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PPAR γ Activation Induces Autophagy in Breast Cancer Cells

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Abstract

It has been previously shown that PPAR γ ligands induce apoptotic cell death in a variety of cancer cells. Given the evidence that these ligands have a receptor-independent function, we further examined the specific role of PPAR γ activation in this biological process. Surprisingly, we failed to demonstrate that MDA-MB-231 breast cancer cells undergo apoptosis when treated with sub-saturation doses of troglitazone and rosiglitazone, which are synthetic PPAR γ ligands. Acridine orange (AO) staining showed acidic vesicular formation within ligand-treated cells, indicative of autophagic activity. This was confirmed by autophagosome formation as indicated by redistribution of LC3, an autophagy-specific protein, and the appearance of double-membrane autophagic vacuoles by electron microscopy following exposure to ligand. To determine the mechanism by which PPAR γ induces autophagy, we transduced primary mammary epithelial cells with a constitutively active mutant of PPAR γ and screened gene expression associated with PPAR γ activation by genome-wide array analysis. HIF1 α and BNIP3 were among 42 genes up-regulated by active PPAR γ . Activation of PPAR γ induced HIF1 α and BNIP3 protein and mRNA abundance. HIF1 α knockdown by shRNA abolished the autophagosome formation induced by PPAR γ activation. In summary, our data shows a specific induction of autophagy by PPAR γ activation in breast cancer cells providing an understanding of distinct roles of PPAR γ in tumorigenesis.

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INTRODUCTION

The peroxisome proliferator-activator receptors are a family of ligand-activated nuclear receptors that include PPAR α , PPAR γ and PPAR δ (Rosen and Spiegelman, 2001). Upon ligand activation, PPAR γ forms a heterodimer with retinoid X receptor (RXR) at peroxisome proliferator responsive element (PPRE) of targeted gene promoters. PPAR γ is expressed mainly in adipose tissue and perform an important role in lipid metabolism and adipocyte differentiation. Ectopic PPAR γ expression promoted NIH-3T3 cell adipogenesis (Tontonoz et al., 1994) and overexpression of PPAR γ in transgenic mouse liver induced hepatic steatosis (Yu et al., 2003). The PPAR γ ligands include naturally occurring fatty acids and the thiazolidinedione (TZD), such as troglitazone and rosiglitazone (BRL 49653).

The role of PPAR γ in tumorigenesis is conflicting. PPAR γ is expressed in breast, prostate and colonic epithelium and addition of ligands to cultured cancer cell lines derived from these tissue types inhibits cellular proliferation (Brockman et al., 1998, Mueller et al., 1998, Elstner et al., 1998a, Ricote et al., 1998, Sarraf et al., 1998, Wang et al., 2001), suggesting a role of PPAR γ as a tumor suppressor. This was further supported by *in vivo* studies using animal models of NMU-induced mammary tumorigenesis in rats showing PPAR γ agonists prevented the development of tumors (Suh et al., 1999) and in another study showing DMBA-induced mammary tumorigenesis was inhibited by troglitazone (Pighetti et al., 2001). In contrast, PPAR γ ligands increased polyp numbers in *Apc^{Min}* model of familial adenomatosis (Lefebvre et al., 1998, Saez et al., 1998), raising the possibility that PPAR γ may serves as collaborative oncogene under certain circumstance. The constitutively active PPAR γ , when targeted to mammary epithelium, enhanced mammary tumorigenesis induced by polyoma middle T antigen (Saez et al., 2004), which perhaps is related in part to the finding that PPAR γ inhibits β -catenin abundance in the presence of wild-type APC (Saez et al., 1998).

