BAG-1 inhibits PPARγ-induced cell death, but not PPARγ-induced transcription, cell cycle arrest or differentiation in breast cancer cells

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Abstract. BAG-1 is a pleiotropic protein that exists as multiple isoforms. BAG-1 overexpression in breast cancer is associated with outcome. BAG-1 modulates the function of various nuclear hormone receptors, including the oestrogen receptor, and BAG-1 can influence the in vitro action of anti-hormonal therapies such as cyproterone acetate in prostate cancer. Activation of PPARγ, a nuclear hormone receptor important for lipid and glucose homeostasis, may present a new therapeutic approach for breast cancer, since PPARγ agonists promote cell cycle arrest, differentiation and apoptosis in breast cancer cells. Here we determined whether BAG-1 also modulated PPARγ function in MCF7 cells. 15-deoxy-Delta12,14-prostaglandin J(2) (15dPGJ 2), an agonistic ligand for PPARγ, induced expression of HSP70, a BAG-1 binding partner, but did not alter BAG-1 isoform expression. Overexpression of BAG-1 isoforms did not alter PPARγ-dependent transcription or interfere with 15dPGJ 2-induced cell cycle arrest or differentiation. However, overexpression of BAG-1 isoforms did interfere with induction of cell death by 15dPGJ2. Thus, BAG-1 is unlikely to directly modulate PPARγ function, but the overexpression of BAG-1 in some breast cancers may limit the efficacy of PPARγ agonists as cancer therapies, by suppression of PPARγ-induced cell death pathways.

Introduction

BAG-1 (Bcl-2 associated athanogene 1) is a multifunctional protein with growth potentiating activity in a range of cancer cell types (1,2). It exists as two major isoforms in human cells, BAG-1S and BAG-1L, which are predominantly localised to the cytoplasm and nucleus, respectively. A third isoform, BAG-1M, is generally less abundant and is not expressed in mice. BAG-1 isoforms are generated by alternate translation from a single BAG-1 mRNA and possess variable functions, localisation and expression patterns. Overexpression of BAG-1 isoforms modulates survival, signalling, transcription, proliferation and metastasis, and the expression of BAG-1 is altered in various cancers (2,3). For example, BAG-1 expression is increased in malignant breast epithelium and can correlate with prognosis (4-8). High levels of BAG-1 expression have also been detected in ductal carcinoma in situ (DCIS) suggesting that changes in BAG-1 expression might be important at an early stage in tumour development (2,4,7,9,10). BAG-1 exerts its pleiotropic cellular effects via protein:protein interactions. Key binding partners for BAG-1 include the 70 kDa heat shock proteins, HSC70 and HSP70, and binding to these molecular chaperones is required for the pro-survival effects of BAG-1 (2,11,12). The precise mechanism by which BAG-1 proteins promote cell survival are not fully understood, but may involve interactions with the proteasome system (2,3).

In addition to promoting cell survival, modulation of nuclear hormone receptor (NHR) function is also likely to contribute to the growth promoting effects of BAG-1 in cancer cells (2,13). For example, BAG-1 potentiates the function of the oestrogen receptor (ER) in breast cancer and the androgen receptor (AR) in prostate cancer (4,14). Other NRs modulated by BAG-1 include the vitamin D3 receptor (VDR), which is activated or repressed by BAG-1 dependent on cellular environment, and the retinoic acid receptor α (RARα), glucocorticoid receptor (GR) and thyroid receptor, which are inhibited by BAG-1 (15-18). Like cell survival, modulation of NHR is frequently dependent on interactions with HSC70/ HSP70 heat shock proteins (4,14,17,19), consistent with the role of heat shock proteins in NHR function (13). Other receptors, such as the mineralocorticoid receptor, and retinoid X receptor (RXR) are unaffected by BAG-1 (15,16,20,21).

Endocrine therapies are well established as means to improve cure rates and palliate advanced disease in breast and prostate carcinoma, and in some leukaemias. However,
these diseases are characterised by progression to a hormone refractory state in a proportion of cases, with subsequent poor prognosis for such patients. Thus, hormonal breast cancer therapy remains sub-optimal for many patients and better understanding of hormonal mechanisms in cancer development and options for treatment are required (22, 23).

