Peroxisome Proliferator-Activated Receptor-γ Activates p53 Gene Promoter Binding to the Nuclear Factor-κB Sequence in Human MCF7 Breast Cancer Cells

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The aim of the present study was to provide new mechanistic insight into the growth arrest and apoptosis elicited by peroxisome proliferator-activated receptor (PPAR)γ in breast cancer cells. We ascertained that PPARγ mediates the inhibition of cycle progression in MCF7 cells exerted by the specific PPARγ agonist rosiglitazone [BRL4653 (BRL)], because this response was no longer notable in the presence of the receptor antagonist GW9662. We also provided evidence that BRL is able to up-regulate mRNA and protein levels of the tumor suppressor gene p53 and its effector p21WAF1/Cip1 in a time- and dose-dependent manner. Moreover, in transfection experiments with deletion mutants of the p53 gene promoter, we documented that the nuclear factor-κB sequence is required for the transcriptional response to BRL.

Interestingly, EMSA showed that PPARγ binds directly to the nuclear factor-κB site located in the promoter region of p53, and chromatin immunoprecipitation experiments demonstrated that BRL increases the recruitment of PPARγ on the p53 promoter sequence. Next, both PPARγ and p53 were involved in the cleavage of caspases-9 and DNA fragmentation induced by BRL, given that GW9662 and an expression vector for p53 antisense blunted these effects. Our findings provide evidence that the PPARγ agonist BRL promotes the growth arrest and apoptosis in MCF7 cells, at least in part, through a cross talk between p53 and PPARγ, which may be considered an additional target for novel therapeutic interventions in breast cancer patients. (Molecular Endocrinology 20: 3083–3092, 2006)

Peroxisome proliferator-activated receptor γ (PPARγ) is a prototypical member of the nuclear receptor superfamily and integrates the control of energy, lipid, and glucose homeostasis (1–4). PPARγ regulates differentiation and induces cell growth arrest and apoptosis in a large variety of cells (Ref. 5 and references therein), including both primary and metastatic breast malignancy (6, 7). However, the molecular mechanisms involved in the inhibitory effects mediated by PPARγ remain to be elucidated.

It is well known that the p53 tumor suppressor gene regulates the transcription of effectors that are also responsible for growth arrest and apoptosis (reviewed in Ref. 8). Among the p53 target genes, the p21WAF1/Cip1 has been recognized to exert an essential role in mediating cell cycle arrest at both G1 and G2-M checkpoints (9–11). p21WAF1/Cip1 inhibits cyclin D1 or E/cyclin-dependent kinase in G1, and cyclin B/cdc2 in G2-M arrest, eliciting regulatory effects on DNA replication and repair (12). Moreover, it has been reported that p53 is able to promote apoptosis in certain cell types in a transcription-independent manner (13).

The function of p53 as a tumor suppressor is finely tuned through an interaction with other transduction pathways regulating the cell network (14–18). For instance, striking evidence has recently emerged for a cross talk between p53 and relevant transcription factors, such as the glucocorticoid, androgen, and estrogen receptors (19). It was therefore proved that these nuclear receptors are able to induce a cytosolic accumulation of p53, altering its stability and, consequently, its function (19).

In the present study, we provide new insight into the molecular mechanisms by which the specific PPARγ ligand rosiglitazone [BRL4653 (BRL)] induces the growth arrest and apoptosis in MCF7 human breast cancer cells. By performing a panel of different assays, we have demonstrated that the biological effects of BRL are triggered, at least in part, by PPARγ binding to the nuclear factor-κB sequence located within the p53 promoter region. Our findings have provided ev-
idence of a cross talk between p53 and PPARγ, which assumes a biological relevance for possible new pharmacological strategies in breast cancer.

RESULTS

BRL Induces G0-G1 Cycle Arrest in MCF7 Cells

On the basis of our (20) and other (21, 22) studies demonstrating the inhibitory effects of the PPARγ agonists on proliferation of breast cancer cells, we first investigated the activity of BRL on MCF7 cell cycle progression. A 48-h exposure to BRL caused the inhibition of G0-G1→S phase progression in a dose-dependent manner with concomitant decrease in the proportion of cells entering in S phase (Table 1). Of note, this effect was mediated by PPARγ, because it was no longer notable in the presence of the specific antagonist GW9662 (GW).