Although the precise role of PPAR γ in tumorigenesis needs to be further investigated, it has been well documented that PPAR γ ligands inhibit cellular proliferation and induce cell death in various cancer cell types (Elstner et al., 1998b, Clay et al., 1999, Nwankwo and Robbins, 2001, Martelli et al., 2002, Shimada et al., 2002, Toyoda et al., 2002, Wang et al., 2002, Yoshizawa et al., 2002) including breast cancer. PPAR γ targets cell cycle regulators including cyclin D1 to reduce the cell proliferation rate, however, the mechanism by which ligand activated PPAR γ induced cell death is still unclear. Given the fact that 15d-PG-J2 also functions independent of PPAR γ activation (Chawla et al., 2001, Clay et al., 2002, Peraza et al., 2006), investigators tested the hypothesis that PPAR γ mediates ligand-induced apoptosis. Surprisingly, both chemical antagonist and dominant negative mutants of PPAR γ failed to rescue the apoptotic cell death induced by 15d-PG-J2 (Clay et al., 2002). A subsequent study demonstrated that decreased viability and enhanced apoptosis in MCF-7 cells treated with 15-PG-J2 were accompanied by an impairment of mitochondrial function and increased ROS production (Pignatelli et al., 2001). The induction of cell death by synthetic ligands such as troglitazone and rosiglitazone was only seen when cells were treated with either over-saturation doses (Yu et al., 2008, Mody et al., 2007) or in combination with other agents (Mody et al., 2007).

In the present study, we show that PPAR γ activation induces autophagy, a vacuolar process responsible for bulk protein or cytoplasmic organelle degradation, through transcriptional up-regulation of HIF1 α and BNIP3 expression.

MATERIALS AND METHODS

Preparation of primary mammary epithelial cells (MECs) and cell culture conditions

Mouse mammary glands were removed from 8-10 weeks old female FVB mice. Glands were minced with scalpel blades to an average fragment size of 1mm³ in the presence of 1.0 mg/ml of collagenase (Sigma, type 3) in DMEM supplemented with 10% fetal calf serum, 5 µg/ml bovine insulin, 10 ng/ml mouse EGF and 1X penicillin/streptomycin followed by collagenase digestion. Organoids attached to plastic dishes and grew as a mammary epithelial cell monolayer. Fibroblast contamination was removed by brief trypsin digestion. Cell monolayers were then trypsinized and seeded at 50% percent confluence for viral infection.

HEK293T, MCF-7, MDA-MB-231 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 1X penicillin/streptomycin and supplemented with 10% fetal bovine serum (Wang et al., 2003) in humidified atmosphere with 5% CO₂ at 37°C. MCF10A cells were cultured in DMEM/F12 supplemented with 5% horse serum, 5% fetal bovine serum, 5 µg/ml bovine insulin, 10 ng/ml recombinant human EGF. 24 hours before PPAR γ ligand treatment, cells were maintained in phenol red-free medium supplemented with 5% charcoal-dextran stripped fetal bovine serum.

Plasmids

The luciferase reporter constructs containing the multimeric acetyl CoA oxidase PPAR γ response element (AOx)₃-TK-Luc) was previously described (Wang et al., 2003). HIF1 α promoter reporter was a gift from Dr. Gregg L. Semenza (Hirota et al., 2004), and BNIP3 promoter reporter was kindly provided by Dr. Andrea Bacon (Bacon and Harris, 2004). The pshRNA against HIF1 α was a gift from Holger K. Eltzschig (Morote-Garcia et al., 2008). GFP-LC3 cDNA was amplified by PCR from pEGFP-LC3 vector provided by Dr. Noboru Mizushima and subcloned into retro-viral vector MSCV-IRES-GFP to substitute GFP cDNA from the vector. Mouse PPAR γ cDNA was subcloned into pACT vector (Promega) in order to express the VP16-PPAR γ fusion protein (P γ CA). Both cDNAs for PPAR γ and P γ CA were subsequently subcloned into MSCV-IRES-GFP, which were designated respectively as MSCV-PPAR γ -IRES-GFP and MSCV-P γ CA-IRES-GFP.

Luciferase reporter assays

MDA-MB-231 cells were seeded at a density of 1.5×10⁵ cells per well respectively in DMEM in a 24-well plate on the day prior to transfection. The following day the cells were transiently transfected with the appropriate combination of the reporter, expression vectors, and control vector with Superfect (Qiagen, Valencia, CA) or Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 5 hours post-transfection, cell culture medium was replaced with fresh medium with ligand or vehicle control. 24 hours post transfection, luciferase assays were performed at room temperature using an Autolumat LB 953 (EG&G Berthold) as previously described (Wang et al., 2003).