One potential area of interest in endocrine manipulation of malignancies, including breast cancer, is peroxisome proliferator activated receptor γ (PPARγ) which belongs to the PPAR family of NHRs. Following ligand binding, PPARγ heterodimerises with RXR and modulates the expression of downstream target genes by binding to specific DNA response elements (PPRE). Target genes for PPARγ are directly linked to lipogenesis and PPARγ plays a pivotal role in lipid metabolism (24, 25) by regulating the storage of fatty acids in adipose tissue and stimulating maturation and differentiation of adipocytes (26-28). PPARγ was initially discovered as an orphan receptor, but can be activated by the agonist prostanoid 15-deoxy-Delta12,14-prostaglandin J(2) (15dPGJ2) and thiazolidinediones, such as troglitazone, used widely to treat type II diabetes (29).

Increasing evidence points to PPARγ having a role in breast cancer development. PPARγ expression is decreased in breast cancer samples compared to normal tissue and lower levels of PPARγ correlate with increasing disease stage, risk of local recurrence and disease specific mortality (30, 31). Moreover, PPARγ stimulation by 15dPGJ2, or thiazolidinediones induces growth arrest, differentiation and apoptosis in breast cancer cell lines (32-37). PPARγ can also interact with oestrogen response elements and cause transactivation of certain ER targets, but inhibit ER activation of others (38). Clinical trial work to assess PPARγ agonists in breast cancer is restricted to a single phase II study of troglitazone in 22 chemotherapy refractory patients in whom no objective responses were seen (39).

The aim of this study was to determine whether BAG-1 modulated the activity of PPARγ, to increase our understanding of NHR function and to determine whether over-expression of BAG-1 in breast cancer might influence the efficacy of PPARγ agonists.

Materials and methods

Reagents and cell culture. MCF7 and MDA-MB-231 breast cancer cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Paisely, UK) with 10% (v/v) foetal calf serum (FCS) (PAA laboratories, Pasching, Austria), 100 μg/ml Penicillin G, 100 μg/ml streptomycin and 292 μg/ml L-glutamine (all from Invitrogen) at 10% (v/v) CO2. MCF7 and MDA-MB-231 breast cancer cell lines (32-37). PPARγ diones induces growth arrest, differentiation and apoptosis in breast cancer development. PPARγ agonists have a role in breast cancer development. PPARγ expression is decreased in breast cancer samples compared to normal tissue and lower levels of PPARγ correlate with increasing disease stage, risk of local recurrence and disease specific mortality (30, 31). Moreover, PPARγ stimulation by 15dPGJ2, or thiazolidinediones induces growth arrest, differentiation and apoptosis in breast cancer cell lines (32-37). PPARγ can also interact with oestrogen response elements and cause transactivation of certain ER targets, but inhibit ER activation of others (38). Clinical trial work to assess PPARγ agonists in breast cancer is restricted to a single phase II study of troglitazone in 22 chemotherapy refractory patients in whom no objective responses were seen (39).

For H376 cells, 3x10³ cells per well were plated per well. Transfection was performed the following day using Transfast reagent (Promega) at 3 μl:1 μg ratio of transfection reagent to total-DNA per sample, in serum-free medium for 1 h. A typical transfection mixture per 35 mm/well would contain 1 μg PPRE-luc or tk-luc control reporter, 2 μg of pcDNA3-based BAG-1 expression plasmid (or empty pcDNA3 plasmid as a control) and 1 μg of PPRE-luc control plasmid or pcDNA3 control and 0.5 μg pRL.tk.luc to act as an internal control for transfection efficiency. The transfection mixture was replaced by fresh medium containing 15dPGJ2, typically to a concentration of 10 μM, or an equivalent concentration of DMSO as a solvent control. After 16 h samples, cells were collected by scraping in phosphate-buffered saline (PBS), re-suspended in 100 μl of luciferase/β-galactosidase lysis buffer [10 mM Tris-HCl, 1 mM, EDTA buffered saline (PBS), re-suspended in 100 μl of luciferase/β-galactosidase lysis buffer [10 mM Tris-HCl, 1 mM, EDTA pH 8, 150 mM NaCl, 0.65% (v/v) Nonidet P40]. After 5 min, lysates were clarified by centrifugation and supernatant collected. Twenty microlitres was assessed for luciferase activity in a Sirius single tube luminometer (Berthold Detection Systems, Redbourn, UK) using luciferase assay reagent (Promega) according to the manufacturer's instructions. β-galactosidase assays were performed in a 96-well plate with each well containing 50 μl assay reagent (120 mM Na2HPO4,12H2O, 80 mM NaH2PO4,2H2O, 2 mM MgCl2,6H2O, 7.5, 100 mM 2-β-Mercaptoethanol, 1.33 g/l o-nitrophenyl-β-D-galactopyranoside), 10 μl of sample and 40 μl luciferase/β-galactosidase lysis buffer. The plate was incubated at 37°C for approximately 2 h and absorbance at 405 nm determined on a BioRad 550 microplate reader using Microplate Manager 5.0 software (BioRad, Hemel Hempstead, UK).

