



SPECIAL REPORT

Bisphenol A diglycidyl ether (BADGE) is a PPAR γ agonist in an ECV304 cell line*¹David Bishop-Bailey, ²Timothy Hla & ¹Timothy D. Warner

¹Department of Cardiac, Vascular and Inflammation Research, William Harvey Research Institute, St. Bartholomew's and the Royal London School of Medicine and Dentistry, Charterhouse Square, London, EC1M 6BQ and ²Center for Vascular Biology, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, Connecticut, CT 06030-3505, U.S.A.

Peroxisome proliferator activated receptors (PPAR)s are nuclear transcription factors of the steroid receptor super-family. One member, PPAR γ , a critical transcription factor in adipogenesis, is expressed in ECV304 cells, and when activated participates in the induction of cell death by apoptosis. Here we describe a clone of ECV304 cells, ECV-ACO.Luc, which stably expresses a reporter gene for PPAR activation. ECV-ACO.Luc respond to the PPAR γ agonists, 15-deoxy- $\Delta^{12,14}$ PGJ₂, and ciglitizone, by inducing luciferase expression. Furthermore, using ECV-ACO.Luc, we demonstrate that a newly described PPAR γ antagonist, bisphenol A diglycidyl ether (BADGE) has agonist activities. Similar to 15-deoxy- $\Delta^{12,14}$ PGJ₂, BADGE induces PPAR γ activation, nuclear localization of the receptor, and induces cell death.

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Abbreviations: BADGE, bisphenol A diglycidyl ether; 15d-PGJ₂, 15-deoxy- $\Delta^{12,14}$ PGJ₂; PPAR, peroxisome proliferator-activated receptor; PPRe, PPAR response element; RXR, retinoid X receptor

Introduction Peroxisome proliferator-activated receptors (PPAR) are a family of three nuclear receptors, $-\alpha$ (NR1C1), $-\delta$ (also referred to as NUC1; NR1C2), and $-\gamma$ (NR1C3), which heterodimerize with the retinoid X receptors (RXR; Kliewer *et al.*, 1992). PPAR α is found predominantly in the liver, heart, kidney, brown adipose and stomach mucosa; PPAR γ is found primarily in adipose tissue, where it plays a critical role in the differentiation of pre-adipocytes into adipocytes; while PPAR δ is almost ubiquitously expressed, with a function that is relatively unknown (Kliewer *et al.*, 1994; Mukherjee *et al.*, 1997). PPAR receptors can be activated by a number of ligands (see Bishop-Bailey, 2000), including WY-14643 (selective for PPAR α), the anti-diabetic thiazolidinediones (PPAR γ selective), and a number of eicosanoids, including, 12-HETE, 15-HETE, 13-HODE, and the prostaglandins, PGA₁, PGA₂, PGI₂, and PGD₂ and the PGD₂ dehydration product 15-deoxy- $\Delta^{12,14}$ PGJ₂ (15d-PGJ₂; Forman *et al.*, 1995; Kliewer *et al.*, 1995). Very few PPAR antagonists have been described. One notable exception, BADGE, is a PPAR γ antagonist in 3T3-L1, and 3T3-F442A preadipocyte cells (Wright *et al.*, 2000).

ECV304 cells were considered to be an immortalized version of a human umbilical vein endothelial cell line, though recent evidence suggests they are a derivative of T24 bladder carcinoma cells. ECV304 cells contain PPAR α , $-\delta$, and $-\gamma$, and respond to PPAR γ agonists; these cause receptor activation, translocation of the receptors from the cytoplasm to nucleus, and induce cell death (Bishop-Bailey & Hla, 1999). PPAR activation can be measured by a reporter gene assay, which utilizes the DNA binding site to which all PPAR: RXR heterodimers bind, termed a 'PPAR response element' (PPRE; see Forman & Evans, 1995). In this study we look at the effects of BADGE on a clone of ECV304 cells that stably expresses the rat acyl CoA PPRe linked to drive the expression of

luciferase (Tugwood *et al.*, 1992; Roberts *et al.*, 1998; Issemann & Green, 1990).

