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

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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_1 phase and apoptosis induction. Moreover, we found a correlation between p53, p21 mRNA expression and nuclear factor-κB activation and we observed a time-dependent increase in PGE_2 synthesis after diosgenin treatment.

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_2 (PGE_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 4×10^3 cells/cm^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 μg/ml streptomycin. Cultures were maintained in a humidified atmosphere with 5% CO_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 (5α-spirosten-3β-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 μM. 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 μM) 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 μM). 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 3.6×10^4 cells in 6-well culture plates, cultured in 10% FCS medium without or with diosgenin (40 μM) 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, 10^6 cells were fixed in 70% etha-

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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 ELISA$^+$, 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 without or with diosgenin (40 μM) for 6, 12 and 24 h by a single-step guanidium thiocyanate–phenol chloroform method using Trizol reagent (Gibco BRL, Cergy-Pontoise, France). 2 μg of total RNA were transcribed into cDNA according to the Omniscript™ RT kit (Qiagen), and 2 μl of the reverse-transcribed cDNA were used for PCR according to the HotStarTaq DNA polymerase mix kit (Qiagen) with 20 pmol of different human sense and antisense primers (Table 1).

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

Cells were cultured in 75 cm^2 flasks and treated with 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 ^{32}P-labeled nuclear factor-κB (NF-κB) or activator protein-1 (AP-1) probes [13]. The samples were loaded on a 5% native polyacrylamide gel, and run in 0.5×TBE buffer. NF-κB 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-p50 for NF-κB; anti-c-fos or anti-c-jun for AP-1).

2.8. Bax and Bcl-2 Western blot analysis

Cells were cultured in 150-cm^2 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 Bax, 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. PGE_2 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. PGE_2 release by cell monolayers was measured by PGE_2 competitive immunoassay (Cayman Chemicals) carried out according to the manufacturer's protocol. PGE_2 production was normalized with respect to the number of viable cells present in the particular culture at the time of sampling.

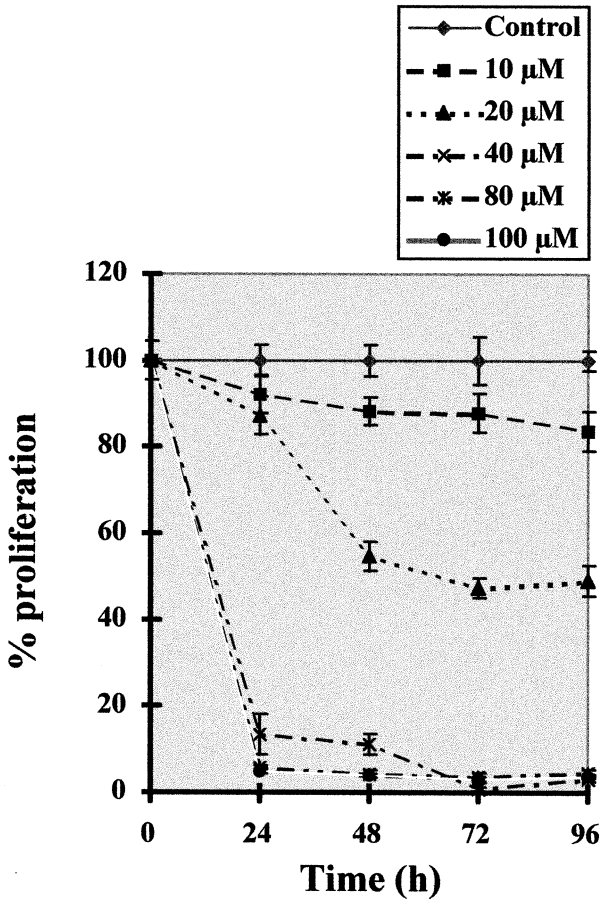

Fig. 1. Effect of diosgenin on 1547 cell growth. After 72 h adherence, a cell line was cultured in 10% FCS medium and treated with diosgenin (10–100 μM) for 24–96 h. Results are presented as percentage of control (untreated cells). Values were expressed as mean ± S.D. of six experiments ($n = 6$) (P-value relative to control group: $P < 0.05$).