For H376 cells, 3x10³ cells per well were plated per well of a 24-well plate. Transfection was performed two days later using Fugene reagent (Roche Diagnostics, Lewes, UK) according to the manufacturer's instructions. A typical transfection mixture would contain 2 μg VDRE-luc or PPRE-luc reporter constructs, 2 μg pcDNA3-based BAG-1 expression plasmid or pcDNA3 control and 0.5 μg pRL.tk.luc to act as...
in internal control for transfection efficiency. Transfection mixture was replaced by fresh cell culture medium at 6 h containing either 100 nM 1α,25 dihydroxyvitamin D3 or 10 μM 15dPGJ2, or the relevant solvent control. After 24 h cells were then lysed and analysed for firefly and renilla luciferase activity using the Dual Luciferase Assay System (Promega) and a Sirius single tube luminometer (Berthold Detection Systems) according to the manufacturer's instructions.

**Immunoblotting.** Immunoblotting was performed as described previously (40), using rabbit polyclonal antibody TB2 for human BAG-1 (9), the HSC70-specific mouse monoclonal antibody B6 (Santa Cruz Biotechnology, CA, USA), HSP70-specific mouse monoclonal (Stressgen, MI, USA), the HSP40-specific rabbit polyclonal (Stressgen) or the PCNA-specific mouse monoclonal antibody PC10 (Cancer Research UK Research Services, London, UK).

**Differentiation analysis.** Cells were plated on to microscope cover slips at a density of 3x10⁵ per 35 mm/well. The following day cells were treated with 10 μM PGJ2. For analysis of Nile Red staining (41), the cover slips were washed twice with PBS and then fixed with PBS containing 4% (w/v) paraformaldehyde for 20 min at room temperature. Cover slips were then washed twice again followed by incubation in the dark for 5 min in freshly made PBS supplemented with 100 ng/ml Nile Red (Sigma). Cover slips were washed twice prior to mounting on microscope slides with Fluorescent Mounting Medium (Dako, Cambridgeshire, UK). Slides were incubated in the dark at 4˚C overnight prior to imaging using a fluorescent microscope (Zeiss Axiovert 200 microscope) and Openlab 3.0.8 software (Improvision, Coventry, UK). The average number of lipid droplets per cell was determined by selecting fields at random and counting the number of lipid droplets in five adjacent cells. This was performed twice for each slide and the mean lipid droplet count (of ten counted cells) was determined.

**Cell cycle analysis.** Cells were plated at a density of 3x10⁵ per 35 mm/well. The following day, cells were treated with 15dPGJ2 or DMSO. To analyse cell cycle phases, cells were washed twice in Hank’s modified salt solution without calcium or magnesium (Invitrogen) and then collected by trypsinisation. The original culture medium, trypsinised cells and each wash solution were combined and cell collected by centrifugation. Cells were washed twice in PBS, resuspended in cold 70% (v/v) ethanol and incubated at 4˚C for a minimum of 24 h. Samples were re-suspended in 500 μl PBS supplemented with propidium iodide (PI) (Sigma) at 80 μg/ml and ribonuclease A (Sigma) at 10 μg/ml. Samples were then analysed on a FACScaliber FACS counter (Becton-Dickinson, Oxford, UK) using CellQuest software. Cell cycle fractions were determined as percentages of the total number of cells in the cell cycle (G0/G1, S and G2/M phases). Dead cell fractions were determined as the percentages of all cells with sub-G1 content.

**Statistics.** Statistical analysis was performed using SPSS for windows version 10 (SPSS Inc., Chicago, USA). Two-sample t tests were used to test the significance of differences in transcriptional activity in the reporter assays.

**Results**

**15dPGJ2 stimulation increases HSP40 and HSP70 expression.** To investigate interactions between PPARγ function and BAG-1, we first analysed the effect of 15dPGJ2 on expression of BAG-1 isoforms and chaperone molecules. 15dPGJ2 is a potent agonist of PPARγ that has been used widely to study the function of this receptor (34,35) although it may also exert independent effects (42). Dose response experiments (data not shown) demonstrated that 10 μM 15dPGJ2 produced robust activation of PPARγ-dependent transcription in breast cancer cells and this concentration of ligand was used in subsequent experiments. MCF7 cells were incubated with 15dPGJ2 for various times and expression of BAG-1, HSC70, HSP70 and HSP40 analysed by immunoblotting (Fig. 1). The expression of BAG-1 isoforms and the constitutively expressed heat shock protein HSC70 were unaltered by 15dPGJ2. By contrast, expression of the inducible heat shock proteins HSP40 and HSP70 was increased from as early as 4 h. Similar results were obtained in MDA-MB-231 breast cancer cells (data not shown). Therefore, PPARγ stimulation increases heat shock protein expression in breast cancer cell lines.

