A plant steroid, diosgenin, induces apoptosis, cell cycle arrest and COX activity in osteosarcoma cells

Sandra Moalic\textsuperscript{a}, Bertrand Liagre\textsuperscript{a,}* , Cécile Corbière\textsuperscript{a}, Arnaud Bianchi\textsuperscript{b}, Michel Dauça\textsuperscript{b}, Karim Bordji\textsuperscript{c}, Jean L. Beneytout\textsuperscript{a}

\textsuperscript{a}Laboratoire de Biochimie, UPRES EA 1085, Faculté de Pharmacie, 2 rue du Docteur Marcland, 87025 Limoges Cedex, France
\textsuperscript{b}Laboratoire de Biologie Cellulaire du Développement, Université Henri Poincaré, Vandoeuvre-lès-Nancy, France
\textsuperscript{c}Laboratoire de Pharmacologie, UMR 7561 CNRS-Université Henri Poincaré Nancy I, Faculté de Médecine, 54505 Vandoeuvre-lès-Nancy, France

Received 2 August 2001; accepted 12 September 2001

First published online 25 September 2001

Edited by Ulf-Ingo Filigge

Abstract Cyclooxygenases (COXs) are key enzymes in the conversion of arachidonic acid into prostanoids which are involved in apoptosis and inflammation. Two distinct COXs have been identified: COX-1 which is constitutively expressed and COX-2 which is induced by different products such as tumor promoters or growth factors. Previously, we demonstrated that a plant steroid, diosgenin, was a new megakaryocytic differentiation inducer of human erythroleukemia cells. In our study, we investigated the effect of diosgenin on the proliferation rate, cell cycle distribution and apoptosis in the human osteosarcoma 1547 cell line. The effects of this compound were also tested on COX expression and COX activities. Diosgenin treatment caused an inhibition of 1547 cell growth with a cycle arrest in G\textsubscript{1} phase and apoptosis induction. Moreover, we found a correlation between p53, p21 mRNA expression and nuclear factor-\kappaB activation and we observed a time-dependent increase in PGE\textsubscript{2} synthesis after diosgenin treatment. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Diosgenin; Apoptosis; Cell cycle; Cyclooxygenase; Osteosarcoma cell line

1. Introduction

Cyclooxygenases (COXs) are key enzymes in the conversion of arachidonic acid into prostanoids which are involved in apoptosis, inflammation, mitogenesis and immunomodulation. Two distinct COX isoforms have been identified: COX-1 which is considered to be the constitutively expressed form and thought to serve housekeeping functions and COX-2 which is expressed at very low basal levels and rapidly induced by different products such as tumor promoters, growth factors or inflammatory cytokines. Many studies report an increase in COX-2 expression in numerous cancer cell lines especially in colorectal cancer cells [1,2] but also in pancreatic carcinoma cells [3], epidermal cancer cells [4], breast cancer cells [5], glioma cells [6] and osteosarcoma cells [7].

Non-steroidal anti-inflammatory drugs (NSAIDs) have been found to inhibit proliferation and to induce apoptosis in human colorectal cell lines in vitro [8,9]. Recently, we described that under apoptotic conditions, there was a link between the effects of NS-398, a selective COX-2 inhibitor, on prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) release, cell apoptosis and COX-2 expression in the human osteosarcoma 1547 cell line [7].

Previously, we demonstrated that a plant steroid, diosgenin, was a new megakaryocytic differentiation inducer of human erythroleukemia cells [10]. In this study, we investigated the effect of diosgenin on the proliferation rate, cell cycle distribution and apoptosis in the human osteosarcoma 1547 cell line. Moreover, the effects of this compound were tested on COX expression and activity.