Western blot

Whole cell lysates (50 µg) were separated by SDS-PAGE and the proteins transferred to nitrocellulose membrane for Western blotting as previously described (Bromberg et al., 1999). The following antibodies were used for protein detection: α -Bcl-xL, α -cytochrome C, α -HIF1 α , α -BNIP3; guanine nucleotide dissociation inhibitor (GDI) (Lee et al., 1999) and vinculin (Santa Cruz) were used as internal protein loading control.

TUNEL staining

TUNEL staining was conducted using a kit according to the manufacturer's instructions (TACS 2 TdT in situ apoptosis detection kit; Trevigen, Inc., Gaithersburg, MD). TUNEL-positive nuclei were scored under a microscope using a 20x objective. Percentage of TUNEL-positive cells was plotted.

Retrovirus preparation and infection

The MSCV-IRES-GFP retrovirus vector and the ecotropic, packaging vector pSV- ψ -E-MLV, which provides ecotropic packaging helper function and infection methods were as described previously (Neumeister et al., 2003). Briefly, the coding region of mouse PPAR γ cDNA, or PyCA (Saez et al., 2004) cDNAs (Wang et al., 2003) was linked in frame to 3 $\times$ FLAG and were inserted into the MSCV-IRES-GFP vector at the *EcoRI* site upstream of the IRES driving expression of GFP. MSCV retroviruses were prepared by transient cotransfection with helper virus into HEK293T cells, using calcium phosphate precipitation. The retroviral supernatants were harvested 24 hours after transfection and filtered through a 0.45 μ m filter. Mammary epithelial cells were incubated with fresh retroviral supernatants in the presence of 8 μ g/ml polybrene for 48 hours.

Transmission electron microscope

Isolated cells were pelleted and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 for 30 minutes at room temperature. Cell pellets were post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4 for 1 hr, followed by dehydration through graded alcohols and propylene oxide. The samples were embedded in EMbed 812 (Electron Microscopy Sciences, Hatfield, PA). Thin sections were cut on an UltraCut E ultramicrotome and stained with uranyl acetate and lead. A modified method was used for lead staining of thin sections (Sato, 1968). Images were collected with an AMT XR41-B 4 megapixel camera on a Hitachi H-7000 electron microscope.

Quantitative PCR

Total RNA was prepared using TriZol reagents (Invitrogen, Carlsbad, CA) by following the manufacturer's manual. 5 μ g of total RNA was subjected to reverse transcription to synthesize cDNA using the SuperScriptTM II Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA). A 25 μ l volume reaction consisted of 1 μ l reverse transcription product and 100 nM of each primer. The 7900 HT Sequence Detection System (Applied Biosystems) was used for quantitative PCR assay. The primer sequences for BNIP3 and HIF1 α were previously described (Ishida et al., 2007, Lang et al., 2007).

Microarray analysis

Total RNA was isolated from primary mouse mammary epithelial cells (MECs) infected with retrovirus vector MSCV-PPAR γ -IRES-GFP, MSCV- PyCA-IRES-GFP or MSCV-IRES-GFP along) using Trizol and used to probe Affymetrix MU74v2 arrays (Affymetrix, Santa Clara, CA). RNA quality was determined by gel electrophoresis. Probe synthesis and hybridization were performed according to the manufacturer's manual (see eukaryotic target preparation section at <http://www.affymetrix.com/support>). Three arrays were used for each condition. Analysis of the arrays was performed using the statistical package R statistics package (Chen et al., 2002) and the limma library (Smyth, 2004) of the Bioconductor software package. Arrays were normalized using Robust Multiarray Analysis (RMA) and the genes were ranked using the log odds ratios for differential expression (Boutros et al., 2004, Renn et al., 2004). The top ranked genes that are differentially expressed in a PyCA-dependent manner were determined based on their log-odds ratio ($B > 3$). These genes were then clustered using hierarchical

clustering with “complete” agglomeration and each cluster was further analyzed based upon known function of the genes contained in the cluster.