**BAG-1 overexpression does not alter PPARγ-dependent transcription.** BAG-1 modulates the activity of a subset of NHR (10). We therefore investigated the ability of BAG-1 to alter PPARγ-dependent transcription using luciferase reporter gene assays. Although 15dPGJ2 robustly increased PPARγ-dependent transcription in MCF7 cells, basal and 15dPGJ2-induced transcription was not significantly altered by over-expression of BAG-1L (Fig. 2a). Further detailed transfection experiments using different cell lines (MDA-MB-231 breast cancer cells and HEK 293 embryonal kidney cells), different 15dPGJ2 concentrations, overexpression of other BAG-1 isoforms (BAG-1S and BAG-1M) and various transfections protocols confirmed that BAG-1 isoforms did not alter PPARγ-
incubated with ligand (closed bars; 10 μM 15dPGJ2) for PPRE-luc transfected as a control) and pRL.tk-luc to control for transfection efficiency. Cells were transfected with VDRE-luc reporter plasmids, the BAG-1L expression plasmid (or pcDNA3 as a control) of multiple experiments. (b) H376 cells were transfected with PPRE-luc or control tk-luc to compare for transfection efficiency. Normalised luciferase activity of PPRE-luc/pcDNA3 transfected cells in the absence of ligand was set to 1.0. Results are representative of two separate experiments. **p<0.001 compared to cells in the absence of 15dPGJ2 or solvent controls (open bars). Firefly and Renilla luciferase activity was determined after 24 h. Results show mean luciferase activity (±SD) of cell lysates from triplicate transfections normalised for Renilla luciferase activity. The normalised luciferase activity of PPRE-luc/pcDNA3 transfected cells in the absence of 15dPGJ2 was set to 1.0. Result shown is representative of two separate experiments. The effect of BAG-1 isoforms on 15dPGJ2-induced differentiation. Activation of PPARγ induces differentiation of breast cancer cell lines (36). Consistent with the role of PPARγ as a key regulator of lipid metabolism, this differentiation process is characterised by the development of intracellular lipid droplets containing milk fat globulins, which can be stained using Nile Red (41). Using this technique, we confirmed that 15dPGJ2 caused the accumulation of cytoplasmic lipid droplets in MCF7 cells (Fig. 3a). 15dPGJ2-treated cells were also larger and flatter than control MCF7 treated cells.

To investigate the effect of BAG-1 on 15dPGJ2-induced differentiation, we used a series of MCF7 cell-derived clones overexpressing BAG-1S or BAG-1L, or containing empty pcDNA3 plasmid as a control (Fig. 3b). The clones were incubated with 15dPGJ2, and the average number of Nile Red positive droplets per cell determined. Overexpression of BAG-1S or BAG-1L did not alter the induction of Nile Red positive cells by 15dPGJ2 (Fig. 3c).

The effect of BAG-1 isoforms on 15dPGJ2-induced cell cycle arrest and cell death. Activation of PPARγ by 15dPGJ2 induces cell cycle arrest and causes activation of caspases, exposure of phosphatidylserine, plasma membrane blebbing and nuclear condensation, hallmarks of apoptosis, in breast cancer cells (33,34). To determine whether overexpression of BAG-1 isoforms altered cell cycle arrest and cell death, MCF7 clones overexpressing BAG-1S or BAG-1L were incubated with 15dPGJ2 and effects on the cell cycle analysed and cell death by flow cytometry. 15dPGJ2 decreased the proportion of cells in the G0/G1 phase and increased the proportion of cells in the G2/M phase of the cell cycle (Fig. 4). However, this cell cycle arrest was not altered by BAG-1 overexpression. 15dPGJ2 also increased the proportion of cells with sub-G1 DNA content, which is indicative of apoptosis (44). In contrast to cell cycle arrest, the accumulation of cells with sub-G1 content was significantly decreased by overexpression of either BAG-1S or BAG-1L (Fig. 4b).
Discussion

BAG-1 is an important co-regulator of certain NHR (2,10,13). The ability of BAG-1 isoforms to counteract inhibition of AR mediated transcription by cyproterone acetate, an anti-androgen used in prostate cancer therapy, in prostate cancer cells (20), and to enhance the activity of ER\(\alpha\) and predict outcome in hormone responsive breast cancer (4), suggest that this activity may be clinically relevant. The aim of this study was to determine whether BAG-1 also influenced the function of PPAR\(\gamma\), which can induce cell cycle arrest, cell death and differentiation in breast cancer cells (32-37). PPAR\(\gamma\) ligands, such as troglitazone have been assessed clinically for anti-tumour activity in breast cancer and it is therefore clearly important to investigate factors which might impact on their efficacy, especially since BAG-1 overexpression has frequently been described in breast cancer (10). Moreover, this study adds to our general understanding of the effects of BAG-1 on NHR.

