: 10 μL Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 1 μL Assay on Demand (Applied Biosystems), 8 μL water, and 1 μL cDNA. β_2 -microglobulin (B2M, housekeeping control) and target genes were assessed using Assays on Demand from Applied Biosystems. All primers were validated by the manufacturer for efficiency and did not detect homologs. Primers were designed across an exon-exon junction to avoid detecting genomic DNA. Thus, no DNase treatment of samples was necessary. All samples were run in duplicates using a 7900HT Sequence Detection System (Applied Biosystems). The PCR was cycled for 2 minutes at 50 $^{\circ}\text{C}$, 10 minutes at 95 $^{\circ}\text{C}$, 40 cycles at 95 $^{\circ}\text{C}$ for 15 seconds and then 60 $^{\circ}\text{C}$ for 1 minute.

Data Calculations and Statistical Analysis

Data from qPCR study were analyzed using the $-2^{\Delta\Delta CT}$ method, as described previously.^[16] Data were removed when the CT values of technical replicates differed by more than 0.5. All data are presented as mean $\pm$ SEM. Differences between groups were determined with Students' t-test for two groups or one-way analysis of variance and a post hoc Newman-Keuls test for multiple comparisons for 3 groups or more using Graph Pad Prism 5 (Graph Pad Software Inc., La Jolla, CA, USA). Differences were considered significant when $p < 0.05$.

RESULTS

Total Nuclear Protein in Adipose and Spleen Tissues

Figure 1 shows total nuclear protein determined based on the Bradford method in spleen and adipose tissue. Spleen had substantially more nuclear protein than that of adipose tissue. Rosiglitazone did not affect total nuclear protein in spleen or adipose tissue. Because the accuracy of the protein assay is critical in the PPAR γ immunoassay, we compared the measured protein content to the calculated values (Figure 2). We found that there was ~75 to 80 % of calculated nuclear protein of 100 to 300 μ g in spleen and ~85 to 95 % in adipose tissue. Taken all 3 protein contents together, there was 77 ± 2 % and 91 ± 3 % of calculated protein content in spleen and adipose tissue, respectively. Because the measured protein contents were substantially lower than the calculated values, we used these data to calculate the actual nuclear protein added to the PPAR γ immunoassay.

Determination of PPAR γ Activity Using DNA Binding Immunoassay

Figure 3 shows PPAR γ activity determined by the PPAR γ binding immunoassay. We selected samples with similar amount of nuclear protein to minimize variability. PPAR γ activity increased with total nuclear protein of spleen from vehicle-treated group, although the increase in PPAR γ activity was not in the same proportion with the amount of nuclear protein in the sample (Figure 3A). Rosiglitazone treatment significantly decreased PPAR γ activity in spleen regardless of protein added ($p < 0.05$) and PPAR γ activity remained similar despite increased nuclear protein. On the contrary, PPAR γ activity was low in adipose tissue (Figure 3B) and ~10-fold lower when compared to similar nuclear protein in spleen. PPAR γ activity was not positively correlated to nuclear protein content and rosiglitazone did not affect PPAR γ activity in adipose tissue, according to this assay.

Determination of PPAR γ Target Gene Expression

To confirm whether rosiglitazone increased transcriptional activity of PPAR γ that was not detected by the immunoassay, we determined PPAR γ target gene expression in spleen and adipose tissue from the same animals and the results are shown in Figure 4. Rosiglitazone significantly increased expression of CD36 (Figure 4A) and LXRA (Figure 4B) in adipose tissue ($p < 0.05$). Rosiglitazone also increased CD36 or LXRA expression in spleen, but this was not statistically significant.

DISCUSSION

Accurately determining of PPAR γ activity in various tissues, particularly in spleen and adipose tissue where PPAR γ is highly expressed,^[7, 8] is critical in understanding the role of PPAR γ in many biological processes. In this study, using a newly developed PPAR γ immunoassay, we found that rosiglitazone, a widely used PPAR γ activator, unexpectedly decreased and had no impact on PPAR γ activity in spleen and adipose tissue, respectively. On the contrary, using qPCR, we found that rosiglitazone increased PPAR γ target gene expression significantly in adipose tissue and nonsignificantly in spleen. Because it is

expected that rosiglitazone would increase expression of PPAR γ target genes as the qPCR showed, it does not appear that the PPAR γ DNA binding immunoassay provided accurate results in either tissue.