RESULTS

PPAR γ activation does not associate with apoptotic cell death in breast cancer cells

Although the results from several laboratories indicate that PPAR γ ligands induce apoptosis (Elstner et al., 1998b, Clay et al., 1999, Nwankwo and Robbins, 2001, Martelli et al., 2002, Shimada et al., 2002, Toyoda et al., 2002, Wang et al., 2002, Yoshizawa et al., 2002), whether this occurs strictly through PPAR γ activation remains to be determined. It has previously been reported that 15d-PG-J2 induction of apoptosis in breast cancer cells does not require PPAR γ activation (Clay et al., 2002). Several studies using thiazolidinediones demonstrated these agents induce cell death at over-saturation doses (Yu et al., 2008, Mody et al., 2007), raising the possibility that PPAR γ -unrelated pathways are involved. We examined the effect of troglitazone (Tro.) and rosiglitazone (Rosi.), both synthetic ligands of PPAR γ , in MDA-MB-231 breast cancer cells. Since the concentration of troglitazone used in previous studies ranges from below 1 μ M to 100 μ M, we first optimized the concentration of troglitazone and rosiglitazone in MDA-MB-231 cells for maximal induction of PPAR γ transactivation as measured by luciferase reporter assay system (AOx)₃-TK-Luc. Troglitazone and Rosiglitazone induced reporter activity in a dose dependent manner and the dose for full activation of PPAR γ reporter was observed at 10 μ M and 20 μ M respectively (data not shown). Consistent with previous findings we also showed that apoptosis was induced by PPAR γ ligands at a dose, which is 5-10 times higher than that required for full activation of PPAR γ (Fig. 1A). We failed to observe a significant increase of apoptotic cell death as measured by TUNEL staining when cells were treated with 5 μ M of troglitazone or 10 μ M of rosiglitazone.

PPAR γ induces autophagy

Given the fact that autophagy is required for necrotic cell death, we next examined the effect of troglitazone on autophagy. The development of acidic vesicular organelles (AVOs) in the cell cytoplasm is one of the hallmarks of autophagy. Acridine orange (AO) stains the acidic compartment within the cell. MDA-MB-231 cells were maintained in phenol red-free DMEM supplemented with 5% charcoal-dextran stripped fetal bovine serum for 24 hours. 5 μ M troglitazone was added for 24 hours. AO staining of MDA-MB-231 cells treated with troglitazone clearly showed AVOs within the cytoplasm compared to cells treated with vehicle control (DMSO) (Fig. 2A). To determine whether the AVOs were caused by autophagy, cells were pre-incubated with 3-methyladenine (3-MA), a known inhibitor of autophagy through repression of phosphatidylinositol-3 kinase (PI3K). We observed that 3-MA completely abolish the troglitazone-induced AVOs (Fig. 2A).

Microtubule-associated protein 1 light-chain 3 (LC3) is a specific membrane marker for the detection of early autophagosome formation. GFP-LC3 was developed to monitor autophagosome in real time, which can be recognized as punctate dots or ring-shaped structures by fluorescence microscopy. Accordingly, we have developed retroviral expression vectors encoding GFP-LC3 and generated a stable MDA-MB-231 cell line (designated as 231/GFP-LC3) that ectopically expresses the fusion protein. We have confirmed that GFP-LC3 specifically relocates to the autophagosome membrane to form ring-shaped structures upon induction of autophagy by culturing cells in nutrition-free, balanced salt solution (data not shown). By treating 231/GFP-LC3 cells with rosiglitazone under the same condition as described earlier, the ring-shaped structures representing autophagosomes were observed (red arrows point to the “ring”) (Fig. 2B). We further confirmed this observation with electron microscopic (EM) analysis, which showed accumulation of double membrane-bound autophagosome vesicles in cells treated with PPAR γ ligand (Fig. 3A).