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 1
Oligonucleotides and PCR product size

cDNA species	GenBank accession number	Corresponding 5′-primer nucleotides	Corresponding 3′-primer nucleotides	Size of PCR product (bp)
Human p21WAF1/CIP1	AF265443	430–454	849–873	444
Homo sapiens p53	AH002918	129–151	609–632	504
Human Bcl-2	M14745	1386–1405	1829–1848	463
Human Bax	L22473	90–110	541–563	474
Homo sapiens caspase-3	4757911	68–89	499–521	454
Human Hsp70	35223	19–40	492–513	495
Homo sapiens COX-1	11386140	89–111	390–411	323
Homo sapiens COX-2	NM_000963	447–469	867–887	441
Homo sapiens β-actin	XM_004814	590–611	1132–1158	569

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 G_1 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-G_1 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 G_1 and accumulation of cells in a G_1 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, Pellizzaro et al. [16] showed that sodium butyrate blocked the growth of both cell lines by induction of

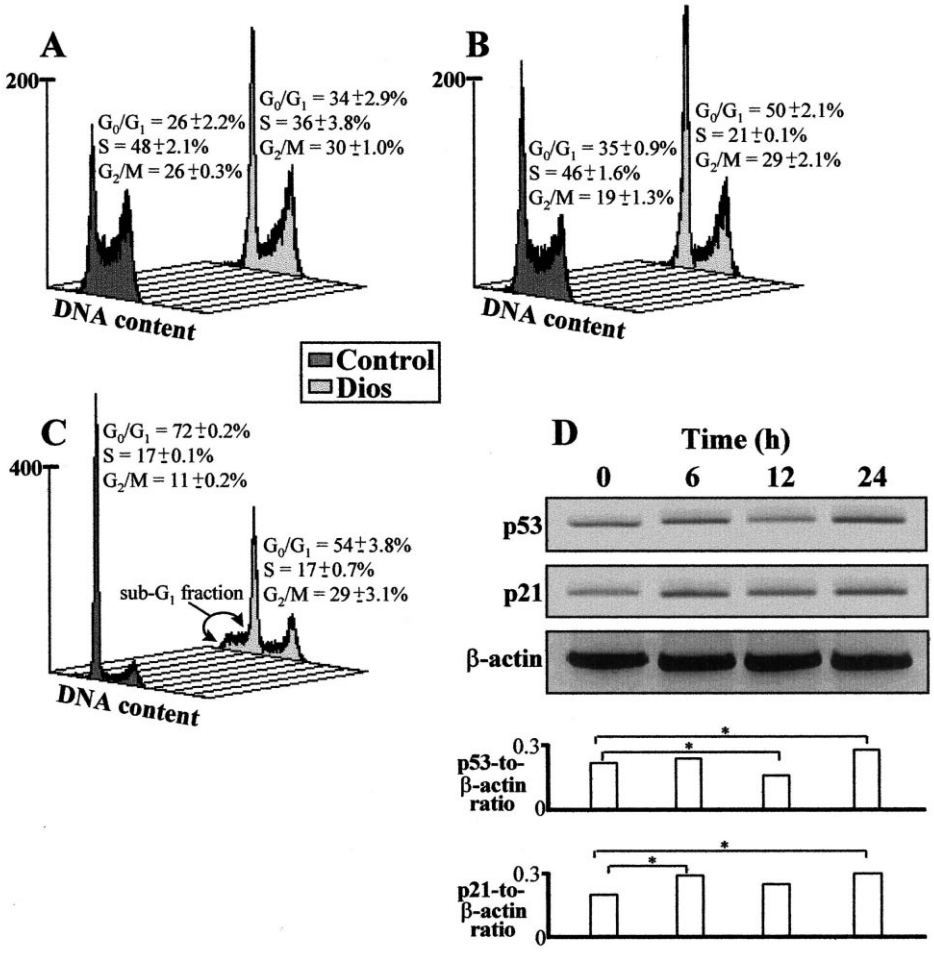

Fig. 2. Cell cycle analysis of 1547 cells cultured in 10% FCS medium without (control) or with 40 μM diosgenin for 12 h (A), 24 h (B) and 48 h (C). Cell phase distribution was determined by PI staining and Facs analysis as previously described [7]. The experiments were performed three times; representative results are shown. Diosgenin treated cells, showing a G_1 block (A and B), a S decrease (A and B) and an appearance of a sub-G_1 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.