BRL Up-Regulates p53 and p21WAF1/Cip1 Expression in MCF7 Cells

Considering that the tumor suppressor gene p53 is mainly involved in the growth arrest promoted by different factors, we aimed to examine the potential ability of PPARγ to modulate the expression of p53 along with its natural target gene p21WAF1/CIP1. The mRNA levels of both p53 and p21WAF1/CIP1 were up-regulated in a time- and dose-dependent manner in MCF7 cells treated with BRL. These stimulations were abrogated by GW (Figs. 1 and 2) suggesting a direct involvement of PPARγ.

BRL Transactivates p53 Gene Promoter

The aforementioned observations prompted us to investigate whether PPARγ is able to transactivate an expression vector encoding p53 promoter gene. Thus, MCF7 cells were transiently transfected with a luciferase reporter construct (named p53-1) containing the upstream region of the p53 gene spanning from −1800 to +12 (Fig. 3A) and treated with increasing concentrations of BRL for 24 h. Interestingly, the dose-dependent activation of p53−1 by BRL was reversed in the presence of GW, indicating that a PPARγ-mediated mechanism was involved in the transcriptional response to BRL (Fig. 3B).

To identify the region within the p53 promoter responsible for transactivation, we used deletion constructs expressing different binding sites such as CTF-1/YY1, nuclear factor-Y (NF-Y), and NFκB (Fig. 3A). In transfection experiments performed using the mutants p53-6 and p-53-13 encoding the regions from −106 to +12 and from −106 to −40, respectively, the responsiveness to BRL was still observed, whereas using the mutant p53−14 encoding the sequence from −106 to −49 we did not detect an increase in luciferase activity (Fig. 3C). Consequently, the region from −49 to −40, which corresponds to the NFκB site (Fig. 3A), was required for the transactivation of p53 by BRL.

PPARγ Binds to NFκB Sequence in EMSA

To further evaluate whether the NFκB site is responsible for the action triggered by BRL, we performed EMSA experiments. Using synthetic oligodeoxyribonucleotides corresponding to the NFκB sequence, we observed the formation of a single band in nuclear extracts from MCF7 cells (Fig. 4A, lane 1), which was abrogated by 100-fold molar excess of unlabeled probe (Fig. 4A, lane 2), demonstrating the specificity of the DNA binding complex. Of note, BRL treatment induced a strong increase in the specific band (Fig. 4A, lane 3), which was immunodepleted and supershifted using anti-PPARγ (Fig. 4A, lane 4) and anti-NFκB (Fig. 4A, lane 5) antibodies. Interestingly, the PPARγ-transcribed and translated protein was able to bind to [32P]NFκB oligonucleotide (Fig. 4A, lane 6). The specificity of the band was proved by a 100-fold excess of cold probe (Fig. 4A, lane 7) and confirmed by a consensus PPAR response element (PPRE) used as a cold competitor (Fig. 4A, lane 8). In addition, the immunodepleted band obtained using the anti-PPARγ antibody (Fig. 4A, lane 9), but not observed with the anti-NFκB antibody (Fig. 4A, lane 10), confirmed that PPARγ binds in a specific manner to the NFκB site present in the promoter of p53. As next controls, we used NFκB protein alone (Fig. 4B, lane 1) and in com-

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Table 1. BRL Induces G0-G1 Cycle Arrest in MCF7 Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>μM</th>
<th>G0-G1</th>
<th>S</th>
<th>G2-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td></td>
<td>53 ± 7.2</td>
<td>30 ± 4.4</td>
<td>17 ± 2.1</td>
</tr>
<tr>
<td>BRL</td>
<td>1</td>
<td>67 ± 7.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20 ± 3.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13 ± 2.2</td>
</tr>
<tr>
<td>BRL</td>
<td>10</td>
<td>76 ± 8.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14 ± 3.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10 ± 2.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BRL</td>
<td>50</td>
<td>82 ± 8.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GW</td>
<td>10</td>
<td>54 ± 6.5</td>
<td>29 ± 3.5</td>
<td>17 ± 2.1</td>
</tr>
<tr>
<td>BRL + GW</td>
<td>1</td>
<td>53 ± 6.1</td>
<td>29 ± 3.2</td>
<td>18 ± 2.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.05, BRL-treated vs. untreated cells.
<sup>b</sup> P < 0.01, BRL-treated vs. untreated cells.
bination with either cold competitor (Fig. 4B, lane 2) or the anti-NFkB antibody (Fig. 4B, lane 3).

