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PAPER

Okadaic acid induces morphological changes, apoptosis and cell cycle alterations in different human cell types

Vanessa Valdiglesias,[ab] Blanca Laffon,[*a] Eduardo Pásaro[a] and Josefina Méndez[b]

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Okadaic acid (OA) is a marine toxin produced by dinoflagellate species which is frequently accumulated in molluscs usual in the human diet. The exact action mechanism of OA has not been described yet and the results of most reported studies are often conflicting. The aim of this work was to evaluate the OA effects on morphology, cell cycle and apoptosis induction by means of light microscopy and flow cytometry, in three different types of human cells (leukocytes, HepG2 cells and SHSY5Y cells). Cells were treated with a range of OA concentrations in the presence and absence of S9 fraction. OA induced morphological changes in all the cell types studied, and cell cycle disruption only in leukocytes and neuronal cells. SHSY5Y cells were the most sensitive to OA insult. Results obtained in the presence and absence of metabolic activation were similar, suggesting that OA acts both directly and indirectly. Furthermore, OA was found to increase the subG$_1$ region in the flow cytometry cell cycle analysis, suggesting induction of apoptosis. These results were confirmed by the employment of specific methodologies for studying apoptosis such as caspase 3 activation and annexin V staining. Increases in the apoptosis rate were obtained in all the cells treated in the absence of S9 fraction, accompanied by increases in caspase 3 activation, suggesting that apoptosis induced by OA is a caspase 3-dependent process. Nevertheless, in the presence of S9 fraction no apoptosis was detected, indicating a metabolic detoxifying activity, although necrosis was observed in neuroblastoma cells.

Introduction

Harmful algal blooms have emerged as a worldwide concern due to the perceived increases in occurrence and severity, and the known acute and chronic toxicity to plants, animals and humans.[1] The marine polyether okadaic acid (OA) is a phycotoxin produced by several types of dinoflagellates, mainly of the genus *Dynophysis* and *Prorocentrum*, which are often responsible for these toxicity phenomena.[2] The ingestion of shellfish contaminated with OA induces acute and strong gastrointestinal symptoms known as diarrheic shellfish poisoning (DSP). The minimum dose of OA necessary to induce DSP symptoms in adults has been estimated to be 40 mg.[3] These symptoms begin within 4 h and continue for about 3 days, but they do not appear to be fatal. The current European regulation on the level of DSP toxins in shellfish for human consumption (REF) establishes 160 μg kg^{-1} as the maximum limit of OA;[4] but this value is exclusively focused on reduction of the gastrointestinal symptoms.

In addition to DSP, OA also represents other potential threats to human health even at concentrations within the nanomolar range.[5] Previous studies have shown that OA is a specific potent

[a]*Toxicology Unit, Psychobiology Department, University of A Coruña, Edificio de Servicios Centrales de Investigación, Campus Elviña s/n, 15071 A Coruña, Spain. E-mail: blaffon@udc.es; Fax: +34 981167172; Tel: +34 981167000*
[b]*Department of Cell and Molecular Biology, University of A Coruña, A Coruña, Spain*

Environmental impact

Marine algal blooms are natural contamination phenomena that have become a public health concern due to their increasing occurrence and intensity. Okadaic acid (OA) is a phycotoxin produced by several dinoflagellates which are often responsible for these toxicity phenomena. OA, the most widely distributed and most common marine toxin in Europe, is responsible for the diarrheic shellfish poisoning (DSP) in molluscs consumers. But, in addition to DSP, it has been reported that OA poses a threat to human health even at concentrations within the nanomolar range. Nevertheless, results of these studies are often differing and the OA behaviour at the cellular and molecular levels is not well understood yet. This fact justifies carrying out further studies on the mechanism of OA cytotoxicity.

inhibitor of Ser/Thr protein phosphatases and protein synthesis.[6,7] The number of physiological processes in which those phosphatases are involved is immense, including regulation of glycogen metabolism and coordination of the cell cycle and gene expression.[8] In fact, as well as causing embryotoxicity, genotoxicity and DNA repair modulations,[9–12] OA has been shown to induce cell cycle arrest, apoptosis, cell proliferation and alterations in gene expression in many previous studies.[13–16] However, not very consistent inter-relationships have emerged on this regard, largely due to the diversity of responses to OA seen among different systems.[17] Although OA can elicit premature induction of mitosis,[13,18] induce proto-oncogene expression[15,19] or stimulate cell cycle progression[20] in several cell systems, most studies report inhibitory proliferative effects over the concentration range 5–100 nM.[14,21–25]

Protein phosphorylation and dephosphorylation events have been also established as key factors in the regulation of cytoskeleton structure and function.[26] In fact, it has been reported that the cytoskeleton is one of the main cellular targets of OA since it was found to induce filamentous actin (F-actin) disorganization, cell rounding, and loss of cell polarity,[27–30] to produce the abnormal hyperphosphorylation of τ, inhibiting its binding and the promotion of microtubule assembly *in vitro*,[31] and to promote the hyperphosphorylation of major intermediate filament (IF) proteins, leading to the disassembly of IF networks, solubilisation of IF proteins, and disruption of desmosomes.[32,33]

Although the effects of OA both *in vitro* and in experimental organisms have been previously reported at different levels, the behaviour of this toxin at the cellular and molecular levels is not well understood, in part due to its complicated biochemical mechanisms.[34] Besides, several previous studies found that OA showed different effects according to the cell type studied.[35,36] Both facts justify carrying out further studies on the mechanism of OA cytotoxicity in different cell lines. Furthermore, the specific effects of OA on cell transformation, cell proliferation and apoptosis vary widely, especially between different cell types, and the molecular events underlying these effects of OA have not been described yet.

In the present study the effects of OA on cell morphology, cell cycle and apoptosis were examined in three different types of human cells: peripheral blood leukocytes, hepatoma cells (HepG2 cell line) and neuroblastoma cells (SHSY5Y cell line). Cells were treated with a range of OA concentrations (5 to 1000 nM), in the presence and absence of S9 fraction. The morphological changes after OA exposure were visualized by phase-contrast microscopy and the OA-induced cell cycle, the rate of apoptosis/necrosis (phosphatidylserine detection in the outer leaflet of the plasma membrane) and the active caspase 3 levels were evaluated by means of flow cytometry methodologies.