PPAR γ is required and sufficient for ligand-induced autophagy

We have shown here that PPAR γ ligands induce autophagy, however it is still unclear whether this biological process occurs exclusively through PPAR γ activation. We next determined whether ligand-induced autophagy is mediated by PPAR γ activation. Retroviral vector expressing constitutive active mutant PyCA was used in autophagy analysis to eliminate non-specific effects of ligands. When we expressed PyCA in MCF10A cells, we observed a dramatic increase in the number of cells forming AVOs (Fig. 3B). In summary, we have shown that PPAR γ activation is sufficient for autophagy induction in breast cancer cells.

Profiling of PPAR γ target genes in mammary epithelial cell

Genetic profiles regulated by PyCA were assessed by genome-wide array analyses, which allow us to determine the molecular mechanisms by which activated PPAR γ functions to promote autophagy at a higher level of resolution. Primary murine mammary epithelial cells were prepared from FVB mice and transduced by either PPAR γ or active PPAR γ , the molecular genetic pathways regulated by these two related molecules in primary epithelial cells, showed differential regulation and quantitative gene expression amongst approximately 42 up-regulated genes (Fig. 4A). Functional annotation of the genes differentially regulated by the active PPAR γ included angiogenesis (Angiopoietin-like 4, HIF1 α , BNIP3), growth and proliferation (dual specificity phosphatase 9, MKP9, prolactin receptor, proliferin, proliferin 2), metabolism (lipoprotein lipase, fatty acid binding protein 4, adipocyte, stearyl-Coenzyme A desaturase 1, microsomal glutathione, S-transferase 3, acetyl-Coenzyme A, acetyltransferase1, pyruvate dehydrogenase kinase, isoenzyme 4,3-Ketoacyl-CoA thiolase peroxisomal precursor) and genes involved in cellular adhesion and migration (Keratin complex 2, basic, gene 6A, CEA-related cell adhesion molecule 1, integral membrane protein 2A) (Fig. 4B).

HIF1 α and BNIP3 are transcriptional targets of PPAR γ

BNIP3 (BCL2/adenovirus E1B 19kDa interacting protein 3) is a mitochondrial protein. Through forming heterodimers with Bcl-2/Bcl-x(L), BNIP3 induces caspase-independent autophagic cell death by permeabilizing the mitochondrial membrane. Both BNIP3 mRNA and protein levels increase in response to hypoxia, which is mediated through activated hypoxia-inducible factor 1 (HIF1 α). Quantitative PCR was performed to confirm that BNIP3 and HIF1 α are bona fide targets of activated PPAR γ using MDA-MB-231 cells treated with 20 μ M rosiglitazone (Fig. 5A). This was further confirmed by Western Blot showing both troglitazone and rosiglitazone induced BNIP3 and HIF1 α expression in a dose dependent manner (Fig. 5B). To avoid receptor-independent effects of ligand, we used active PPAR γ (PyCA) to determine the specific effect of PPAR γ activation, which was associated with the induction of BNIP3 expression. (Fig. 5C). To determine if activated PPAR γ regulates BNIP3 and HIF1 α at the transcriptional level, luciferase gene reporter assays were conducted. MDA-MB-231 cells were transfected with reporter plasmid and treated with troglitazone or rosiglitazone for 24 hours. Both BNIP3 and HIF1 α promoter reporters were induced by PPAR γ ligands (Fig. 5D).

HIF1 α is required for PPAR γ -induced autophagy

It has been previously determined that overexpression of HIF1 α is required for hypoxia-induced autophagy (Bellot et al., 2009). Given the ability of PPAR γ to promote autophagy and to induce HIF1 α expression, we sought to determine whether HIF1 α expression is required for PPAR γ -induced autophagy. MDA-MB-231 cells were transduced with pshRNA against HIF1 α . Knockdown of HIF1 α expression (Fig. 6A) inhibited punctate GFP-LC3 distribution induced by PPAR γ ligand (Fig. 6B), suggesting that HIF1 α is essential for autophagy induced by PPAR γ activation.