Methods *Construction of ECV-ACO.Luc* ECV304 cells have endogenous PPAR receptors, whose activation can be measured by reporter gene assay. In ECV304 cells we have previously used transient transfections with the pACO.g.Luc (Bishop-Bailey & Hla, 1999); a plasmid that contains the rat acyl CoA PPRe linked to drive the expression of luciferase (Tugwood *et al.*, 1992; Roberts *et al.*, 1998; Issemann & Green, 1990). We therefore created a clone of ECV304 cells that stably expresses this reporter gene for PPAR activation. ECV304 were maintained as previously described (Ristimäki *et al.*, 1994). For stable transfections, ECV304 in 10-cm dishes were transiently transfected overnight with 10 μ g pACO.g.LUC, and 1.5 μ g pEGFPN-1, which contains a neomycin resistance cassette, using 12 μ l of NovaFector. Clones were then selected in 1 μ g ml⁻¹ G418 sulphate. Once isolated, clones were seeded in 6- or 24-well plates. After 24 h incubation with agonist (30 μ M ciglitizone) in serum-free medium, cells were lysed with 200 μ l of distilled H₂O for 10–15 min. Luciferase activity was measured in 50–100 μ l of lysates according to the manufacturer's recommended protocol (Promega). This protocol usually gives a basal reading in untreated control cells of 1–10 relative light units. For normalization of luminescence readings, due to the fact that high levels of PPAR γ agonists also induce cell death in ECV304 cells (Bishop-Bailey & Hla, 1999), protein levels were determined in cell lysates using the Bradford assay (Bradford, 1976). The clone with the highest signal with ciglitizone, relative to background basal luciferase activity was subsequently used to study the effects of other PPAR agonists. This clone was termed ECV-ACO.Luc.

For the experiments described in this report, ECV-ACO.Luc were seeded in 24-well plates, such that 24 h later they were approximately 20% confluent; preliminary results suggest this cell density gave optimal agonist induced luciferase induction (data not shown). Carbaprostacyclin or 15d-PGJ₂ were dissolved in ethanol, while ciglitizone or BADGE were

*Author for correspondence.

E-mail: d.bishop-bailey@mds.qmw.ac.uk

dissolved in DMSO. Vehicles did not exceed a final concentration of 0.1%, amounts that had no effect on any end-point measured (data not shown).

Immunofluorescence and cell viability Immunofluorescent staining for PPAR γ was as previously described (Bishop-Bailey & Hla, 1999), and images were taken using a BioRad MRC600 confocal microscope. For viability studies, confluent monolayers of ECV-ACO.Luc in 96-well plates were used. Cell viability was measured after 24 h of drug treatment by the MTT assay as previously described (Mosmann, 1983; Bishop-Bailey & Hla, 1999).

Materials ECV304 were from ATCC (Manassas, VA, U.S.A.), pACO.gLuc was a gift from Dr Ruth Roberts (AstraZeneca, Macclesfield, U.K.), pEGFPN-1 was from Clontech (Palo Alto, CA, U.S.A.), goat anti-PPAR γ was from Santa Cruz (Autogen Bioclear UK Ltd., Wiltshire, U.K.), FITC-conjugated anti-goat IgG was from Cappel (ICN Chemicals, Basingstoke, Hampshire, U.K.), cell culture media and supplements were from Life Technologies Ltd. (Paisley, U.K.), Nova-Fector was from Venn-Nova (Pampono Beach, FL, U.S.A.), ciglitizone was from BioMol (Affiniti Research Products Ltd., Exeter, U.K.), 15d-PGJ₂ and carbaprostacyclin were from Cayman Chemicals (SPI-BIO-Europe, Massy, France), Luciferin, ATP, DTT, and co-enzyme A (Luciferase assay reagents) were from Roche (Roche Diagnostics Ltd., Lewes, U.K.); all other reagents were from Sigma (Poole, Dorset, U.K.).