Methods

Chemicals

OA (CAS No. 78111-17-8), mytomycin C (MMC) (CAS No. 50-07-7), benzo(*a*)pyrene (B(*a*)P) (CAS No. 50-32-8), camptothecin (CAS No. 7689-03-4), RNase A and propidium iodide (PI) were purchased from Sigma-Aldrich Co. (Madrid, Spain). OA was dissolved in dimethyl sulfoxide (DMSO), and MMC, B(*a*)P and camptothecin were dissolved in sterile distilled water; from a stock solution prepared in DMSO in the case of B(*a*)P. The metabolic activation fraction used was S9 from male Sprague-Dawley rats from Sigma-Aldrich Co. (Madrid, Spain). The freshly prepared S9 mix consisted of 10% S9, 3.3% 1 M KCl, 3.2% 0.25 M $MgCl_2 \cdot 6H_2O$, 2.5% 0.2 M glucose-6-phosphate, 10% 0.04 M NADP, 21% distilled water and 50% phosphate buffer (pH 7.4).

Cell culture

Human peripheral blood was collected using heparinised vials from three healthy non-smoker male donors aged 23–30. The University of A Coruña Research Ethics Committee approved the investigations. Written consent was obtained from each donor prior to joining the study. Mononuclear cells (lymphocytes and monocytes) were isolated on a Ficoll–Hypaque density gradient as described earlier[37] and cultured in supplemented RPMI 1640 medium containing 15% heat inactivated foetal bovine serum, 1% phytohaemagglutinin (PHA), 1% L-glutamine (200 mM) and 1% penicillin (5000 U ml^{-1})/streptomycin (5000 μg ml^{-1}), all from Invitrogen (Barcelona, Spain). SHSY5Y cells (human neuroblastoma cell line) and HepG2 cells (human hepatocellular carcinoma cell line) were obtained from the European Collection of Cell Cultures. SHSY5Y cells were grown in nutrient mixture EMEM/F12 (1 : 1) medium with 1% non-essential amino acids, 1% antibiotic and antimycotic solution and supplemented with 10% heat-inactivated foetal bovine serum, all from Invitrogen (Barcelona, Spain). HepG2 cells were cultured in DMEM medium with 1% antibiotic and antimycotic solution and supplemented with 10% foetal bovine serum, all from Invitrogen (Barcelona, Spain). The cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

For the SHSY5Y and HepG2 cell assays, 96 well plates (flat bottom) were prepared with cells obtained from a 90 to 100% confluence flask. Cell suspension (200 μl) was added to each well and cells were cultured at 37 °C for 24 h prior to the experiment. Cell densities were approximately in the range of 6–8 × 10^5 cells per well.

Cell treatments

For the OA treatments, all cells were incubated at 37 °C in the presence of OA (5, 10, 20, 50, 100 and 1000 nM) or the control solutions at 1% of final volume. All the treatments for morphology and cell cycle assessment were performed in the presence and absence of metabolic activation in leukocytes and SHSY5Y cells, following the protocol described by Pérez-Machado *et al.*[38] for the treatment with S9 fraction. HepG2 cells were not cultured in the presence of S9 fraction in any case due to their demonstrated ability to activate compounds without the addition of exogenous enzymes.[39] DMSO was used as negative control in all cases; camptothecin (10 μM) or MMC (3 μM for leukocytes and 1.5 μM for HepG2 and SHSY5Y cells) were used as positive controls in experiments without metabolic activation, and B(*a*)P (50 μg ml^{-1} for leukocytes and 10 μg ml^{-1} for HepG2 and SHSY5Y cells) was used as the positive control in experiments with metabolic activation.

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Cell morphology

The changes in cell morphology in leukocytes, SHSY5Y cells and HepG2 cells, induced by OA were observed with a light microscope (Nikon TMS, Japan). After 3 h of treatment with the different OA concentrations, cells were visualized and phase-contrast photographs of control and treated cells were obtained.

Cell cycle analysis

Cells (8×10^5) were cultured with OA for 48 h. Then cells were centrifuged, resuspended in cold phosphate buffer solution (PBS) and fixed with cold 70% (v/v) ethanol (−20 °C). Fixed cells were then stored overnight at 4 °C. For analysis, cells were centrifuged, resuspended in PBS containing 0.1 mg ml^{-1} RNase A and 50 μg ml^{-1} PI and incubated at 37 °C in the dark for 30 min. Samples were kept on ice prior to analysis. The analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson). At least 10^4 events were acquired, and the DNA content was assessed from the PI signal detected by the FL2 detector. Cell cycle histograms were evaluated using Cell Quest Pro software (Becton Dickinson) to provide information on the percentage of occupancy of subG_1, G_0/G_1, S and G_2/M regions.

Activation of caspase 3

To evaluate the cleavage activation of caspase 3, cells were treated with OA (5–1000 nM) for 3 h and cultured in fresh OA-free medium for another 3 h in the case of SHSY5Y and HepG2 cells, and for another 21 h in the case of leukocytes. Then, the levels of cleaved caspase 3 were determined by flow cytometry using the BD Pharmingen™ FITC active caspase-3 apoptosis kit (Becton Dickinson, Madrid, Spain) following manufacturer's instructions. In brief, 6×10^5 cells were trypsinized and washed with phosphate buffer solution (PBS). The cells were fixed/permeablised with cytofix/cytoperm solution for 20 min at 4 °C, and then the intracellular active caspase 3 was stained with fluorescein-isothiocyanate (FITC)-conjugated rabbit anti-active caspase 3 for 30 min at room temperature in the dark. Cells were analyzed in a FACSCalibur flow cytometer using Cell Quest Pro software (Becton Dickinson). A minimum of 10^4 events were acquired.

Analysis of apoptosis by PI–annexin V staining

Annexin V–FITC and propidium iodide (PI) staining was used to evaluate by means of flow cytometry the apoptosis/necrosis induction by OA, using the BD Pharmingen™ Annexin V–FITC apoptosis detection kit I, according to the provided protocol. Briefly, cells were treated with OA (5–1000 nM) for 3 h and cultured in fresh medium for another 3 h or 21 h, according to the cell type. Then the treated cells were washed in PBS and suspended in binding buffer at 6×10^5 cells per 100 μl. Annexin V–FITC and PI were added and incubated for 15 min at room temperature in the dark. Flow cytometric analysis was performed in a FACSCalibur flow cytometer (Becton Dickinson). At least 10^4 events were acquired, obtaining data from FL1 (FITC) and FL2 (IP) detectors. Data were analyzed using Cell Quest Pro software (Becton Dickinson). The redistribution of phosphatidylserine from the inner to the outer leaflet of the plasma membrane is one of the early physiological changes in cells undergoing apoptosis.[40] Annexin V is a protein that exhibits specific affinity for the phosphatidylserine, so cells in the early stages of apoptosis bind annexin V–FITC in the absence of PI uptake, while those in the late stages of apoptosis, or in necrosis, bind annexin V–FITC in the presence of PI uptake. Early apoptosis and late apoptosis/necrosis were expressed as the percentages of annexin V^+/PI^- and annexin V^+/PI^+ cells, respectively.