2. Materials and methods

2.1. Cell line, cell culture and treatment

The 1547 human osteosarcoma cell line was kindly provided by Professor M. Rigaud (Laboratoire de Biochimie, Faculté de Médecine de Limoges, France). Freshly trypsinized cells were seeded at 4 \times 10^{3} cells/cm\textsuperscript{2} and grown in Eagle’s minimum essential medium (Gibco BRL, Cergy-Pontoise, France) supplemented with 10% fetal calf serum (FCS) (Gibco BRL), 100 U/ml penicillin and 100 \mu g/ml streptomycin. Cultures were maintained in a humidified atmosphere with 5% CO\textsubscript{2} at 37°C. Cell viability was determined by the trypan blue dye exclusion method. For all experiments cells were allowed to adhere and grow for 3 days in culture medium prior to exposure to diosgenin (5\alpha-spirost-3\beta-ol, Sigma). A stock solution of 10^{-2} M diosgenin was prepared in ethanol and diluted in culture medium to give a final concentration of 10–100 \mu M. The same amount of ethanol was added to control cells.

2.2. Cell proliferation assay

Measurement of cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, trypsinized cells were plated (1200 cells/well) in 96-well culture plates. 3 days later, the seeding medium was removed and replaced by 10% FCS medium containing diosgenin (0–100 \mu M) for 24–96 h. MTT test was carried out daily as previously described [11]. Experiments were performed in sextuple assays.

2.3. Lactate dehydrogenase (LDH) test

Cells were seeded in 96-well plates at a density of 1200 cells/well and treated without or with diosgenin (20 and 40 \mu M). Cytotoxicity detection kit (Boehringer Mannheim) measured the LDH activity released from the cytosol of damaged cells into the supernatant which evaluated the percentage of cell death according to the manufacturer’s protocol.

2.4. Cell cycle analysis

Cells were seeded at 3.6 \times 10^{4} cells in 6-well culture plates, cultured in 10% FCS medium without or with diosgenin (40 \mu M) for 12–48 h. Adherent and floating cell populations were combined and counted, and cell viability was determined by the trypan blue dye exclusion method. For DNA content analysis, 10\textsuperscript{6} cells were fixed in 70% ethan-
nol (in phosphate-buffered saline (PBS)), washed in PBS and stained with propidium iodide (PI) (50 μg/ml final concentration) [12]. Flow cytometric analyses were performed as previously described [7].

2.5. Measurement of apoptosis

1547 cells were cultured in 6-well culture plates. After diosgenin treatment (40 μM) for 6, 12 and 24 h, we observed an increasing proportion of floating cells. As we found with other compounds [7,11] these cells were apoptotic. To accurately determine the extent of apoptosis, we first evaluated the amount of floating cells in culture supernatants. Secondary, apoptosis was quantified by 'cell death' enzyme-linked immunosorbent assay ELISA (Cell Death Detection ELI- SA+, Roche Diagnostics) on pooled fractions (adherent and floating cells). Cytosol extracts were obtained according to the manufacturer’s protocol and apoptosis was measured as previously described [7].

2.6. RNA extraction and semi-quantitative RT-PCR analysis of 1547 culture extracts

Total RNA was extracted from cells cultured in 10% FCS medium with or without 40 μM diosgenin for 24 h. EMSA experiments were performed as previously described [13]. Briefly, cells were scraped and lysed: nuclei were collected and 10 μg of nuclear proteins were incubated with 32P-labeled nuclear factor-xB (NF-xB) or activator protein-1 (AP-1) probes [13]. The samples were loaded on a 5% native polyacrylamide gel and run in 0.5 x TBE buffer. NF-xB and AP-1-specific bands were confirmed by competition with a 100-fold excess of the respective unlabeled probe which resulted in no shifted band. For super-shift experiments, the extracts were incubated with the specific antibodies (anti-p65 or anti-c-fos or anti-c-jun for AP-1).

2.7. Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Cells were cultured in 75 cm² tissue culture flasks. After 40 μM diosgenin treatment, adherent cells were trypsinized and pooled with the floating cell fraction. Western blot analysis was performed as previously described [7] using the primary monoclonal antibodies Bcl-2 (mouse anti-human Bcl-2, Dako) or Bax (mouse anti-human Bcl-2, Immunotech) and the secondary polyclonal antibody conjugated with peroxidase (Dako). Blots were visualized using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech) and immediately exposed to X-ray film.