Table 2
Apoptosis in 1547 cells treated with 40 μM diosgenin

Time (h)	Ratio of floating cells (%)	Apoptotic ratio[a]
0 (control)	0	0
6	2.4 ± 0.9#	0.5 ± 0.1*
12	9.2 ± 1.8#	2.5 ± 1.2*
24	25.1 ± 5.1#	5.5 ± 1.2*

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

p21 through a p53-dependent or p53-independent mechanism. Levine [17] has also shown that activated p53 causes G_1 arrest by inducing expression of p21 and the consequent inhibition of cyclin D/cyclin-dependent kinases. Moreover, p53-dependent arrest of cells in the G_1 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 antiproliferative 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), which could be due to stress conditions. Hsp proteins function as molecular chaperones in regulating cellular homeostasis and promoting survival. If the stress is too severe, a signal that leads to programmed cell death is activated, thereby providing a finely timed balance between survival and death [19]. Moreover, Hsps such as hsp70 transiently associate with key molecules of the cell cycle control system such as p53 and are involved in the nuclear localization of regulatory proteins [20]. In addition, the expression of caspase-3 mRNA was not modified (Fig. 3).

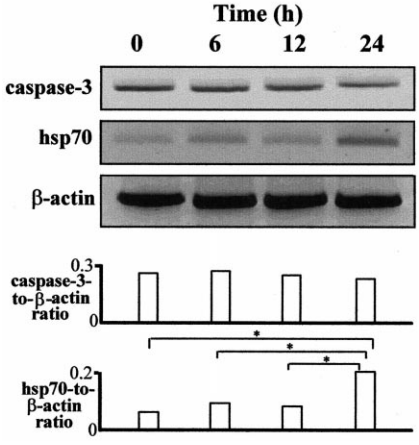

Fig. 3. Top: Effect of diosgenin on caspase-3 and hsp70 mRNA expression. 1547 cells were cultured in 10% FCS-containing medium in the absence (time 0) or presence of 40 μM diosgenin for 6, 12 and 24 h. Total RNA was immediately extracted for RT-PCR experiments. Bottom: Caspase-3 and hsp70 transcripts were quantified using β-actin as an internal control. Quantification of each band was performed by densitometry analysis software and results were expressed as the ratio (caspase-3/β-actin or hsp70/β-actin) in relative arbitrary units. Results represent mean ± S.D. ($n = 3$). After 24 h treatment, hsp70 mRNA expression was strongly increased whereas caspase-3 mRNA expression was not modified.

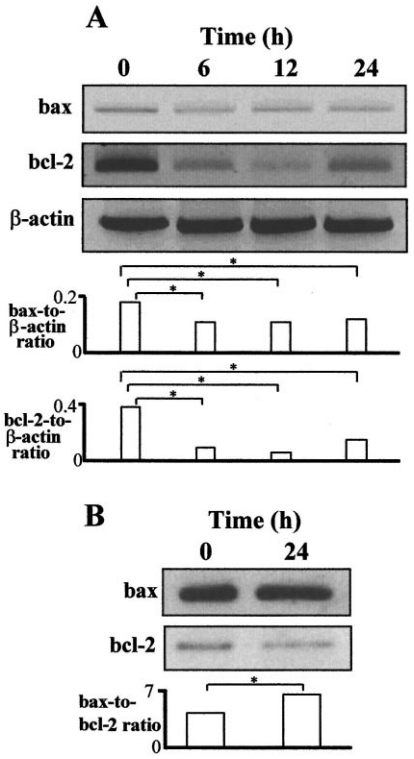

Fig. 4. Time course for the expression of bax and bcl-2 during diosgenin-induced apoptosis in 1547 cells. A: RT-PCR analysis. Cells were treated with 40 μM diosgenin for 6, 12 and 24 h. Bax and bcl-2 transcripts were quantified using β-actin as an internal control. Quantification of each band was performed by densitometry analysis software and results were expressed as the ratio (bax/β-actin or bcl-2/β-actin) in relative arbitrary units. Quantifications are the result of three independent experiments. B: Western blot analysis. Cells were treated or not (time 0) with 40 μM diosgenin for 24 h. Proteins were extracted from the cells and separated on 15% SDS–PAGE gel. Each lane contains 35 μg of total cell lysates. Cellular expression of bax and bcl-2 were estimated using mouse anti-human bax and mouse anti-human bcl-2 antibodies. After 24 h treatment, Western blot analysis showed that the bax/bcl-2 ratio was increased 1.53-fold compared to control cells ($^{*}P < 0.05$).

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).