Statistical analysis

Three independent experiments were performed for each experimental condition tested. Experimental data were expressed as mean ± standard error. Distribution of the response variables departed significantly from normality (Kolmogorov–Smirnov goodness of fit test) and therefore non-parametric tests were considered adequate for the statistical analysis of these data. Differences between groups were tested with Kruskal–Wallis test and Mann–Whitney *U*-test. Dose–response relationships for OA were determined by Pearson's correlation. A *P*-value of <0.05 was considered significant. Statistical analysis was performed using the SPSS for Windows statistical package (version 16.0).

Results

Fig. 1–3 show the comparative morphologies of unexposed and OA-exposed leukocytes (Fig. 1), SHSY5Y cells (Fig. 2) and HepG2 cells (Fig. 3). In leukocytes treated with OA in the absence of S9 fraction, the cell shape changed from spherical to irregular at 1000 nM (Fig. 1a–c); in the presence of S9 fraction these cells did not show morphological alterations (Fig. 1d–f). SHSY5Y cells exposed to OA with and without metabolic activation showed morphological changes from the 50 nM concentration on, with rounding of the cells, loss of neurites and detaching from the surface forming clusters (Fig. 2a–f). Morphological alterations were also observed in HepG2 cells; after 3 h of OA treatment at 50, 100 and 1000 nM cells retracted into spherical shape and also detached from the substratum forming clusters (Fig. 3a–c).

Results obtained in the cell cycle analyses by flow cytometry in the three types of human cells evaluated, in the absence and presence of S9 fraction, are shown in Fig. 4. Four distinct phases are distinguished in a proliferating cell population: G_1, S (DNA synthesis phase), G_2 and M (mitosis) phases. However, G_2 and M phases, which both have an identical DNA content, could not be discriminated on the basis of their different DNA contents. When the cell cycle of leukocytes exposed to OA in the absence of S9 fraction was analyzed, the proportion of cells in the S phase decreased significantly at 50 nM, and the G_2/M phase increased at 100 nM (Fig. 4a). In the presence of S9 fraction, a significant increase in the G_0/G_1 phase and a significant decrease in the G_2/M phase were found at 10 and 1000 nM, respectively (Fig. 4b). When SHSY5Y cells were incubated with OA in the absence of S9 fraction, statistically significant alterations were observed in their cell cycle at 100 and 1000 nM, with both decreases in the G_0/G_1 phase and increases in the S phase (Fig. 4c). Indeed, significant dose–response relationships were obtained for the G_0/G_1 phase ($r = -0.721$, $P < 0.01$) and S phase ($r = 0.838$, $P < 0.01$).

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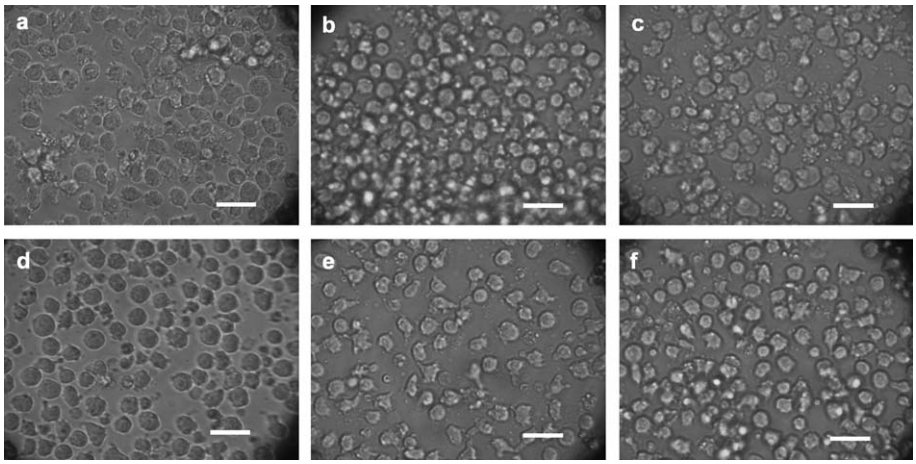

Fig. 1 Morphology of human leukocytes: control cells (a and d), and cells treated for 3 h with 50 nM OA (b and e) and 1000 nM OA (c and f). Treatments were performed in the absence of S9 (a–c) or in the presence of S9 (d–f). Scale bar: 100 μm.

When these same cells were treated in the presence of S9 fraction, the G_2/M phase decreased at 20 and 1000 nM OA. Furthermore, an increase in the S phase at this last concentration and significant dose–response relationships for the G_0/G_1 phase ($r = -0.316$, $P < 0.05$) and S phase ($r = -0.612$, $P < 0.01$) were observed (Fig. 4d). Besides, no alterations were found when HepG2 cells were exposed to OA at any concentration (Fig. 4e).

The subG_1 region was also evaluated in the cell cycle analyses since DNA fragmentation, indicative of the late stages of apoptosis, results in the appearance of PI-stained events containing subG_1 levels.[41] Results of OA-induced apoptosis, measured as the cell percentage in the subG_1 region, are shown in Fig. 5. An increase in the percentage of apoptosis was found in leukocytes treated with OA in the absence of S9 fraction at 100 and 1000 nM, with a statistically significant dose–response relationship ($r = 0.555$, $P < 0.01$) (Fig. 5a), and in the presence of S9 fraction at 5, 10 and 1000 nM (Fig. 5b). OA was also observed to induce apoptosis in a dose-dependent manner in SHSY5Y cells without metabolic activation ($r = 0.573$, $P < 0.01$), with significant increases at 100 and 1000 nM (Fig. 5c), and with metabolic activation ($r = 0.533$, $P < 0.01$), with a significant increase at 1000 nM (Fig. 5d), and in HepG2 cells ($r = 0.356$, $P < 0.05$), with significant increases at 100 and 1000 nM (Fig. 5e).