Results *PPAR γ ligands and BADGE induce luciferase expression in ECV-ACO.Luc cells* Cells were treated with optimum concentrations (data not shown) of a range of PPAR ligands (see Bishop-Bailey, 2000); 15d-PGJ₂ (1 μ M; non specific PPAR ligand), ciglitizone (30 μ M; a thiazolidinedione PPAR γ ligand), carbaprostacyclin (30 μ M; a PPAR α/δ ligand) (Figure 1A), or BADGE (10–100 μ M; Figure 1B). Luciferase enzyme expression was induced by 15d-PGJ₂, ciglitizone, (i.e. activators of PPAR γ) or BADGE, but not carbaprostacyclin (Figure 1). At high concentrations BADGE induced an apparent decrease in luciferase activity. However, under microscopic examination this effect was seen to be due to the induction of cell death.

BADGE induces ECV cell death The PPAR γ agonists 15d-PGJ₂ and ciglitizone, as previously reported for the parental line ECV304 (Bishop-Bailey & Hla, 1999), induce cell death in ECV-ACO.Luc cells (Figure 2). BADGE similarly induced the death of ECV-ACO.Luc cells (Figure 2), with a potency similar to ciglitizone.

BADGE induces nuclear localization of PPAR γ Under control culture conditions, PPAR γ was expressed throughout ECV-ACO.Luc (Figure 3A). Following incubation of ECV-ACO.Luc for 24 h, with either 15d-PGJ₂ (Figure 3B) or BADGE (Figure 3C), PPAR γ became localized to the nucleus with virtually undetectable staining in the cytoplasm. Without primary antibody no specific staining was observed (Figure 3D); results that are consistent, with experiments showing the specificity of this PPAR γ antibody in the parental cell line (Bishop-Bailey & Hla, 1999).

Discussion ECV304 cells express PPAR α , δ , and γ , though responses have only been detected to PPAR γ agonists (Bishop-Bailey & Hla, 1999). We made a clone of ECV304 that stably expresses the PPARE of the rat acyl CoA oxidase promoter linked to drive luciferase expression (pACOg.Luc; Roberts et

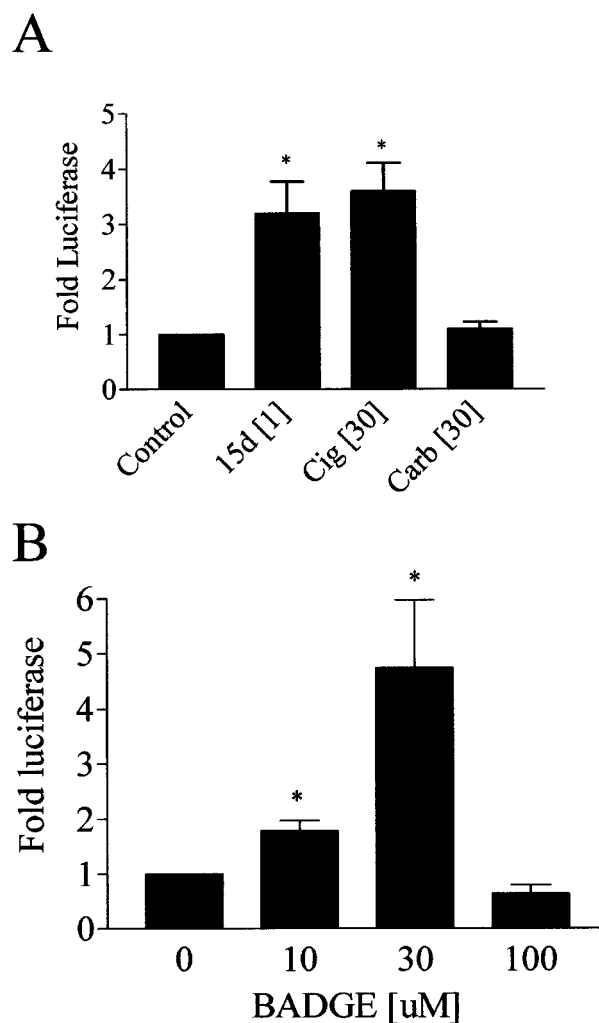


Figure 1 Characterization of the induction of luciferase by PPAR agonist in ECV-ACO.Luc, a cell line which stably expresses a reporter gene for PPAR activation. Cells at 15–20% confluence in 24-well plates were treated for 24 h with (A) the PPAR γ agonists, 15d-PGJ₂ (15d; 1 μ M) and ciglitizone (Cig; 30 μ M), or the PPAR α , and - δ agonist carbaprostacyclin (Carb; 30 μ M), or (B) with BADGE (10–100 μ M). The results represent fold increase of luciferase activity induced by ligands compared to untreated cells, and are expressed as mean $\pm$ s.e.mean for $n=6-12$. *Denotes significance ($P < 0.05$) of drug treatment compared to control by one sample t -test.