Functional Interaction of PPARγ with p53 in Chromatin Immunoprecipitation (ChIP) Assay

The interaction of PPARγ with p53 was further elucidated by ChIP experiments. MCF7 cells were treated with formaldehyde to form DNA-protein cross-links and then sonicated. Thereafter, using anti-PPARγ, anti-NFkB, and anti-RNA polymerase II (Pol II) antibodies, we immunoprecipitated the complexes, and the binding of PPARγ, NFkB, and RNA Pol II, respectively, to the NFkB site within the p53 promoter was revealed by PCR. As shown in panel A of Fig. 5, BRL increased the recruitment of PPARγ to the promoter of p53. The BRL-induced effect was slightly reduced by TGFβ, but not altered in presence of the specific inhibitor of NFkB parthenolide (P) (23) (Fig. 5A). As it concerns the recruitment of NFkB to p53, evaluated using the anti-NFkB antibody, TGFβ enhanced such interaction that was abolished by P (Fig. 5A). Moreover, P was able to prevent the binding of RNA Pol II to p53 induced by TGFβ, but not that determined by BRL (Fig. 5A). These findings confirmed the ability of PPARγ to stimulate the transcription of p53 in a NFkB-independent manner (Fig. 5A). Next, the anti-PPARγ antibody did not immunoprecipitate a region upstream the NFkB site located within the p53 promoter gene (Fig. 5B).

BRL Induces Caspase-9 Cleavage and DNA Fragmentation in MCF7 Cells

Having demonstrated that PPARγ mediates p53 expression induced by BRL, we investigated the cleavage of caspase 9, which is an important component of the intrinsic apoptotic process (24). Notably, the treatment of MCF7 cells with BRL for 48 h promoted the caspase-9 activation, which was prevented by GW and in presence of an expression vector encoding p53 antisense (AS/p53) (Fig. 6A), which abolished p53 expression (Fig. 6B). On the contrary, the effect of BRL on the cleavage of caspase 9 was still notable using the NFkB inhibitor P (Fig. 6A), which abrogating the NFkB protein levels (Fig. 6C) excluded the contribution of such factor in the action elicited by BRL. As evidenced in DNA fragmentation assay, PPARγ was also involved in the apoptotic process triggered by BRL because this effect was completely and partially reversed by GW and the AS/p53, respectively (Fig. 6D). Taken together, these results indicate that, at least in part, a cross talk between PPARγ and p53 may be responsible for the growth arrest and apoptosis induced by BRL in MCF7 cells.

DISCUSSION

In recent years, a great deal of attention focused on the antiproliferative effects of PPARγ in a variety of
cancer cell types. Treatments with PPARγ ligands have been demonstrated to induce cell cycle arrest and apoptosis in different cancer models (6, 7, 25). In addition, an interaction between PPARγ and p53 was hypothesized, but not clarified, at molecular level in cholangiocarcinoma (26), in human gastric cancer cells (27), and even in rat vascular smooth muscle cells (28). In addition, from our and other studies emerged the ability of PPARγ to up-regulate the expression of the tumor suppressor gene phosphatase and tensin analog, which is required for both a negative modulation of phosphatidylinositol 3-kinase/Akt-dependent cell proliferation (20, 29, 30) and a p53-mediated regulation of cell survival and apoptosis (31). Consequently, PPARγ and p53 may converge in a tumor suppressor activity that remains to be further elucidated.