Data of active caspase 3 estimated by flow cytometry in leukocytes, SHSY5Y cells and HepG2 cells treated with OA in the presence and absence of S9 fraction are shown in Fig. 6. A statistically significant increase of active caspase 3 was found in leukocytes exposed to OA at 10, 50, 100 and 1000 nM in the absence of S9 fraction (Fig. 6a). On the contrary, in the presence of S9 fraction decreases in active caspase 3 values were observed at all the OA concentrations investigated, significant at 50, 100 and 1000 nM (Fig. 6b). The incubation of SHSY5Y cells with OA resulted in statistically significant increases in active caspase 3 at 1000 nM in the absence of S9 fraction (Fig. 6c), and at all the concentrations tested in the presence of S9 fraction (Fig. 6d). Furthermore, statistically significant dose–response relationships were obtained in both cases ($r = 0.668$, $P < 0.01$ with metabolic activation, and $r = 0.591$, $P < 0.01$ without metabolic activation). Similarly increased active caspase 3 levels were observed in HepG2 cells after treatment with OA, significant at 1000 nM, and also a statistically significant dose–response relationship was found in these cells ($r = 0.519$, $P < 0.01$) (Fig. 6e).

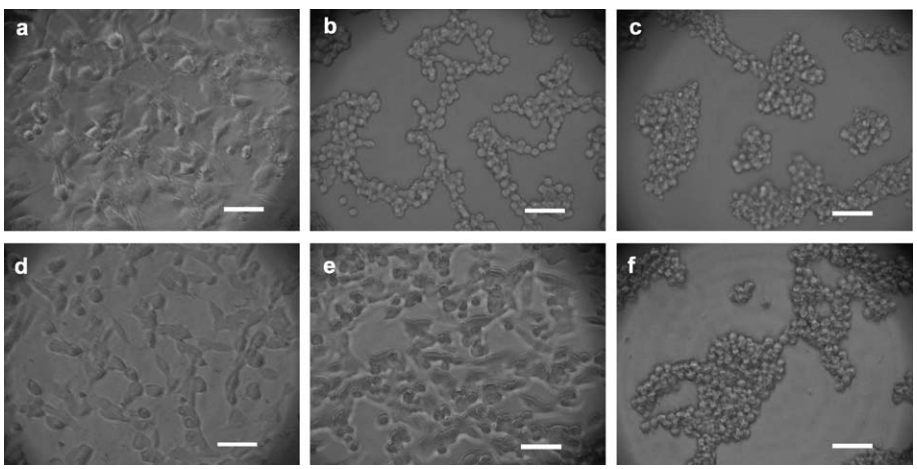

Fig. 2 Morphology of SHSY5Y cells: control cells (a and d), and cells treated for 3 h with 50 nM OA (b and e) and 1000 nM OA (c and f). Treatments were performed in the absence of S9 (a–c) or in the presence of S9 (d–f). Scale bar: 100 μm.

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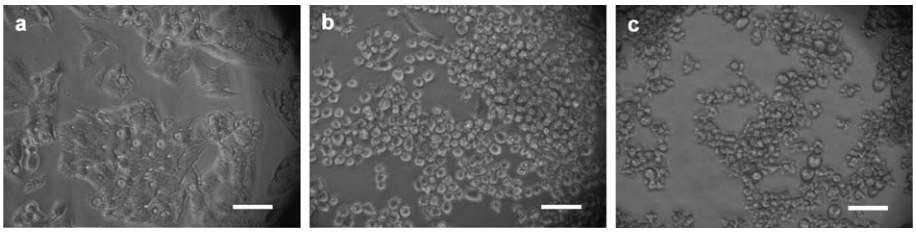

Fig. 3 Morphology of HepG2 cells: control cells (a), and cells treated for 3 h with 50 nM OA (b) and 1000 nM OA (c). Scale bar: 100 μm.

Fig. 7 and 8 show the results of the flow cytometric analysis of annexin V/PI staining of cells exposed to OA in the absence and presence of S9 fraction, with regard to the rates of apoptosis and necrosis, respectively. An increase in the percentage of apoptosis was found in the absence of S9 fraction in leukocytes at 100 and 1000 nM OA (Fig. 7a), and in SHSY5Y cells at 1000 nM OA (Fig. 7c). In HepG2 cells the apoptotic rate was also higher in OA exposed cells, significant at 100 and 1000 nM (Fig. 7e). No apoptosis induction was observed in leukocytes and SHSY5Y cells in the presence of S9 fraction (Fig. 7b and d). Furthermore, statistically significant dose–response relationships were obtained for HepG2 cells ($r = 0.426$, $P < 0.01$) and for leukocytes ($r = 0.401$, $P < 0.05$) treated with OA in the absence of S9 fraction, while an inverse dose–response relationship was found in these last cells with metabolic activation ($r = -0.396$, $P < 0.01$).

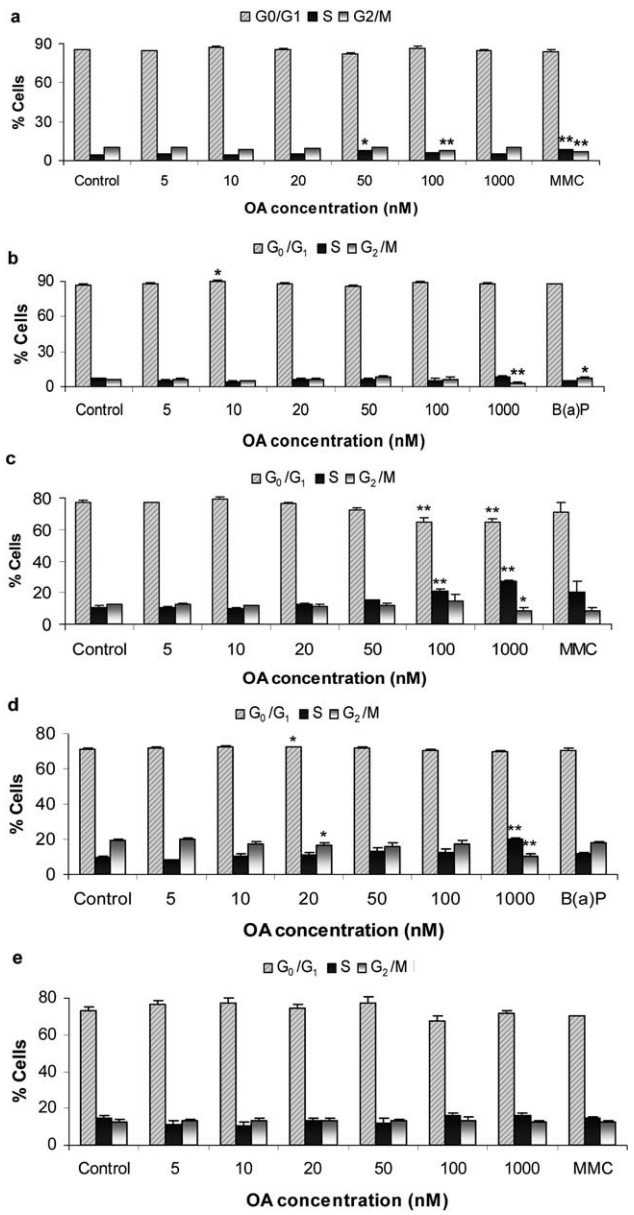

Fig. 4 Cell cycle analysis in human cells treated with OA for 48 h: leukocytes (a and b), SHSY5Y cells (c and d), and HepG2 cells (e). Treatments were performed in the absence (a, c and e) or presence (b and d) of S9 fraction. *: $P < 0.05$ and **: $P < 0.01$, significant difference with regard to the control.