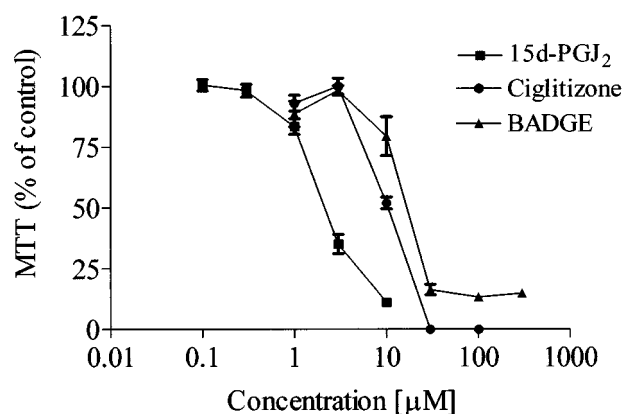


Figure 2 Effect of PPAR agonists and BADGE on ECV-ACO.Luc viability. ECV-ACO.Luc were treated with 15d-PGJ₂ (0.1–10 μ M), ciglitizone (1–100 μ M), or BADGE (1–300 μ M) for 24 h, and cell viability measured by MTT assay. Results, expressed as per cent of control, are the mean $\pm$ s.e.mean from 9–15 determinations from 3–5 separate experiments.

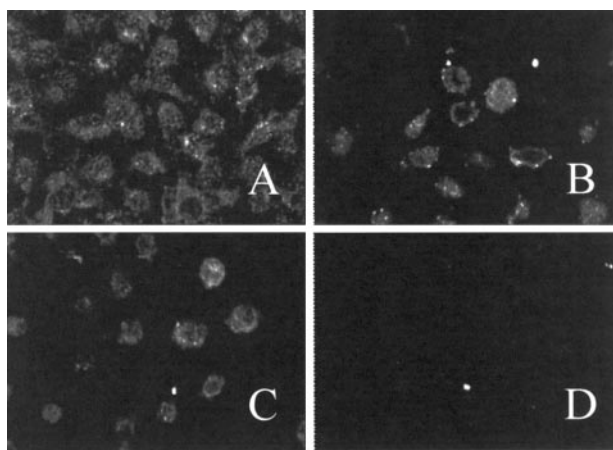


Figure 3 PPAR expression and activation in ECV-ACO.Luc. Immunofluorescence micrographs of PPAR γ in ECV-ACO.Luc under control culture conditions (A), and following treatment with either 3 μ M 15d-PGJ $_2$ (B), or 30 μ M BADGE (C) for 24 h. In the absence of primary antibody against PPAR γ (D), no specific staining was observed. This data is representative of $n=3$ separate experiments.

et al., 1998; Tugwood *et al.*, 1992). This clone termed ECV-ACO.Luc, like the parental cell line, only responds to PPAR γ agonists, which cause activation of luciferase expression and induce cell death. Although the maximum fold increase we saw with ligand induced luciferase was only 5–6 fold above basal levels, this is comparable to other cell systems that rely on endogenous receptors for the activation of the PPRE (Brun *et al.*, 1996). PPAR γ is present throughout ECV-ACO.Luc, both in the nucleus and the cytosol, which is in slight contrast to ECV304, where expression is primarily peri-nuclear and cytosolic (Bishop-Bailey & Hla, 1999). Whether this slight difference in PPAR γ expression is a clonal difference, or due to the presence of the transgene is uncertain. However, similar to ECV304, ECV-ACO.Luc has a strong predominantly nuclear expression of PPAR γ when treated with 15d-PGJ $_2$ for 24 h.