PPAR γ is highly expressed in adipose tissue and TZD treatment increases PPAR γ target gene expression.^[13, 14] The qPCR results of the present study agree with these previous findings, however, similar results were not obtained from the PPAR γ immunoassay. There are a few possibilities for this discrepancy. First, unlike using adipocytes culture, the amount of PPAR γ in the nuclear extract from adipose tissue may have been very low and close to the detection limit of the immunoassay. Changes of PPAR γ binding to PPRE may not be detectable in such a low signal. Second, the PPAR γ immunoassay was designed to determine human PPAR γ DNA binding. Although species and tissue specificity of the assay were demonstrated in cell lines and other tissues from rat and mouse,^[9, 10, 12] the assay may not have high specificity in nuclear extract from fresh adipose tissue. Third, this immunoassay only measures PPAR γ DNA binding to PPRE, but not a direct determination of PPAR γ transcriptional activity. The affinity of PPAR γ binding to PPRE may be affected by conditions in the buffer solution, such as pH and salt concentration, which may be altered by nuclear extracts from different kinds of tissues. Lastly, it has been suggested that PPAR γ is also distributed throughout the cytosol of adipocyte,^[17] thus we may not be able to detect rosiglitazone-induced increase in PPAR γ activity because we only determined activity in nuclear extracts.

In addition to adipose tissue, PPAR γ is also highly expressed in spleen.^[8] However, the role of PPAR γ in spleen is less studied, with studies mainly focused on the anti-inflammatory properties of PPAR γ .^[18–20] To our knowledge, the present study was the first to use this PPAR γ immunoassay in spleen tissue. We showed that rosiglitazone decreased PPAR γ binding to PPRE. Previous study showed that rosiglitazone decreased spleen weight.^[21] It is therefore possible that the decreased PPAR γ activity detected with the immunoassay was due to migration of PPAR γ -containing immune cells out of the spleen and/or PPAR γ -induced inhibition of T-cell proliferation.^[18] This would suggest that PPAR γ activation may decrease PPAR γ protein in spleen, however, we did not find a decrease in protein content with rosiglitazone treatment. In addition, qPCR analysis suggested that PPAR γ transcriptional activity was increased, but not statistically significant. The difference between the result obtained from immunoassay and qPCR is unknown. One possibility is that the rosiglitazone-induced increase in PPAR γ transcriptional activity may be masked by the various cell types in spleen, which may have differential PPAR γ activity and sensitivity to rosiglitazone.

Results from the present study showed that investigators must be cautious when using a PPAR γ immunoassay, particularly with fresh tissue homogenates. First, measurement of total nuclear protein is critical to accurately determine how much nuclear protein is added into the PPAR γ immunoassay. The Bradford protein assay is recommended by the manufacturer and other studies,^[22] but other protein assays may have advantages that investigators may also consider.^[22, 23] Second, in order to have an appropriate signal, sufficient amount of nuclear protein must be added into the assay with minimal changes to the optimal condition of the reaction mixture for PPAR γ binding to PPRE. This can be achieved by having a more concentrated nuclear extract. This is particularly important when using tissues where PPAR γ is not highly expressed. However, increased nuclear protein content in the sample may interfere with PPAR γ binding to PPRE, as suggested by our result that increased total protein did not proportionally increase the signal of the assay. Thus, an optimal amount of nuclear protein should be used for the immunoassay. Third, the manufacturer only provides a sample of cell lysate as positive control but did not include a standard curve. This may be because endogenous coactivator and corepressor of PPAR γ in

different kinds of nuclear extract samples make comparison to the standard curve not appropriate. But the disadvantage is that the immunoassay is only semi-quantitative. Lastly, the validity of the results in different tissue homogenates should be confirmed with another approach, such as qPCR analysis of PPAR γ target genes. The advantage of using the PPAR γ immunoassay is the ability to determine PPAR γ activity, while other methods, such as determination of PPAR γ mRNA or protein in cells or tissues reported in other studies, only examined expression levels.^[24, 25] With appropriate target gene expression study as validation, the results have implications on the biological effects of PPAR γ in a particular cell type or tissue.

In conclusion, while a PPAR γ transcription factor immunoassay can be used with cell lysates, investigators should be cautious when using with fresh tissue homogenates. Validation of the results in each tissue and comparison to other methods, such as qPCR, are recommended.

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Figure 1.

Graph showing total nuclear protein of spleen and adipose tissues from vehicle- or rosiglitazone (Rosi)-treated animals. Note that rosiglitazone did not significantly affect total nuclear protein in either tissue.

Figure 2. Graph showing the percentage of measured versus calculated protein content in spleen and adipose tissues. Note that the measured protein contents were between 75 to 95 % of the calculated values.

Figure 3.

Graph showing PPAR γ DNA binding activity in nuclear extractions of A) spleen and B) adipose tissue from vehicle- or rosiglitazone (Rosi)-treated animals. Note that PPAR γ activity increased with total protein only in spleen from vehicle-treated rats. Rosiglitazone significantly decreased PPAR γ activity in spleen, but had no effect in adipose tissue.

*p<0.05 versus corresponding vehicle group.

Figure 4. Graph showing relative quantification (RQ) of mRNA expression of A) adipose CD36, B) adipose liver X receptor- α (LXR α) C) spleen CD36, and D) spleen LXR α from vehicle- or rosiglitazone (Rosi)-treated animals. *p<0.05 versus vehicle group. Note that the expression of both PPAR γ target genes was significantly increased by rosiglitazone treatment.