The rate of necrosis was observed to increase in leukocytes exposed to OA in the absence of metabolic activation, significant at 1000 nM (Fig. 8a), and a significant dose–response relationship was obtained ($r = 0.491$, $P < 0.01$). Besides, SHSY5Y cells treated with OA at 5, 50 and 100 nM in the presence of S9 fraction showed significantly higher necrosis percentage than the control (Fig. 8d). In contrast, no differences were found in OA exposed leukocytes with metabolic activation (Fig. 8b), SHSY5Y cells without metabolic activation (Fig. 8c) or HepG2 cells (Fig. 8e).

Discussion

OA is a phycotoxin produced by marine dinoflagellates and involved in DSP in humans.[42] This toxin is known to produce gastrointestinal symptomathology but it is also a well-known inhibitor of protein phosphatases 1 and 2A, and exerts its toxicity through general overphosphorylation of cellular proteins.[43] OA has been reported to induce genotoxic,[9,10] cytotoxic[44,45] and embryotoxic effects.[11] Furthermore, this marine toxin has been demonstrated to be a potent promoter of skin and stomach tumours in rodents[46–48] and is suspected to participate in causing digestive tumours in humans.[49] Nevertheless, it is also known to induce apoptosis or growth inhibition in some cell transformation systems;[17,34,50] protein phosphorylation and caspases were suggested to play a role in OA-induced apoptosis.[51] This apparently dualistic response of OA has been of greater concern since its complicated biochemical mechanisms have not been well defined and its effects cannot always be explained by phosphatases inhibition.[5]

In this study, the OA-induced morphological changes were investigated in three types of human cells (peripheral blood leukocytes, HepG2 hepatoma cells, and SHSY5Y neuroblastoma cells) by light microscopy. Furthermore, a flow cytometric analysis of the DNA content was performed to assess the cell cycle phase distribution after OA exposure (5–1000 nM), and the

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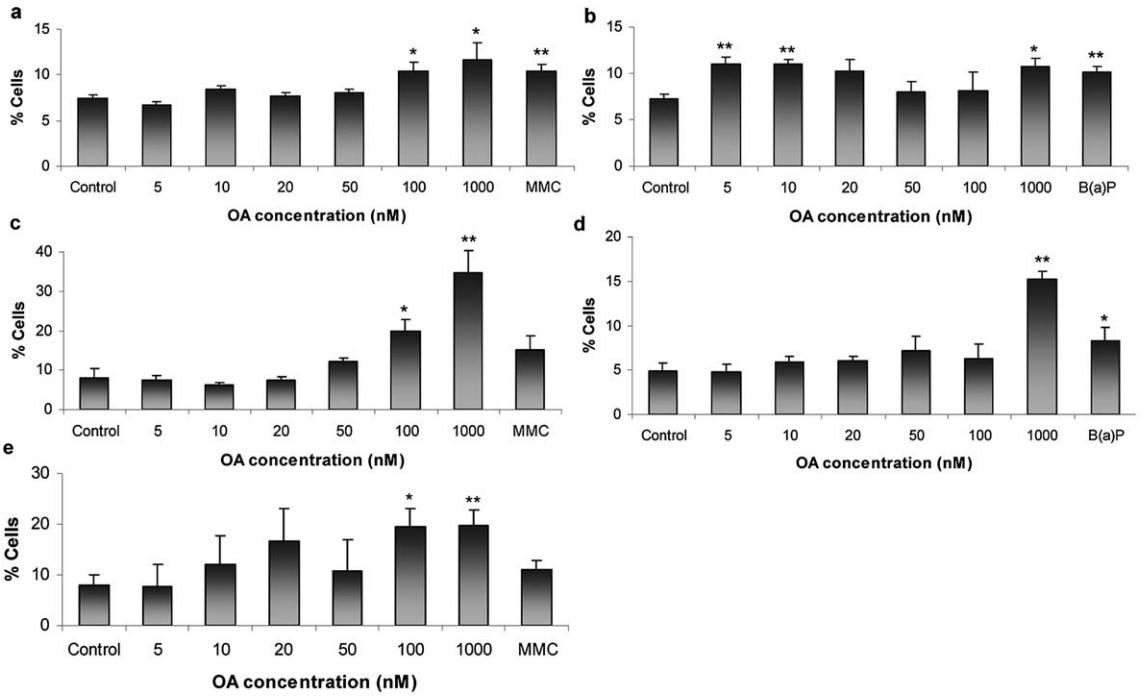

Fig. 5 Apoptosis ($subG_1$ region) in human cells treated with OA for 48 h: leukocytes (a and b), SHSY5Y cells (c and d), and HepG2 cells (e). Treatments were performed in the absence (a, c and e) or presence (b and d) of S9 fraction. *: $P < 0.05$ and **: $P < 0.01$, significant difference with regard to the control.

role of OA as a possible apoptosis inductor was also studied by means of flow cytometry methodologies. The different types of cells employed were selected on the basis of the results of previous OA studies (reviewed in ref. 12). Cytoskeletal structures play key roles in the maintenance of cell architecture, adhesion, migration, differentiation, division, and organelle transport.[8]