Having seen that the ECV-ACO.Luc has responses similar to the parental ECV304 cells we looked at the effects of BADGE. BADGE is a compound used in the manufacture of industrial plastics that has recently been identified as an antagonist of PPAR γ with μ M affinity (Wright *et al.*, 2000). BADGE inhibits both transcriptional activation mediated by PPAR γ and RXR α transfected in NIH-3T3 cells, and PPAR γ ligand induced adipocyte differentiation in 3T3-L1, and 3T3-F442A preadipocytes (Wright *et al.*, 2000). Surprisingly, when tested in ECV-ACO.Luc BADGE alone induced transcriptional activation of the PPAR reporter gene to a similar level seen with the well-characterized PPAR γ ligands 15d-PGJ $_2$, and ciglitizone. Furthermore, at the highest concentration of 100 μ M, transcriptional activation was reduced due to high levels of cell death. These results were confirmed in experiments with 96-well plates, indicating that BADGE

concentration-dependently induced cell death, with a similar potency to ciglitizone. It is unclear why ciglitizone, and BADGE were apparently more potent in the MTT cell viability assay (>90% cell death at 30 μ M), while still inducing optimal transcriptional response at this concentration. A possible explanation may be that these PPAR γ agonists are slightly more potent on confluent cells (in the MTT assay) compared to sub-confluent cells (approximately 20%) used in the transcriptional assay.

15d-PGJ $_2$ induces nuclear localization of PPAR γ as measured by confocal microscopy; an effect we suggest may be an initial part of its activation. Similarly, BADGE also induces the nuclear localization of PPAR γ . With these similarities to known PPAR γ agonists, that of PPRE activation, induction of cell death, and the ability to induce nuclear translocation, coupled with the fact that BADGE is known to bind PPAR γ in the μ M range in which we see these effects (Wright *et al.*, 2000). It appears clearly that BADGE acts as a PPAR γ agonist in ECV-ACO.Luc cells.

Although the previous report showing that BADGE is an antagonist is in stark contrast to this report, it is tempting to speculate that this is explained by PPAR γ acting in a cell type specific manner. Control of the activation state of nuclear hormone receptors/transcription factors such as the PPAR γ :RXR heterodimer is due to a large dynamic complex of proteins (see Torchia *et al.*, 1998). For example, it is known that at least two thiazolidinedione compounds, MC-555 (Reginato *et al.*, 1998), and GW0072 (Oberfield *et al.*, 1999) are partial agonists of PPAR γ by virtue of their inability to effectively recruit co-activator proteins to the receptor complex. Moreover, it is not known whether these complexes are identical for every cell type. The mechanism by which BADGE causes inhibition of PPAR γ is not known, though this type of mechanism of action may help to explain a cell type specific difference, such as the one we observe.

In conclusion, we have used a newly created cell line of ECV304 cells, which stably expresses a luciferase reporter gene for PPAR activation. This cell line may be of use to bioassay new compounds for PPAR γ activation. Furthermore, using this cell line we have identified that BADGE, the only compound to be previously described as a pure PPAR γ antagonist, has PPAR γ agonist activity. These results indicate that care must be taken when using BADGE as a pharmacological tool to look at the role of PPAR γ . Furthermore, these results indicate that the activation of PPAR γ may be regulated with greater cell type specificity than previously thought.

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References

- BISHOP-BAILEY, D. (2000). Peroxisome proliferator-activated receptors in the cardiovascular system. *Br. J. Pharmacol.*, **129**, 823–834.
- BISHOP-BAILEY, D. & HLA, T. (1999). Endothelial cell apoptosis induced by the peroxisome proliferator-activated receptor (PPAR) ligand 15-deoxy- $\Delta^{12,14}$ prostaglandin J $_2$. *J. Biol. Chem.*, **274**, 17042–17048.
- BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **7**, 248–254.
- BRUN, R.P., TONTONZOZ, P., FORMAN, B.M., ELLIS, R., CHEN, J., EVANS, R.M. & SPIEGELMAN, B.M. (1996). Differential activation of adipogenesis by multiple PPAR isoforms. *Genes Dev.*, **10**, 974–984.