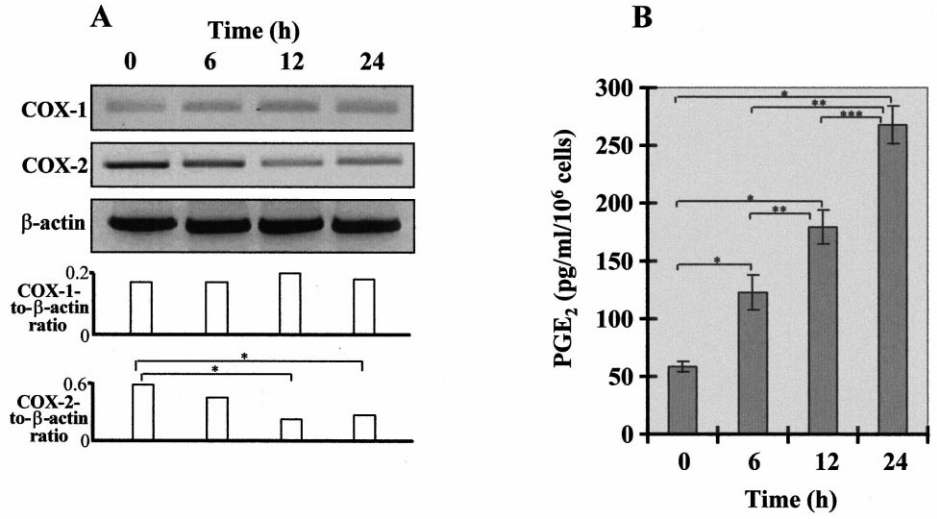

Fig. 5. Effect of diosgenin on COX mRNA expression (A) and PGE_2 release (B) in 1547 cells. A: RT-PCR analysis. Cells were treated with 40 μM diosgenin for 6, 12 and 24 h. COX-1 and COX-2 transcripts were quantified using β-actin as an internal control. Quantification of each band was performed by densitometry analysis software and results were expressed as the ratio (COX-1/β-actin or COX-2/β-actin) in relative arbitrary units. Quantifications are the result of three independent experiments. B: The amounts of PGE_2 released by cell monolayers in culture supernatants were quantified by EIA. PGE_2 levels were normalized to the number of adherent cells. Values are expressed as mean ± S.D. of three experiments ($n = 3$). *Significant difference from control group (time 0) ($P < 0.05$), **significant difference from group (time 6 h) ($P < 0.05$), ***significant difference from group (time 12 h) ($P < 0.05$). PGE_2 synthesis was time-dependent and significantly increased over time after diosgenin treatment.

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 is 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 PGE_2 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 local-

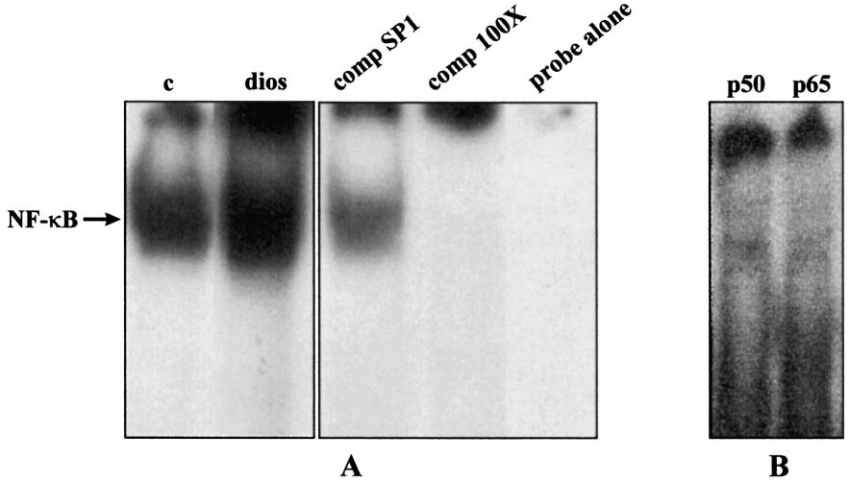

Fig. 6. Diosgenin enhanced NF-κB DNA-binding activity in cultured human 1547 osteosarcoma cells. Cells were cultured in 10% FCS-containing medium with diosgenin (dios, 40 μM) or vehicle (c, ethanol 0.1%) for 24 h. Nuclear proteins were extracted and 10 μg of each sample were subjected to EMSA using NF-κB (A) consensus site radiolabeled probe. Complexes were visualized by autoradiography. Comp SP1 = competitor SP1; Comp 100× = 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 G_1 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 PGE_2 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.

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