To provide new insight into the inhibitory action exerted by the cognate PPARγ-ligand BRL, we first demonstrated that PPARγ mediates the growth arrest in G0-G1 phase induced by BRL in MCF7 cells. In addition, considering the key role elicited by p53 in the growth inhibition and apoptosis (14, 17), we have evaluated whether PPARγ signaling converges on p53 transduction pathway in MCF7 cells. Of interest, we found that BRL exposure up-regulates both p53 mRNA and protein levels with a concomitant increase of p21WAF1/Cip1 expression. These effects were abrogated in the presence of the specific antagonist GW, addressing a PPARγ-mediated mechanism. Therefore, investigating the potential ability of BRL to modulate p53 promoter gene, we performed transient transfections in MCF7 cells using diverse deletion mutants of p53 promoter gene (32). The dose-dependent transactivation of p53 by BRL involved PPARγ directly because the transcriptional activity was prevented by GW treatment. Moreover, we documented that the region spanning from −49 to −40, which corresponds to the NFκB site, is required for the responsiveness to BRL.

It deserves to be mentioned that the transcription factor NFκB can regulate both pro- and antiapoptotic signaling pathways depending on cell type, the extent of NFκB activation, and the nature of the apoptotic stimuli (33). NFκB was reported to physically interact with PPARγ (34), which in some circumstances binds to DNA cooperatively with NFκB (35, 36), further enhancing the NFκB-DNA binding (37). Furthermore, PPARγ agonists were able to enhance the binding of NFκB to the upstream xB regulatory element site of c-myc (38). Our EMSA experiments extended the aforementioned observations because nuclear extracts of MCF7 cells treated with BRL showed an increased binding to the NFκB sequence located in the p53 promoter region. Given that the anti-PPARγ and anti-NFκB antibodies were both able to induce shifted bands, we performed an EMSA study using a cell-free system to ascertain the potential direct interaction of PPARγ with the NFκB site. Interestingly, we observed the formation of a single DNA-binding complex, which was again shifted by the anti-PPARγ antibody. These findings were supported by ChIP assay in MCF7 cells demonstrating the ability of BRL to enhance the recruitment of PPARγ and RNA Pol II to the promoter of p53 even in presence of the NFκB inhibitor P. Overall, these data indicate that the
PPARγ-mediated growth arrest upon addition of BRL in MCF7 cells involves, at least in part, the direct stimulation of p53 transcription.

p53 acts as a tumor suppressor depending on its physical and functional interaction with diverse cellular proteins (39), like some nuclear receptors that, in turn, exert an inhibitory activity on p53 biological outcomes (19). In the supplemental data, published on The Endocrine Society’s Journals online web site at http://mend.endojournals.org, we show an evident coimmunoprecipitation and colocalization of PPARγ/H9253 and p53 after BRL treatment. However, additional experiments are required to better characterize such interaction and its functional consequences.

A large body of evidence has suggested the straightforward role of p53 signaling in the apoptotic cascades that include the activation of caspases, a family of cytoplasmic cysteine proteases (40). The intrinsic apoptotic pathway involves a mitochondria-dependent process, which results in cytochrome c release and, thereafter, activation of caspase-9 (24). Furthermore, apoptosis is characterized by distinct morphological changes including the internucleosomal cleavage of DNA, which is recognized as a DNA ladder (Ref. 24 and references therein). Notably, we evidenced that in a consecutive series of events BRL 1) up-regulates the expression of p53 and 2) its effector p21WAF1/Cip1, 3) triggers the cleavage of caspases-9, and 4) induces DNA fragmentation in a PPARγ-mediated manner. Given the ability of AS/p53 to reduce the last two biological effects of BRL, an involvement of p53 in such PPARγ-dependent activity may be argued. On the contrary, the cleavage of caspase-9 and DNA fragmentation observed upon BRL treatment did not show changes suppressing the NFκB at protein level with P, suggesting that this factor is not required for the apoptotic events elicited by BRL.

In the present study we have provided a new insight into the molecular mechanism through which PPARγ mediates the growth arrest and apoptosis induced by BRL in MCF7 cells. Our findings suggest that a cross talk between p53 and PPARγ may assume biological relevance in setting novel therapeutic interventions in breast cancer.
MATERIALS AND METHODS

Reagents

BRL49653 was a gift from GlaxoSmithKline (West Sussex, UK), the irreversible PPARγ-antagonist GW was purchased from Sigma (Milan, Italy), human recombinant TGFβ was obtained from ICN Biomedicals (DBA, Milan, Italy), and P was purchased from Alexis (San Diego, CA).