2.8. Bax and Bcl-2 Western blot analysis

Cells were cultured in 150 cm² tissue culture flasks. After 40 μM diosgenin treatment, adherent cells were trypsinized and pooled with the floating cell fraction. Western blot analysis was performed as previously described [7] using the primary monoclonal antibodies Bcl-2 (mouse anti-human Bcl-2, Dako) or Bax (mouse anti-human Bcl-2, Immunotech) and the secondary polyclonal antibody conjugated with peroxidase (Dako). Blots were visualized using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech) and immediately exposed to X-ray film.

2.9. PGE2 EIA analysis

1547 cells were cultured and treated (6, 12 and 24 h) as described above in 6-well culture plates. Undiluted culture supernatants were centrifuged (2000 rpm for 5 min at 4°C) before being stored at −80°C until analysis. PGE2 release by cell monolayers was measured by PGE2 competitive immunoassay (Cayman Chemicals) carried out according to the manufacturer’s protocol. PGE2 production was normalized with respect to the number of viable cells present in the particular culture at the time of sampling.

2.10. Statistical analysis

Statistical analysis of differences was carried out by analysis of variance (ANOVA). A P-value of less than 0.05 was considered to indicate significance.

3. Results and discussion

3.1. Effect of diosgenin on cell growth

Cells were cultured in 10% FCS-containing medium with or

<table>
<thead>
<tr>
<th>Oligonucleotides and PCR product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human p21WAF1/CIP1 AF265443 430–454</td>
</tr>
<tr>
<td>Homo sapiens p53 AH002918 129–151</td>
</tr>
<tr>
<td>Human Bcl-2 M14745 1386–1405</td>
</tr>
<tr>
<td>Human Bax L22473 90–110</td>
</tr>
<tr>
<td>Homo sapiens caspase-3 4757911 68–89</td>
</tr>
<tr>
<td>Human Hsp70 35223 19–40</td>
</tr>
<tr>
<td>Homo sapiens COX-1 11386140 89–111</td>
</tr>
<tr>
<td>Homo sapiens COX-2 NM_000963 447–469</td>
</tr>
<tr>
<td>Homo sapiens β-actin XM_004814 590–611</td>
</tr>
</tbody>
</table>
without diosgenin (10–100 μM) during 4 days and cell proliferation was evaluated by the MTT test. Under our experimental conditions, a dramatic decrease in proliferation was observed until 24 h after diosgenin treatment (40, 80 and 100 μM) (Fig. 1), especially at 24 h for 40 μM diosgenin where the percentage of inhibition was 86% \( (P < 0.05) \). As the percentage of inhibition did not strongly increase for 80 or 100 μM diosgenin, we choose 40 μM for the following experiments. These results were confirmed by counting cells and, in order to verify cell viability after 40 μM diosgenin treatment, we used the LDH test which did not show any cytotoxicity (data not shown).

### 3.2. Cell cycle analysis and p21, p53 mRNA expression

To ascertain potential mechanisms by which diosgenin inhibited 1547 cell proliferation rate, we studied the effect of diosgenin on the cell cycle distribution (Fig. 2). 1547 cells were treated with 40 μM diosgenin for 12, 24 and 48 h. After 12 h, we observed a significant accumulation of cells in the G1 phase (26 to 34%) \( (P < 0.05) \) (Fig. 2A). This effect was markedly enhanced at 24 h (35 to 50%) \( (P < 0.05) \) (Fig. 2B). Consequently, the fraction of S phase cells decreased at 12 h (48 to 36%) \( (P < 0.05) \) and at 24 h (46 to 21%) \( (P < 0.05) \) (Fig. 2A,B). At 48 h, a sub-G1 population, normally associated with apoptotic cells, appeared compared to controls (Fig. 2C). Moreover, RT-PCR analysis showed that p53 and p21 mRNA expression were significantly increased after 24 h of diosgenin treatment (1.3- and 1.5-fold versus control respectively, \( P < 0.05 \) (Fig. 2D).

