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SPECIAL REPORT

Bisphenol A diglycidyl ether (BADGE) is a PPARγ agonist in an ECV304 cell line

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Peroxisome proliferator activated receptors (PPAR)s are nuclear transcription factors of the steroid receptor super-family. One member, PPARγ, 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 PPARγ agonists, 15-deoxy-$\Delta^{12,14}$ PGJ_2, and ciglitizone, by inducing luciferase expression. Furthermore, using ECV-ACO.Luc, we demonstrate that a newly described PPARγ antagonist, bisphenol A diglycidyl ether (BADGE) has agonist activities. Similar to 15-deoxy-$\Delta^{12,14}$ PGJ_2, BADGE induces PPARγ activation, nuclear localization of the receptor, and induces cell death.
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Abbreviations: BADGE, bisphenol A diglycidyl ether; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$ PGJ_2; 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). PPARα is found predominantly in the liver, heart, kidney, brown adipose and stomach mucosa; PPARγ is found primarily in adipose tissue, where it plays a critical role in the differentiation of pre-adipocytes into adipocytes; while PPARδ 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 PPARα), the anti-diabetic thiazoldine-diones (PPARγ selective), and a number of eicosanoids, including, 12-HETE, 15-HETE, 13-HODE, and the prosta-glandin's, PGA_1, PGA_2, PGI_2, and PGD_2 and the PGD_2 dehydration product 15-deoxy-$\Delta^{12,14}$ PGJ_2 (15d-PGJ_2; Forman *et al*., 1995; Kliewer *et al*., 1995). Very few PPAR antagonists have been described. One notable exception, BADGE, is a PPARγ antagonist in 3T3-L1, and 3T3-F442A preadiopocyte 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 PPARα, $-\delta$, and $-\gamma$, and respond to PPARγ 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 μg pACO.g.LUC, and 1.5 μg pEGFPN-1, which contains a neomycin resistance cassette, using 12 μl of NovaFector. Clones were then selected in 1 μg ml^{-1} G418 sulphate. Once isolated, clones were seeded in 6- or 24-well plates. After 24 h incubation with agonist (30 μM ciglitizone) in serum-free medium, cells were lysed with 200 μl of distilled H_2O for 10–15 min. Luciferase activity was measured in 50–100 μl 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 PPARγ 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-PGJ_2 were dissolved in ethanol, while ciglitizone or BADGE were

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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 PPARγ 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-PPARγ was from Santa Cruz (Autogen Bioclear UK Ltd., Wiltshire, U.K.), FITC-conjugated anti-goat IgG was from Cappell (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-PGJ_2 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 *PPARγ 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-PGJ_2, (1 μM; non specific PPAR ligand), ciglitizone (30 μM; a thiazoldinedione PPARγ ligand), carbaprostacyclin (30 μM; a PPARα/ δ ligand) (Figure 1A), or BADGE (10–100 μM; Figure 1B). Luciferase enzyme expression was induced by 15d-PGJ_2, ciglitizone, (i.e. activators of PPARγ) 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 PPARγ agonists 15d-PGJ_2 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 PPARγ Under control culture conditions, PPARγ was expressed throughout ECV-ACO.Luc (Figure 3A). Following incubation of ECV-ACO.Luc for 24 h, with either 15d-PGJ_2 (Figure 3B) or BADGE (Figure 3C), PPARγ 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 PPARγ antibody in the parental cell line (Bishop-Bailey & Hla, 1999).

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

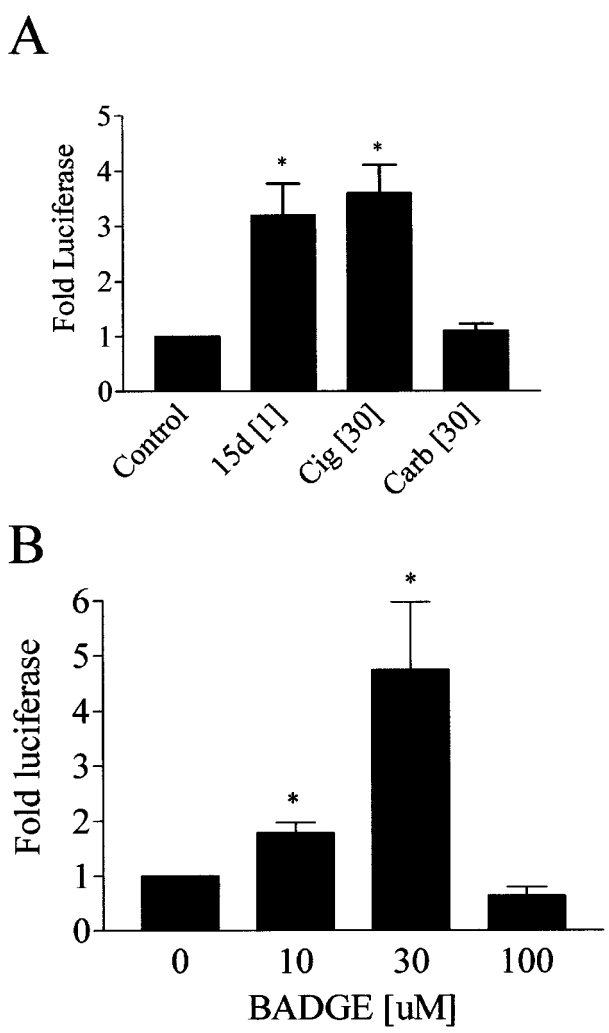

Figure 1 Characterization of the induction of luciferase by PPAR agonist in ECV-ACO.Luc, a cell line which stably expresses a reporter gene for PPAR activation. Cells at 15–20% confluence in 24-well plates were treated for 24 h with (A) the PPARγ agonists, 15d-PGJ_2 (15d; 1 μM) and ciglitizone (Cig; 30 μM), or the PPARα, and -δ agonist carbaprostacyclin (Carb; 30 μM), or (B) with BADGE (10–100 μM). 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.