Plasmids

The p53 promoter-luciferase reporters, constructed using pGL2 for cloning of p53-1 and -6, and TpGL2 for p53-13 and -14 were kindly provided by Dr. Stephen H. Safe (Texas A&M University, College Station, TX). The constructs used were generated by Safe (32) from the human p53 gene promoter as follows: p53-1 (containing the −1800 to +12 region), p53-6 (containing the −106 to +12 region), p53-13 (containing the −106 to −40 region) and p53-14 (containing the −106 to −49 region).

Cell Cultures

Wild-type human breast cancer MCF7 cells (a gift from Dr. Ewa Surmacz, Sbarro Institute for Cancer Research and Molecular Medicine, Philadelphia, PA) were grown in DMEM plus glutamax containing 10% fetal calf serum (Invitrogen, Milan, Italy) and 1 mg/ml penicillin-streptomycin.

Fig. 4. PPARγ Binds to NFκB Site in the p53 Promoter Region in EMSA

A, Nuclear extracts from MCF7 cells (lane 1) or 2 µl of PPARγ translated protein (lane 6) were incubated with a double-stranded NFκB sequence probe labeled with [γ-32P] and subjected to electrophoresis in a 6% polyacrylamide gel. Competition experiments were performed adding as competitor a 100-fold molar excess of unlabeled NFκB probe (lanes 2 and 7) or as cold competitor PPRE (lane 8). In lane 3, nuclear extracts from MCF7 were treated with 10 µM BRL. Anti-PPARγ and anti-NFκB Abs were incubated with nuclear extracts from MCF7 cells treated with 10 µM BRL (lanes 4 and 5, respectively) or added to PPARγ protein (lanes 9 and 10, respectively). Lane 11 contains probe alone, lane 12 contains 2 µl of unprogrammed rabbit reticulocyte lysate incubated with NFκB (URRL). B, NFκB protein (1 µl) (lane 1) was incubated with a double-stranded NFκB sequence probe labeled with [γ-32P] and subjected to electrophoresis in a 6% polyacrylamide gel. A 100-fold molar excess of unlabeled NFκB probe (lanes 2) or anti-NFκB antibody (Ab) (lane 3) was added to NFκB protein.
Materials and Methods

Normal rabbit antiserum was used as negative control (N). To control input DNA, p53 promoter was amplified from 30 μl of initial preparations of soluble chromatin (before immunoprecipitations). Normal rabbit antiserum was used as negative control (N).

RT-PCR Assay

MCF7 cells were grown in 10-cm dishes to 70–80% confluence, shifted to regular growth medium/well the day before transfection. The firefly luciferase activity and data were reported as relative light units. The activity of AS/p53 was verified using Western blot to detect changes in p53 protein levels. Time course analysis revealed that p53 levels were effectively suppressed at 18 h after transfection (data not shown). Empty vector was used to ensure that DNA concentrations were constant in each transfection.

EMS A

Nuclear extracts from MCF7 cells were prepared as previously described for EMSA (42). Briefly, MCF7 cells plated into 10-cm dishes were grown to 70–80% confluence, shifted to SFM for 24 h, and then treated with 10 μM BRL for 6 h. Thereafter, cells were scraped into 1.5 ml of cold PBS. Cells were pelleted for 10 sec and resuspended in 400 μl cold buffer A [10 mM HEPES-KOH (pH 7.9) at 4 C, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonylfluoride (PMSF), 1 mM leupeptin] by flicking the tube. The cells were allowed to swell on ice for 10 min and then vortexed for 10 sec. Samples were then centrifuged for 10 sec and the supernatant fraction was discarded. The pellet was resuspended in 50 μl of cold Buffer B [20 mM HEPES-KOH, pH 7.9; 25% glycerol; 1.5 mM MgCl₂; 420 mM NaCl; 0.2 mM EDTA; 0.5 mM dithiothreitol; 0.2 mM PMSF; 1 mM leupeptin] and incubated in ice for 20 min for high-salt extraction. Cellular debris was removed by centrifugation for 2 min at 4 C, and the supernatant fraction (containing DNA-binding proteins) was stored at −70 C. In vitro- transcribed and translated PPARγ was synthesized using the T7 polymerase in the rabbit reticulocyte lysate system from PPARγ plasmid as recom-
directed by the manufacturer (Promega). The probe was generated by annealing single-stranded oligonucleotides and labeled with \[^{32}P\]ATP (Amersham Pharmacia, Buckinghamshire, UK) and T4 polynucleotide kinase (Promega) and then purified using Sephadex G50 spin columns (Amersham Pharmacia). The DNA sequence of the NF\(^{B}\) used as probe or as cold competitor is the following: NF\(^{B}\), 5'-AGT TGA GGG GAC TTT CCC AGG C-3' (Sigma Genosys, Cambridge, UK). As cold competitor we also used PPRE oligonucleotide: 5'-GGGACCAGGACAAAGGTCACGTT-3' (Sigma Genosys).