It is now established that the tumor suppressor p53 inhibits cell growth through activation of cell cycle arrest and apoptosis. This is effected, at least in part, by transcriptional activation of the p21 gene, a cell cycle inhibitor [14]. Moreover, Katayose et al. [15] demonstrated that an adenovirus vector expressing p53 induced p21, cell cycle arrest at G1 and accumulation of cells in a G1 subgroup. In our study, the growth of 1547 cells was inhibited in a time-dependent manner after 40 μM diosgenin treatment and this process was accompanied by a modulation of cell cycle-related mRNA: p53 and p21 mRNA levels were increased following diosgenin treatment for 24 h. Recently, Pelizzaro et al. [16] showed that sodium butyrate blocked the growth of both cell lines by induction of

---

**Fig. 2.** Cell cycle analysis of 1547 cells cultured in 10% FCS medium without (control) or with 40 μM diosgenin for 12 h (A), 24 h (B) and 48 h (C). Cell phase distribution was determined by PI staining and Facs analysis as previously described [7]. The experiments were performed three times; representative results are shown. Diosgenin treated cells, showing a G1 block (A and B), a S decrease (A and B) and an appearance of a sub-G1 population (C). (D) Top, p53 and p21 mRNA expression in 1547 cells treated or not (time 0) with diosgenin in 10% FCS medium. Cells were treated with 40 μM diosgenin for 6, 12 and 24 h. Bottom, p53 and p21 transcripts were quantified using β-actin as an internal control. Quantification of each band was performed by densitometry analysis software (Quantity One, Bio-Rad) and results were expressed as the ratio (p53/β-actin or p21/β-actin) in relative arbitrary units. Quantifications are the result of three independent experiments. After RT-PCR analysis, p53 and p21 mRNA expression were increased after 24 h of diosgenin treatment.
p21 through a p53-dependent or p53-independent mechanism. Levine [17] has also shown that activated p53 causes G1 arrest by inducing expression of p21 and the consequent inhibition of cyclin D/cyclin-dependent kinases. Moreover, p53-dependent arrest of cells in the G1 phase of the cell cycle is an important component of the cellular response to stress [18].

3.3. Diosgenin induced apoptosis in 1547 cells

Another mechanism by which diosgenin produced an anti-proliferative effect on these cells was induction of apoptosis. Apoptosis was evaluated by counting floating cells and by ELISA performed on pooled cell fractions (floating and adherent cells). The effect of 40 μM diosgenin was observed at 6, 12 and 24 h. Diosgenin treatment induced a significant increase in floating cells over time: 2.4% ± 0.9% (P < 0.05) for 6 h, 9.2% ± 1.8% (P < 0.05) for 12 h and 25.1% ± 5.1% (P < 0.05) for 24 h, compared to controls (Table 2). Moreover, the apoptotic ratio, determined by ELISA, significantly increased over time (Table 2). After 24 h diosgenin treatment, we observed a marked increase of hsp70 mRNA expression (3.3-fold versus control, P < 0.05) by RT-PCR analysis (Fig. 3).

Table 2

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Ratio of floating cells (%)</th>
<th>Apoptotic ratioa</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>2.4 ± 0.9*</td>
<td>0.5 ± 0.1*</td>
</tr>
<tr>
<td>12</td>
<td>9.2 ± 1.8*</td>
<td>2.5 ± 1.2*</td>
</tr>
<tr>
<td>24</td>
<td>25.1 ± 5.1*</td>
<td>5.5 ± 1.2*</td>
</tr>
</tbody>
</table>

Cell counts of adherent and floating cells were determined at 6, 12, and 24 h, and the ratio of floating cells to total cells plotted. These results are mean ± S.D. of three separate wells (P relative to control group: *P < 0.05). Moreover, apoptosis was performed by ELISA and the apoptotic ratio determined. Values are expressed as mean ± S.D. of three experiments (P relative to control group: *P < 0.05).

A sample absorbance/control absorbance.