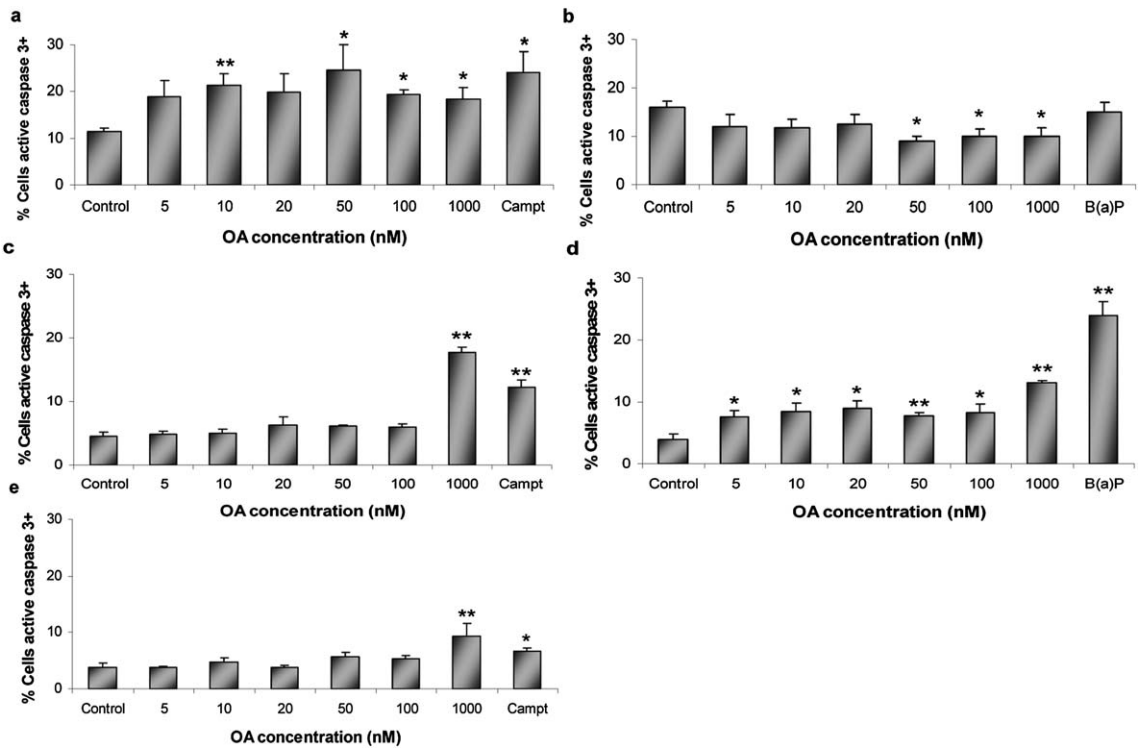

Fig. 6 Results of caspase 3 activation in OA-treated cells: leukocytes (a and b), SHSY5Y cells (c and d), and HepG2 cells (e). Treatments were performed in the absence (a, c and e) or presence (b and d) of S9 fraction. *: $P < 0.05$, **: $P < 0.01$, significant difference with regard to the control.

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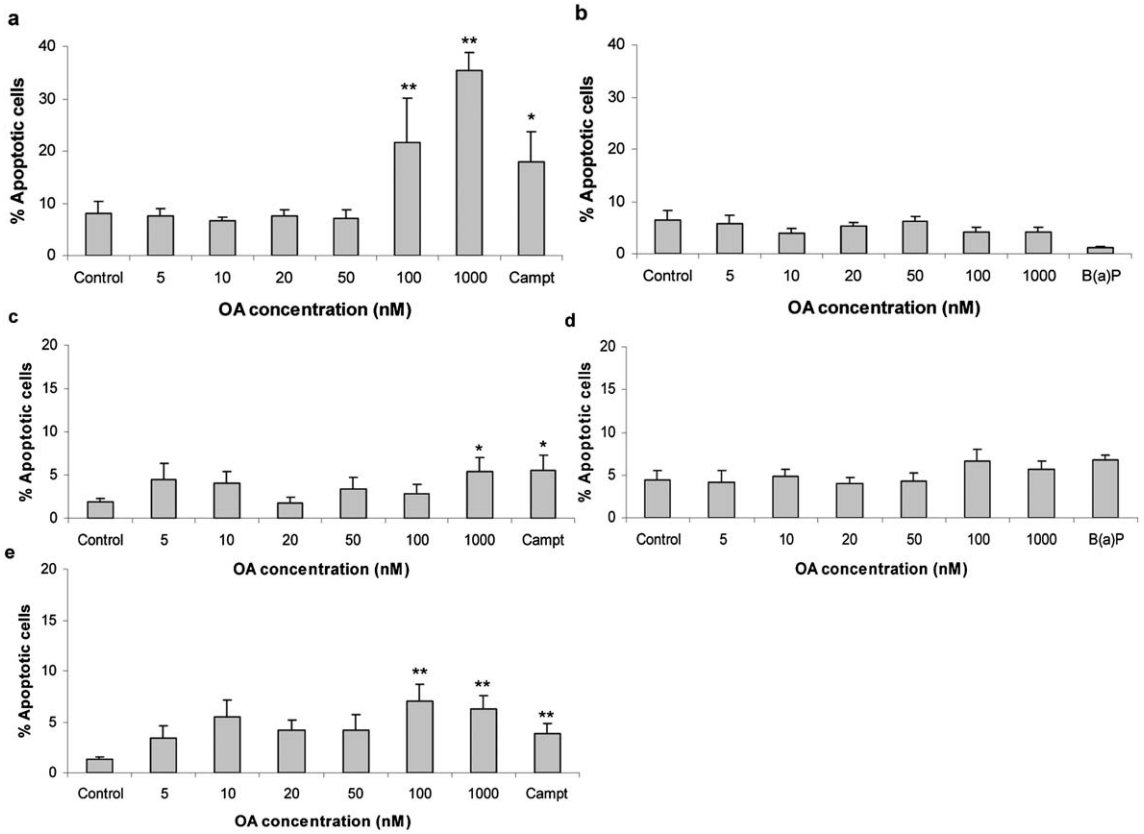

Fig. 7 Percentage of apoptosis estimated by annexin V–FITC/PI staining and flow cytometry in OA-treated cells: leukocytes (a and b), SHSY5Y cells (c and d), and HepG2 cells (e). Treatments were performed in the absence (a, c and e) or presence (b and d) of S9 fraction. *: $P < 0.05$ and **: $P < 0.01$, significant difference with regard to the corresponding control.