The protein-binding reactions were carried out in 20 \(\mu\)l of buffer [20 mM HEPES (pH 8), 1 mM EDTA, 50 mM KCl, 10 mM dithiothreitol, 10% glycerol, 1 mg/ml BSA, 50 \(\mu\)g/ml polydeoxyinosinic deoxycytidylic acid] with 50,000 cpm of labeled probe, 5 \(\mu\)g of MCF7 nuclear protein, or 2 \(\mu\)l of transcribed and translated in vitro PPAR\(\gamma\) protein, or 1 \(\mu\)l of NF\(\kappa\)B protein (Promega), and 5 \(\mu\)g of polydeoxyinosinic deoxycytidylic acid. The mixtures were incubated at room temperature for 20 min in the presence or absence of unlabeled competitor oligonucleotides. For the experiments involving anti-PPAR\(\gamma\) and anti-NF\(\kappa\)B antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), the reaction mixture was incubated with these antibodies at 4 C for 30 min before addition of labeled probe. The entire reaction mixture was electrophoresed through a 6% polyacrylamide gel in 0.25 \(\times\) Tris borate-EDTA for 3 h at 150 V. Gel was dried and subjected to autoradiography at \(-70^\circ\) C.

**Fig. 6. BRL Induces Cleavage of Caspase-9 and DNA Laddering**

A, MCF7 cells were treated with BRL alone or in combination with GW or P for 48 h as indicated, or transfected with an expression plasmid encoding for p53 antisense (AS/p53). Positions of procaspase-9 and its cleavage products are indicated by arrowheads to the right. One of three similar experiments is presented. \(\beta\)-Actin was used as loading control on the same stripped blot. B, p53 protein expression (evaluated by Western blot) in MCF7 cells transfected with an empty vector (v) or a AS/p53 and treated as indicated. \(\beta\)-Actin was used as loading control. C, NF\(\kappa\)B expression in MCF7 cells untreated or treated with P as indicated, \(\beta\)-Actin was used as loading control. D, DNA laddering was performed in MCF7 cells treated for 72 h as indicated, or transfected with AS/p53.

**ChIP**

MCF7 cells were grown in 10-cm dishes to 50–60% confluence, shifted to SFM for 24 h, and then treated with 10 \(\mu\)M BRL for 1 h. Thereafter, cells were washed twice with PBS and cross-linked with 1% formaldehyde at 37 C for 10 min. Next, cells were washed twice with PBS at 4 C, collected and resuspended in 200 \(\mu\)l of lysis buffer (1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris-HCl, pH 8.1), and left on ice for 10 min. Then, cells were sonicated four times for 10 sec at 30% of maximal power (Vibra Cell 500 W; Sonics and Materials, Inc., Newtown, CT) and collected by centrifugation at 4 C for 10 min at 14,000 rpm. The supernatants were diluted in 1.3 ml of immunoprecipitation buffer (0.01% SDS; 1.1% Triton X-100; 1.2 mM EDTA; 16.7 mM Tris-HCl, pH 8.1; 16.7 mM NaCl) followed by immunoclearing with 80 \(\mu\)l of sonicated salmon sperm DNA/protein A agarose (DBA Srl, Milan, Italy) for 4 C. The precleared chromatin was immunoprecipitated with anti-PPAR\(\gamma\), anti-NF\(\kappa\)B, and anti-RNA Pol II antibodies (Santa Cruz Biotechnology). At this point, 60 \(\mu\)l salmon sperm DNA/protein A agarose was added, and precipitation was further continued for 2 h at 4 C. After pelleting, precipitates were washed sequentially for 5 min with the following buffers: Wash A [0.1% SDS, 1% Triton X-100; 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl], WA B [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl],
MCF7 cells were grown in 10-cm dishes to 70% confluence. Cell were then harvested in cold PBS and resuspended in lysis buffer containing 20 mM HEPES (pH 8), 0.1 mM EDTA, 5 mM MgCl2, 0.5 mM NaCl, 20% glycerol, 1% Nonidet P-40, and inhibitors (0.1 mM Na3VO4, 1% PMSF, 20 mM EDTA, 5 mM MgCl2, and 90 sec at 72 C, 40 sec at 55 C, 40 sec at 57 C, 90 sec at 72 C, 40 sec at 55 C, and 90 sec at 72 C). The amplification products obtained in 30 cycles were analyzed in a 2% agarose gel and visualized by ethidium bromide staining. The negative control was provided by PCR amplification without a DNA sample. The specificity of reactions was ensured using normal mouse serum in place of the first antibody and reprobed with anti-