DISCUSSION

In this study, we report the PPAR γ ligands induce autophagy in MDA-MB-231 breast cancer cells by up-regulating the expression of HIF1 α and BNIP3. Autophagy is a ubiquitous process where cytoplasmic materials or organelles are sequestered within double membraned vacuoles, called autophagosomes. Under certain circumstances, autophagy is critical for maintaining cell viability. However, prolonged activation of the autophagy program can eventually lead to cell death independent of apoptosis.

The biological interplay of cellular proliferation, migration, apoptosis, and angiogenesis in the context of tumorigenesis is very well characterized; targeting these processes is the goal of developing new therapeutic agents. Tumorigenesis is a multi-step process involving genetic alterations that lead to tumor initiation and progression. The biological relevance of autophagy during tumorigenesis is controversial, reflecting the complexity of this biological process (Shintani and Klionsky, 2004, Hippert et al., 2006). In many reports, induction of autophagy suppresses tumor formation, which may provide a useful way for preventing tumor initiation, limiting cancer progression, and increasing the efficacy of cancer treatments particularly in cancer cells where apoptotic cell death is defective. Many agents including rapamycin, tamoxifen, and histone deacetylase inhibitors have been reported to induce autophagic cell death (Hoyer-Hansen et al., 2005, Samaddar et al., 2008, Carew et al., 2007, Hrzenjak et al., 2008), indicating autophagy induction to be an important drugable mechanism for anti-cancer agents. Contradictory results have shown that autophagy may promote tumor growth and survival under conditions of nutrient deprivation and hypoxia in solid tumors (Azad et al., 2008, Cosse and Michiels, 2008, Bellot et al., 2009). Autophagy may protect tumor cells from undergoing cell death in response to treatment with anticancer agents.

The role of PPAR γ in tumorigenesis remains poorly understood. Most *in-vitro* studies have shown that PPAR γ ligands suppress tumor cell growth and induce terminal differentiation of cancer cells and cell death. NMU-induced mammary tumorigenesis is prevented by PPAR γ agonists (Suh et al., 1999) and DMBA-induced mammary tumorigenesis was inhibited by PPAR γ ligand (Mehta et al., 2000, Pighetti et al., 2001, Nicol et al., 2004). Rosiglitazone and troglitazone significantly reduces the growth rate of colon and thyroid tumors (Kato et al., 2002, Matthiessen et al., 2005, Aiello et al., 2006) and genetic removal of one allele of the PPAR γ gene predisposes mice to cancer (Lu et al., 2005). Evidence supporting PPAR γ as a collaborative oncogene includes findings that PPAR γ ligands promote colonic tumor growth in Apc^{Min} mice fed a high fat diet (Saez et al., 1998). Targeted expression of PPAR γ to the mammary gland resulted in tumorigenesis with polyoma middle T antigen (Saez et al., 2004). It was proposed that the tumor promoting effects of active PPAR γ in the mammary epithelium was due to enhanced Wnt signaling (Saez et al., 2004). Elevated expression of HIF1 α , regulated by hypoxia or other stimulus in solid tumor, results in enhanced expression of a number of genes including VEGF, which promotes angiogenesis. Whether induction of HIF1 α and BNIP3 expression is responsible for PPAR γ repression of tumor formation or enhanced tumor growth requires more studies in the future. Clarity in this regard could provide fundamental understanding of the contradictory role of PPAR γ in tumor initiation and progression.

The role of PPAR γ activation in chemoprevention has been investigated in mammary cancer rodent models. PPAR γ ligand GW 7845 significantly reduces carcinogen-induced tumor initiation. Clinical trials were conducted in human cancer, including prostate, breast, and colon, although no beneficial outcome was observed, a study using TZD together with chemotherapeutic agents showed a very promising synergistic effect in repressing tumor cell growth (Girnun et al., 2008, Girnun et al., 2007). Our result indicated that autophagy may sensitize the cancer cells to treatments despite the report showing disruption of autophagy

synergizes with SAHA in provoking apoptotic death in Imatinib-refractory chronic myelogenous leukemia (CML) (Carew et al., 2007).