Our findings demonstrate that the function of PPAR\(\gamma\) is not directly modulated by BAG-1 overexpression, at least in the experimental systems studied here. As such, PPAR\(\gamma\) can be grouped with RXR and the mineralocorticoid receptor which are similarly unaffected by BAG-1 overexpression (16,21). 15dPGJ\(_2\) did not alter BAG-1 isoform expression, although it did enhance expression of HSP40 and HSP70, partly confirming a previous microarray study demonstrating induction of HSP40, HSP70 and BAG-1 RNA in 15dPGJ\(_2\)-treated MDA-MB-231 cells (34). The resistance of PPAR\(\gamma\) to BAG-1 modulation is perhaps surprising considering that RAR\(\alpha\), which like PPAR\(\gamma\) requires RXR as an obligate partner, is modulated by BAG-1 (21). The mechanism of regulation of NHR by BAG-1 has been most intensively studied for GR (16,17,45,46). In this case, it is likely that BAG-1 binds the GR after ligand binding and subsequent receptor activation and is then transported with the receptor complex into the nucleus. However, it remains unclear whether this is a general model that applies to other NHRs (10). Given the various effects of BAG-1 overexpression on specific NHR (enhanced activity, decreased activity or no effect), the variable effects of specific BAG-1 isoforms and cell type-dependent effects,
Further studies are required before we have a full understanding of the mechanisms by which BAG-1 modulates NHR activity (10,13).

Consistent with the lack of effects of BAG-1 overexpression of PPARγ-mediated transcription, overexpression of BAG-1L or BAG-1S did not interfere with G2/M cell cycle arrest or differentiation induced by 15dPGJ2. However, overexpression of BAG-S or BAG-1L did decrease induction of cell death by 15dPGJ2. Previous studies have demonstrated that 15dPGJ2-induced cell death is due to apoptosis (33,34) and we believe that this effect is due to the well established pro-survival activities of these BAG-1 isoforms in the face of a pro-apoptotic stimulus (12), rather than direct modulation of PPARγ activity.

The decreased expression of PPARγ and 15dPGJ2 RNA in breast cancer samples compared to normal tissue, and their association with increasing disease stage, risk of local recurrence and disease specific mortality (30,31), has led to clinical trials of PPARγ agonists in this disease. Despite promising pre-clinical data, including significant responses in experimental animal models (37,47,48), these trials have produced conflicting results (29). In breast cancer, a phase II study of troglitazone was terminated prematurely due to toxicity concerns and detected no objective tumour responses in twenty-two patients with advanced refractory disease. Unfortunately, no attempt was made in this study to determine hormonal or PPARγ expression status which, along with the treatment refractory advanced disease in this cohort, may have limited

Figure 4. Effect of BAG-1 on 15dPGJ2-induced cell cycle arrest and cell death. MCF7 cell clones were treated with DMSO or 10 μM 15dPGJ2 for 96 h. The cell cycle distribution of cells was analysed by flow cytometry. (a) Representative histogram plots of DMSO and 15dPGJ2 treated cells. (b) Quantitation of different cell cycle phases. The results show the mean proportion of cells in each phase (±SD) for three pcDNA clones (open bars), three BAG-1S clones (grey bars) and three BAG-1L clones (closed bars). 15dPGJ2 significantly decreased the proportion of cells in G0/G1 and increased the proportion of cells in G2/M in all three groups of clones compared to DMSO treated cells (p<0.05). 15dPGJ2 significantly increased the proportion of cells with sub-G1 content (p<0.05) only in pcDNA3 clones. Experiment shown is representative of four independent experiments.
the opportunity to detect clinical benefit (26,39). Moreover, pharmacodynamic end-points were not assessed. Our studies suggest that the elevated expression of BAG-1 that is frequently observed in breast cancer may not counter PPARγ-induced cell cycle arrest or differentiation, but may limit the cytotoxic effects of agonists. Future clinical studies of novel anti-hormonal agents should attempt to investigate the impact of expression of receptors and their regulatory molecules, including BAG-1.

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References