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and Wash C [0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)], and then twice with TE buffer (10 mM Tris, 1 mM EDTA). The immuno-

Immunoblotting

MCF7 cells were grown in 10-cm dishes to 70–80% conflu-

DNA Fragmentation

DNA fragmentation was determined by gel electrophoresis. MCF7 cells were grown in 10-cm dishes to 70% confluence and treated with 10 μM BRL and/or 10 μM GW and/or 15 μM P. After 72 h cells were collected and washed with PBS and pelleted at 1800 rpm for 5 min. The samples were resus-

**Statistical Analysis**

Statistical analysis was performed using ANOVA followed by Newman-Keuls testing to determine differences in means. P < 0.05 was considered as statistically significant.

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and Wash C [0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)], and then twice with TE buffer (10 mM Tris, 1 mM EDTA). The immuno-

Immunoblotting

MCF7 cells were grown in 10-cm dishes to 70% confluence. Cell were then harvested in cold PBS and resuspended in lysis buffer containing 20 mM HEPES (pH 8), 0.1 mM EDTA, 5 mM MgCl2, 0.5 mM NaCl, 20% glycerol, 1% Nonidet P-40, and inhibitors (0.1 mM Na3VO4, 1% PMSF, 20 mg/ml aprotinin). Protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). A 50-μg portion of protein lysates was used for Western blotting, resolved on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with an antibody directed against the p53, p21(WAF1/Cip1), caspases-9, and NFκB (Santa Cruz Biotechnology). As internal control, all membranes were subsequently stripped (0.2 M glycine, pH 2.6, for 30 min at room temperature) of the first antibody and reprobed with anti-β-actin antibody.

The antigen-antibody complex was detected by incubation of the membranes for 1 h at room temperature with peroxi-
dase-coupled goat antimouse or antirabbit IgG and revealed using the enhanced chemiluminescence system (Amersham Pharmacia). Blots were then exposed to film (Kodak film, Sigma). The intensity of bands representing relevant proteins was measured by Scion Image laser densitometry scanning program.

DNA Fragmentation

DNA fragmentation was determined by gel electrophoresis. MCF7 cells were grown in 10-cm dishes to 70% confluence and treated with 10 μM BRL and/or 10 μM GW and/or 15 μM P. After 72 h cells were collected and washed with PBS and pelleted at 1800 rpm for 5 min. The samples were resus-
pended in 0.5 ml of extraction buffer (50 mM Tris-HCl, pH 8; 10 mM EDTA, 0.5% SDS) for 20 min in rotation at 4 C. DNA was extracted with phenol-chloroform three times and once with chloroform. The aqueous phase was used to precipitate acids nucleic with 0.1 vol or of 3 M sodium acetate and 2 volumes cold EtOH overnight at -20 C. The DNA pellet was resuspended in 15 μl of H2O treated with RNAse A for 30 min at 37 C. The absorbance of the DNA solution at 260 and 280 nm was determined by spectrophotometry. The extracted DNA (40 μg/lane) was subjected to electrophoresis on 1.5% agarose gels. The gels were stained with ethidium bromide and then photographed.

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