Besides, apoptotic processes are often preceded by a reorganization of the cytoskeleton.[44] The maintenance of the cytoskeletal structure in leukocytes, SHSY5Y cells and HepG2 cells after 3 h of OA treatment (5–1000 nM concentrations) was analyzed in this study. The highest OA concentrations employed (50, 100 and 1000 nM) induced morphologic alterations in the SHSY5Y cells, both in the presence and in the absence of S9 fraction, and in the HepG2 cells. Leukocytes resulted less affected by OA, with no alterations at all in the presence of S9 fraction and only morphological changes in the cell shape at 1000 nM in the absence of S9 fraction. Under these specific conditions, cells growing as monolayers rounded up, detached from the substrate and exhibited several morphological alterations as a consequence of cytoskeleton disruption. The different OA responses found in leukocytes with regard to the other two cell types employed may come from their own differential characteristics, which can determine their different response mechanisms. In general, in experimental settings leukocytes have been reported to be one of the most resistant cell types to the potentially harmful substances, since they are supposed to be prepared to be in contact with xenobiotic compounds in the bloodstream.

These results are in line with other previous studies in which several cytoskeletal alterations after OA treatment were reported in different cell systems. It was described that OA can induce noticeable morphological changes in rat hepatocytes, and in MCF-7, SK-N-SH, GH3 and IPC-81 cells at concentrations ranging from 100 to 1000 nM,[44] and destabilization of microtubules in neurons.[52] Also, it was observed that OA caused loss of stabilization of focal adhesions and a consequent loss of cytoskeletal organization in keratinocytes,[53] and cell rounding and loss of barrier properties in human intestinal epithelial T84[54] and Caco2 cells.[55] It has been hypothesized that these alterations could be due to different mechanisms that involve disruption of F-actin and/or hyperphosphorylation and activation of kinases that stimulate tight junction disassembly, but the exact molecular mechanism has not been elucidated yet.[8]

In general, when DNA damage occurs in a cell, two consequences can be postulated: the cell enters apoptosis and dies or growth is arrested at specific cell-cycle checkpoints to allow DNA repair and continued survival.[56] In this study the relative cellular DNA content was determined by flow cytometry in order to examine the cell distribution during the different phases of the cell cycle (G_0/G_1, S, G_2/M) in cells treated with OA. Slight cell cycle alterations were found in leukocytes, both in the presence and in the absence of metabolic activation. Besides, when SHSY5Y cells were incubated with OA in the absence or presence of S9 fraction concentration-dependent modulations were observed in their cell cycle, significant at the highest concentrations employed. However, exposure to the marine toxin did not produce cell cycle alterations in HepG2 cells.

OA acts by inhibiting protein phosphatases which leads to an increase of the phosphorylated forms of proteins such as several kinds of kinases.[57,58] The absence of dephosphorylation control of these kinases can lead to an increase in proliferation, aberrant

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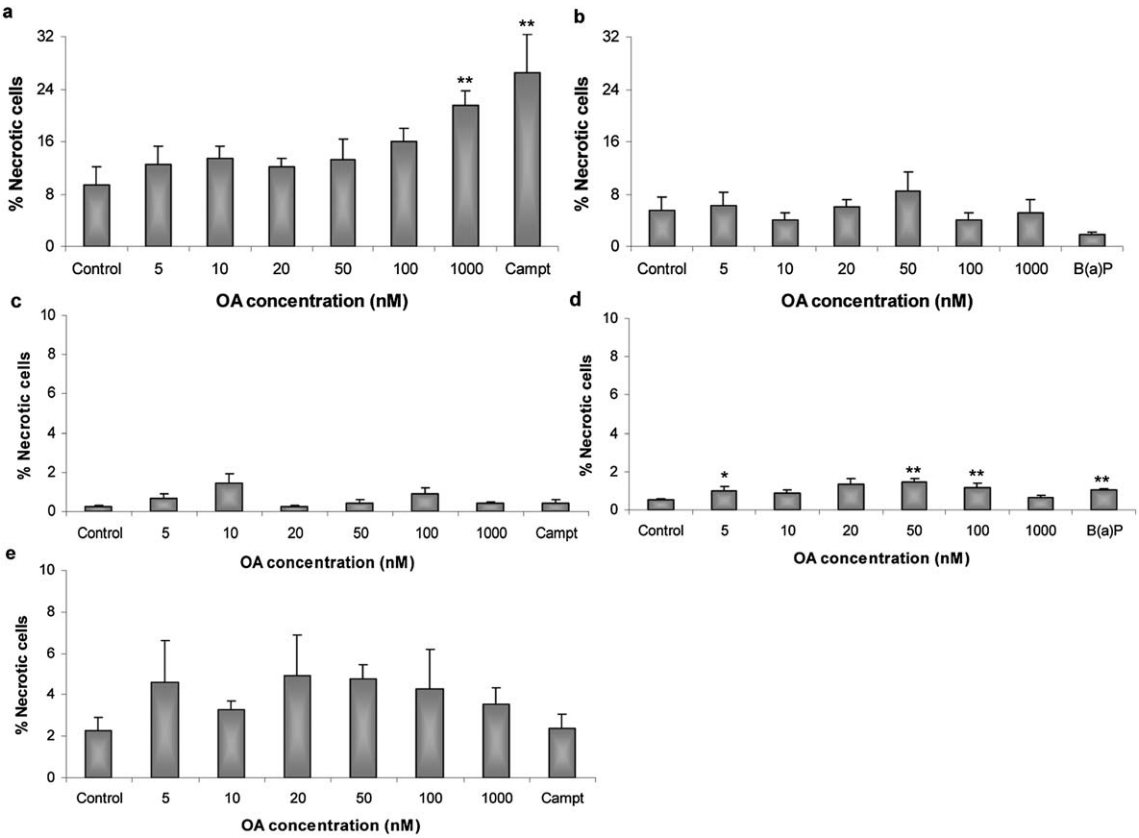

Fig. 8 Percentage of necrosis estimated by annexin V–FITC/PI staining and flow cytometry in OA-treated cells: leukocytes (a and b), SHSY5Y cells (c and d), and HepG2 cells (e). Treatments were performed in the absence (a, c and e) or presence (b and d) of S9 fraction. *: $P < 0.05$ and **: $P < 0.01$, significant difference with regard to the corresponding control.

mitosis, or growth arrest, depending on the cell type.[59] This was confirmed in our study, in which among the three cell types analyzed only the neuronal cells showed a clear cell cycle arrest at the S phase. Furthermore, in previous studies in other different cell types OA has been shown to induce mitotic arrest or premature chromosome condensation. These include HeLa cells,[13] fibroblasts,[18] pituitary cells,[60] neuronal cells,[50] myeloid cells,[12,61] Caco2 cells[9] and lymphocytes.[62] The mechanism by which OA causes mitotic arrest is not well understood, although protein phosphatase 2A (PP2A) activity appears to be required for the metaphase–anaphase transition.[14]