- FORMAN, B.M. & EVANS, R.M. (1995). Nuclear hormone receptors activate direct, inverted, and everted repeats. *Ann. N. Y. Acad. Sci.*, **12**, 29–37.
- FORMAN, B.M., TONTONOS, P., CHEN, J., BRUN, R.P., SPIEGELMAN, B.M. & EVANS, R.M. (1995). 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J_2 is a ligand for the adipocyte determination factor PPAR γ . *Cell*, **83**, 803–812.
- ISSEMAN, I. & GREEN, S. (1990). Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature*, **347**, 645–650.
- KLIEWER, S.A., FORMAN, B.M., BLUMBERG, B., ONG, E.S., BORGMEYER, U., MANGELSDORF, D.J., UMESONO, K. & EVANS, R.M. (1994). Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 7355–7359.
- KLIEWER, S.A., LENHARD, J.M., WILLSON, T.M., PATEL, I., MORRIS, D.C. & LEHMANN, J.M. (1995). A prostaglandin J metabolite binds peroxisome proliferator-activated receptor γ and promotes adipocyte differentiation. *Cell*, **83**, 813–819.
- KLIEWER, S.A., UMESONO, K., NOONAN, D.J., HEYMAN, R.A. & EVANS, R.M. (1992). Convergence of 9-*cis* retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. *Nature*, **358**, 771–774.
- MOSMANN, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, **16**, 55–63.
- MUKHERJEE, R., JOW, L., CROSTON, G.E. & PATERNITI, J.R. (1997). Identification, characterisation, and tissue distribution of human peroxisome proliferator-activated receptor (PPAR) isoforms PPAR γ 2 versus PPAR γ 1 and activation with retinoid X receptor agonists and antagonists. *J. Biol. Chem.*, **272**, 8071–8076.
- OBERFIELD, J.L., COLLINS, J.L., HOLMES, C.P., GOREHAM, D.M., COOPER, J.P., COBB, J.E., LENHARD, J.M., HULL-RYDE, E.A., MOHR, C.P., BLANCHARD, S.G., PARKS, D.J., MOORE, L.B., LEHMANN, J.M., PLUNKET, K., MILLER, A.B., MILBURN, M.V., KLIEWER, S.A. & WILLSON, T.M. (1999). A peroxisome proliferator-activated receptor γ ligand inhibits adipocyte differentiation. *Proc. Natl. Acad. Sci. U.S.A.*, **96**, 6102–6106.
- REGINATO, M.J., BAILEY, S.T., KRAKOW, S.L., MINAMI, C., ISHII, S., TANAKA, H. & LAZAR, M.A. (1998). A potent antidiabetic thiazolidinedione with unique peroxisome proliferator-activated receptor γ -activating properties. *J. Biol. Chem.*, **273**, 32679–32684.
- RISTIMÄKI, A., GARFINKEL, S., WESSENDORF, S., MACIAG, T. & HLA, T. (1994). Induction of cyclooxygenase-2 by interleukin-1 α . Evidence for post-transcriptional regulation. *J. Biol. Chem.*, **269**, 11769–11775.
- ROBERTS, R.A., JAMES, N.H., WOODYATT, N.J., MACDONALD, N. & TUGWOOD, J.D. (1998). Evidence for the suppression of apoptosis by the peroxisome proliferator activated receptor α (PPAR α). *Carcinogenesis*, **19**, 43–48.
- TORCHIA, J., GLASS, C. & ROSENFELD, M.G. (1998). Co-activators and co-repressors in the integration of transcriptional responses. *Curr. Opin. Cell Biol.*, **10**, 373–383.
- TUGWOOD, J.D., ISSEMAN, I., ANDERSON, R.G., BUNDELL, K.R., MCPHEAT, W.L. & GREEN, S. (1992). The mouse peroxisome proliferator-activated receptor recognises a response element in the 5' flanking sequence of the rat acyl CoA oxidase gene. *EMBO J.*, **11**, 433–439.
- WRIGHT, H.M., CLISH, C.B., MIKAMI, T., HAUSER, S., YANAGI, K., HIRAMATSU, R., SERHAN, C.N. & SPIEGELMAN, B.M. (2000). A synthetic antagonist for the peroxisome proliferator-activated receptor γ inhibits adipocyte differentiation. *J. Biol. Chem.*, **275**, 1873–1877.

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