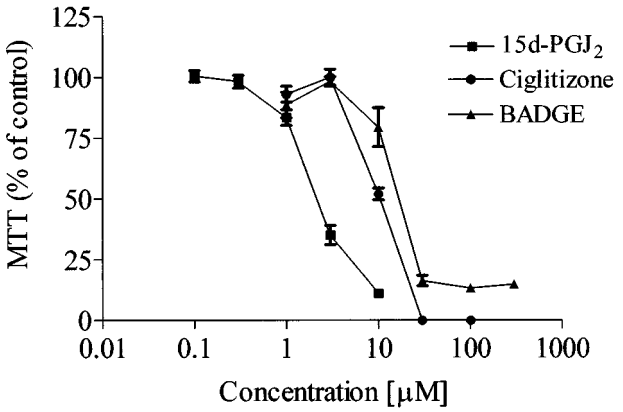

Figure 2 Effect of PPAR agonists and BADGE on ECV-ACO.Luc viability. ECV-ACO.Luc were treated with 15d-PGJ_2 (0.1–10 μM), ciglitizone (1–100 μM), or BADGE (1–300 μM) for 24 h, and cell viability measured by MTT assay. Results, expressed as per cent of control, are the mean$\pm$s.e.mean from 9–15 determinations from 3–5 separate experiments.

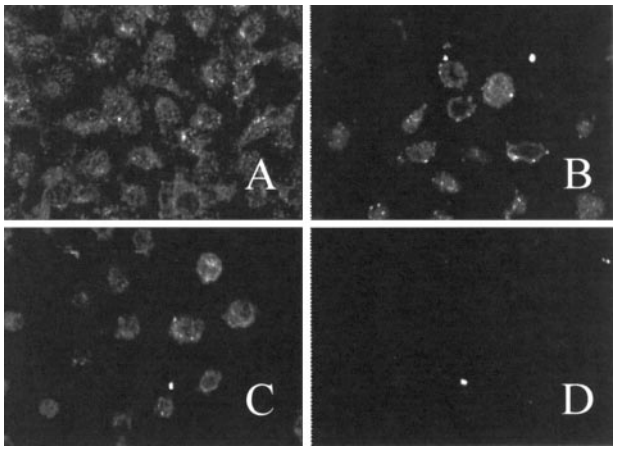

Figure 3 PPAR expression and activation in ECV-ACO.Luc. Immunofluorescence micrographs of PPARγ in ECV-ACO.Luc under control culture conditions (A), and following treatment with either 3 μM 15d-PGJ_2 (B), or 30 μM BADGE (C) for 24 h. In the absence of primary antibody against PPARγ (D), no specific staining was observed. This data is representative of $n=3$ separate experiments.

al., 1998; Tugwood *et al*., 1992). This clone termed ECV-ACO.Luc, like the parental cell line, only responds to PPARγ 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). PPARγ 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 PPARγ 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 PPARγ 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 PPARγ with μM affinity (Wright *et al*., 2000). BADGE inhibits both transcriptional activation mediated by PPARγ and RXRα transfected in NIH-3T3 cells, and PPARγ 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 PPARγ ligands 15d-PGJ_2, and ciglitizone. Furthermore, at the highest concentration of 100 μM, 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 μM), while still inducing optimal transcriptional response at this concentration. A possible explanation may be that these PPARγ 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 PPARγ 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 PPARγ. With these similarities to known PPARγ 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 PPARγ in the μM range in which we see these effects (Wright *et al*., 2000). It appears clearly that BADGE acts as a PPARγ 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 PPARγ acting in a cell type specific manner. Control of the activation state of nuclear hormone receptors/transcription factors such as the PPARγ: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 thiazoldinedione compounds, MC-555 (Reginato *et al*., 1998), and GW0072 (Oberfield *et al*., 1999) are partial agonists of PPARγ 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 PPARγ 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 PPARγ activation. Furthermore, using this cell line we have identified that BADGE, the only compound to be previously described as a pure PPARγ antagonist, has PPARγ agonist activity. These results indicate that care must be taken when using BADGE as a pharmacological tool to look at the role of PPARγ. Furthermore, these results indicate that the activation of PPARγ 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., TONTONOZ, 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., TONTONOZ, 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 PPARγ. *Cell,* **83,** 803–812.

ISSEMANN, 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 PPARγ2 versus PPARγ1 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 thiazoldinedione 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 alpha. 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 alpha (PPAR alpha). *Carcinogenesis,* **19,** 43–48.

TORCHIA, J., GLASSS, 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., ISSEMANN, 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 g inhibits adipocyte differentiation. *J. Biol. Chem.,* **275,** 1873–1877.

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