Autophagy is well recognized as a survival mechanism during conditions of limited nutrient and metabolic stress. Cells utilize autophagy to recycle raw materials through the degradation of cytoplasmic material. PPAR γ promotes cell differentiation in adipocytes as evidenced by accumulation of intracellular triglyceride and the induction of fat-specific markers through insulin-stimulated glucose transport. NIH-3T3 cells ectopically expressing PPAR γ leads to adipogenesis in the absence of insulin-induced glucose transport, suggesting the involvement of other pathways regulated by PPAR γ activation likely exists. Whether this is mediated by autophagy remains to be determined in future studies.

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Figure 1. PPAR γ activation is not required for cell apoptosis

TUNEL staining was performed on MDA-MB-231 cells treated with different doses of PPAR γ ligand for 24 hours. Cells treated with doxorubicin (2 μ M) were included as positive control.

Figure 2. Induction of autophagy by PPAR γ ligands

A). MDA-MB-231 cells treated with troglitazone (5 μ M) for 24 hours were stained with 1 mg/ml Acridine orange (AO) showed acidic vesicular organelles (AVOs). Cells pretreated with autophagy inhibitor 3-methyladenine (3-MA) abolished AVOs formation. B). MDA-MB-231 cells were transduced with MSCV-IRES-GFP or MSCV-IRES-GFP-LC3 followed by 24 hours treatment with 10 μ M rosiglitazone. The ring-shaped structures representing autophagosomes were observed in cells treated rosiglitazone, but not vehicle (DMSO). Area in red box was enlarged and red arrow points to autophagosome.

Figure 3. PPAR γ activation is required and sufficient for autophagy

A). Electron microscopic (EM) analysis was performed on troglitazone-treated MDA-MB-231 cells to confirm the presence of double-membrane autophagosomes. Abundant autophagic vacuoles (arrows) containing cytoplasmic material are present in troglitazone treated cells. The double membrane nature of these autophagosomes can be observed (arrows). Many other vacuoles are also present that contain degraded material most likely representing later stage vacuoles. Bar = 300 nm. B). The constitutively active mutant of PPAR γ (P γ CA) was used to eliminate the potential non-specific effect of PPAR γ ligand. MCF10A cells were transduced with retroviral P γ CA and control vector. Cells were stained with Acridine orange (AO) to show acidic vesicular organelles (AVOs) as indicated by white arrows.

Figure 4. Expression profiling of genes regulated by active PPAR γ

A). Primary mammary epithelial cells were prepared from FVB mice. Cells at passage one were transduced with MSCV-IRES-GFP vector encoding either PPAR γ or P γ CA. Pictures were taken before harvesting; GFP was included to monitor the transduction efficiency. B). Total RNA was prepared and labeled for Affymetrix array hybridization. Comparison was made between PPAR γ and P γ CA. 42 genes were differentially upregulated by active PPAR γ in the absence of troglitazone.

Figure 5. PPAR_γ induction of HIF1α and BNIP-3 expression

A). HIF1α and BNIP3 abundance were determined by real-time PCR. MDA-MB-231 cells were treated with rosiglitazone for 30 hours. B). Western Blot was conducted for HIF1α protein abundance upon rosiglitazone (Rosi.) or troglitazone (Tro.) treatment with increased dose as indicated. C). MCF10A-ErbB2 cells transduced with retroviral vectors expressing PPAR_γ and P_γCA or control. BNIP3 protein expression was determined by Western Blot using specific antibody against BNIP3. D). MDA-MB-231 cells transfected with BNIP3 or HIF1α promoter reporter plasmids were treated with troglitazone or rosiglitazone, luciferase reporter assay was conducted. The PPAR_γ reporter (AOx)₃ was included as positive control for PPAR_γ activation upon ligand treatment.

Figure 6. HIF1 α is required for PPAR γ induction of autophagy

A). HIF1 α shRNA reduced protein abundance in MDA-MB-231/GFP-LC3 cells transduced with the pshRNA against HIF1 α or vector control. B). 231/GFP-LC3 cells were transduced with pshRNA or vector were treated with 10 μ M rosiglitazone. HIF1 α deficiency abolished autophagosome formation induced by PPAR γ activation.