During the cell cycle assessment we could also observe that OA induced apoptosis (as an increase in the subG$_1$ region) in a dose-dependent manner. After 48 h treatment, an increased percentage of apoptotic cells was obtained in all the cell types evaluated in the presence or absence of S9 fraction, mainly at the highest concentrations. Previously reported data on apoptosis induction by OA are especially controversial. Despite the fact that OA is known to induce apoptosis in a variety of cell lines (reviewed in ref. 63), its tumour promoter activity[64] and its protector effect against apoptosis induced by other compounds have also been demonstrated.[65,66] We used annexin-V/PI staining assessed by flow cytometry to investigate specifically the OA-induced apoptosis, since it has been reported that annexin-V staining is able to detect apoptosis in the early stage based on the alteration of the cell membrane.[67] All the cells evaluated in this study showed apoptosis induction after 3 h OA treatment without metabolic activation. As described above, there are a great number of studies that reported the role of OA as an apoptosis inductor in different *in vivo* and *in vitro* systems.[16,30,50,68–71] Our results are in line with these works when cells were treated with OA in the absence of S9 fraction.

Caspases represent cysteine proteases which become activated in early phases of apoptotic responses, and play key roles in execution of cell death by destruction of selected proteins.[72] The involvement of caspases in OA-induced apoptosis has been previously studied in several experimental systems but no clear conclusions were obtained with regard to caspase 3 participation.[73,74] Our results showed that OA induced caspase 3 activation in all the cell types evaluated in the absence of S9 fraction, but only in the SHSY5Y cells in the presence of S9 fraction.

A previous study demonstrated that caspase 3 is important in the process of OA-induced apoptosis;[75] on the contrary, another work showed that OA causes apoptosis in the human breast carcinoma cell line MCF-7, which lacks expression of caspase 3,[74] and a caspase inhibitor was also reported to block OA-induced caspase 3 activation but not apoptosis.[76] So the role of caspase 3 in the OA-induced apoptosis remains unclear. In fact, a recent study in CHO-K1 cells concluded that OA could induce DNA fragmentation, a hallmark of apoptosis, *via* caspase 3-dependent and caspase 3-independent pathways,[73] and another study in HeLa cells reported that at least three distinct signal transduction pathways are involved in OA-induced apoptosis and that, despite their well-differentiated mechanisms, caspase-dependent and

-independent pathways may operate together or in parallel.[51] The results of our study suggest that caspase 3 activation is closely related to apoptosis induction in OA-treated cells, evaluated both as the subG$_1$ region in the flow distribution and annexin V staining, especially in the absence of S9 fraction.

However no increase in the percentage of annexin V$^+$/PI$^-$ cells in the flow distribution was found in leukocytes or in neuronal cells treated for 3 h with metabolic activation, suggesting that OA effects on apoptosis induction are directly induced, and that incubation in the presence of S9 fraction has a detoxifying effect on OA under these conditions. In the specific case of leukocytes, an inverse dose-relationship was obtained for the apoptosis rate in the presence of S9 fraction. These data support those obtained in the same cells for caspase 3 activation, in which a significant decrease of this parameter was found with regard to the control. Other previous studies had already described a protector effect of OA against apoptosis induced by other compounds such as gamma-radiation, tetrandrine, bistratene A, and cisplatin,[65] etoposide,[77] anisomycin,[78] and 1-methyl-4-phenylpyridinium ion.[66] On the basis of the results of this work regarding the apoptosis induction, this different cell behaviour in the presence of OA treatment could be determined by the different experimental conditions, since when the OA-induced apoptosis was estimated as the subG$_1$ region in the cell cycle analysis after 48 h treatment, all the cells tested in the presence or absence of S9 fraction showed increases in the apoptosis rate. This suggests that the detoxifying role of the S9 fraction protects from OA apoptosis induction only at the short term, since the metabolites generated seem to be less effective than the original compound. But when the exposure is longer, *e.g.* 48 h, cells become sensitive to the apoptosis induced by the metabolites. So, according to this, the differences between studies with regard to the apoptosis induction by OA could be due to the different metabolic capacities of the cells, the time of exposure and the OA concentration employed.

Necrosis, another type of cell death, is considered to be a toxic process in which the cell is a passive victim and follows an energy-independent mode of death.[79] Necrotic cell death is characterized by cytoplasmic and organelle swelling, followed by the loss of cell membrane integrity and release of the cellular content into the surrounding extracellular space.[80] The OA-induced necrosis was also evaluated in this study as annexin V$^+$/PI$^+$ cells in the flow distribution. Among all the cells evaluated, only leukocytes in the absence of S9 fraction and neuronal cells with metabolic activation showed an increase in the percentage of necrotic cells after 3 h OA treatment. Following the same procedure, Dogliotti *et al.*[81] also found OA-induced necrosis in Down syndrome fibroblasts after 1 h treatment with this toxin at 100 nM.

Whether a cell dies by necrosis or apoptosis depends in part on the nature of the cell death signal, the tissue type, the developmental stage of the tissue and the physiologic milieu.[82,83] In our study, neuronal cells treated for 3 h with OA were observed to undergo apoptosis in the absence of S9 fraction, but also to die by necrosis in the presence of metabolic activation. However, both in the presence and absence of S9 fraction dose-dependent caspase 3 activation was detected in these cells, suggesting that caspase 3 activation was immediately followed by apoptosis in the presence of metabolic activation, but in the absence of these metabolic enzymes a certain proportion of cells died by necrosis and only after a longer exposure time (48 h) the apoptosis was triggered. Besides, leukocytes without S9 fraction were found to increase both the apoptosis and necrosis frequencies after OA treatment, and also the rate of active caspase 3. It was previously reported that although apoptosis and necrosis are different processes, both can occur simultaneously depending on factors such as the intensity and duration of the stimulus, the extent of ATP depletion and the availability of caspases.[83]

In conclusion, the results of this study showed that OA acts as a cell cycle disruptor in leukocytes and neuronal cells and as an apoptosis inductor in leukocytes, SHSY5Y cells and HepG2 cells, although its effects vary depending on the cell type, the concentration employed, the time of exposure and the presence/absence of metabolic activation. Furthermore, in all the cases these processes were accompanied by morphological changes, characteristic of cytoskeletal disruption, and by caspase 3 activation, suggesting that OA induces apoptosis in the cells evaluated in this study through a caspase 3-dependent pathway.

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