3.4. Time course for the expression of bax and bcl-2 in 1547 cells treated with diosgenin

RT-PCR and Western blot analysis were used to evaluate the time course for bax and bcl-2 expression during diosgenin induction of apoptosis. RT-PCR analysis showed that 40 μM diosgenin treatment down-regulated mRNA expression of bax and bcl-2 (Fig. 4A). After 24 h treatment, the expression of anti-apoptotic bcl-2 protein and pro-apoptotic bax protein was analyzed. Western blot analysis showed that the bax/bcl-2 ratio, which is a critical determinant of apoptosis, was 1.53-fold higher (P < 0.05) than in control cells (Fig. 4B).
3.5. Effect of diosgenin on COX expression and activity

Recently, we showed the modulation of COX expression and COX activity in human 1547 cells by NS-398, a selective COX-2 inhibitor [7]. This phenomenon was well established in other cell types [2,9]. In our study, RT-PCR analysis showed a significant decrease in COX-2 mRNA expression over time after 40 μM diosgenin treatment but, diosgenin was ineffective on COX-1 (non inducible form) mRNA expression (Fig. 5A). Moreover, diosgenin regulated enzymatic COX activities (Fig. 5B). The synthesis of PGE2 was time-dependent and this production was significantly increased over time after diosgenin treatment (2.1-fold at 6 h, 3.1-fold at 12 h and 4.6-fold at 24 h versus control, \( P < 0.05 \)). This synthesis was not correlated with COX-1 and COX-2 mRNA expression. However, cells were under stress conditions (hsp70 mRNA expression increased), a phenomenon which could explain the marked enhancement of COX activity.

3.6. Diosgenin activated NF-κB in 1547 cells

To test if diosgenin could have an effect on NF-κB or AP-1 activation pathways, 1547 cells were treated with 40 μM diosgenin for 24 h. EMSA allowed us to visualize the binding of NF-κB and AP-1 on oligonucleotide probes containing their specific response element. Diosgenin enhanced nuclear localization of NF-κB DNA-binding activity in cultured human 1547 osteosarcoma cells. Cells were cultured in 10% FCS-containing medium with diosgenin (dios, 40 μM) or vehicle (c, ethanol 0.1%) for 24 h. Nuclear proteins were extracted and 10 μg of each sample were subjected to EMSA using NF-κB (A) consensus site radiolabeled probe. Complexes were visualized by autoradiography. Comp SP1 = competitor SP1; Comp 100X = 100-fold concentrated unlabeled probe. B: EMSA ‘super-shift’ assays identifying the subunit components for NF-κB dimer. The experiments were performed three times; representative results are shown.
ization of NF-κB compared to control (Fig. 6A) whereas it was ineffective on AP-1 activation (data not shown). Incubation of nuclear proteins with 100-fold concentrated unlabeled probe was performed to indicate the specificity of binding of NF-κB to the DNA. Moreover, pre-incubation in the presence of specific antibodies identified the components of the protein complex as being p65/p50 heterodimer for NF-κB (Fig. 6B). One of the key proteins that modulates the apoptotic response is NF-κB, a transcription factor that can protect or contribute to apoptosis. Recently, Ryan et al. [21] have shown that induction of p53 causes an activation of NF-κB that correlates with the ability of p53 to induce apoptosis. Moreover, it was shown that the human p21 promoter harbors p53-responsive elements and an NF-κB-binding site. Recently, Hellin et al. [22] demonstrated the binding of NF-κB dimers to the κB site and transcriptional activation of the human p21 promoter by daunomycin and NF-κB subunits, thereby confirming the functionality of this κB-binding site in human breast and colon carcinoma cells.

In conclusion, our study suggests that diosgenin induces an inhibition of 1547 cell growth with a cycle arrest in G1 phase and apoptosis induction. We found a correlation between p53, p21 mRNA expression and NF-κB activation. This activation of NF-κB does not produce an increase in COX-2 mRNA expression in our conditions. The observed enhanced rate of PGE2 production could be explained by cellular stress as shown by the increase in hsp70 mRNA expression. Future work in our laboratory will seek to understand the precise molecular mechanism(s) of diosgenin’s action.

Acknowledgements: We are grateful to Professor M. Rigaud (Laboratoire de Biochimie Médicale, Faculté de Médecine, Limoges, France) for providing the 1547 human osteosarcoma cell line. We would like to thank Dr. C. Jayat-Vignoles (Service Commun de Cytométrie, Université de Limoges) for valuable advice concerning flow cytometry analysis. The expenses of this work were defrayed in part by the Ministère de l’Education Nationale, de la Recherche et de la Technologie, the Conseil Régional du Limousin and by the Ligue Nationale de Recherche contre le Cancer.

References