

## Involvement of PPAR $\alpha$ in the growth inhibitory effect of arachidonic acid on breast cancer cells

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Epidemiological studies suggest that dietary PUFA may influence breast cancer progression. *n*-3 PUFA are generally known to exert antitumour effects, whereas reports relative to *n*-6 PUFA anti-carcinogen effects are controversial. Arachidonic acid (AA; 20:4*n*-6) and its metabolites have been shown to inhibit the growth of human breast cancer cell lines, even if the downstream mechanisms by which AA may influence carcinogenesis remain unresolved. We explored the molecular basis for AA influence on proliferation, signal transduction and apoptosis in two human breast cancer cell lines, MCF-7 and MDA-MB-231. In both cell lines AA inhibited cell growth in a dose-dependent manner, even if MDA-MB-231 was somewhat more growth-inhibited than MCF-7. AA decreased extracellular signal-regulated protein kinase 1/2 phosphorylation level, and positively modulated PPAR $\gamma$  and PPAR $\alpha$  expression, with only a slight effect against PPAR $\beta/\delta$ . In addition, AA increased Bak (an apoptosis-regulating protein) expression and reduced procaspase-3 and -9 levels only in MDA-MB-231 cells, thus indicating that the growth inhibitory effect can be correlated with apoptosis induction. In both cell lines the use of a specific antagonist made it possible to establish a relationship between AA growth inhibitory effect and PPAR $\alpha$  involvement. AA decreases cell proliferation most likely by inducing apoptosis in MDA-MB-231 cells, while in the MCF-7 cell line the growth inhibitory activity can be attributed to the inhibition of the signal transduction pathway involved in cell proliferation. In both cases, the results here presented suggest PPAR $\alpha$  as a possible contributor to the growth inhibitory effect of AA.

**Arachidonic acid: Breast cancer cells: PPAR: Extracellular signal-regulated protein kinase: Apoptosis**

Breast cancer still represents one of the most relevant mortality factors in women throughout the world, despite the significant advancements in its early detection and therapeutic approaches. Some evidence has established dietary essential fats as potential regulators of breast tumour cell growth<sup>(1,2)</sup>. It is known that *n*-3 and *n*-6 fatty acids, the two major families of essential fatty acids, play an important role in tumour proliferation, though their bio-clinical mechanisms on tumour cells are still not properly understood<sup>(2–4)</sup>. *n*-6 Fatty acids were found to stimulate the growth and metastasis of human breast cancer cells<sup>(5,6)</sup>, while *n*-3 fatty acids appeared to exert a protective effect<sup>(7)</sup>. More recently, it has been reported that both *n*-3 and *n*-6 PUFA are able to reduce the growth of different human cancer cells, although to different extents<sup>(8)</sup>.

Literature data suggest that the intake of PUFA may modulate cell behaviour and growth by a variety of mechanisms, including modification of tumour cell membranes which, in turn, can affect cell signalling pathways, lipid peroxidation and oxidative stress<sup>(9)</sup>, eicosanoid production, fatty acid metabolism<sup>(10)</sup> and regulation of gene expression<sup>(11)</sup>.

Among *n*-6 PUFA, 20:4*n*-6 arachidonic acid (AA) is a relatively minor PUFA found in cell membrane glycerolipids<sup>(12)</sup>, which has been reported to inhibit the growth of some breast cancer cell lines<sup>(4)</sup>. In contrast to other more abundant

unsaturated fatty acids (linoleic or linolenic acid), levels of unesterified AA are stringently controlled within mammalian cells, and the pathways of AA uptake, incorporation and remodelling are well documented<sup>(13)</sup>. The intracellular levels of AA are regulated through distinct and non-overlapping mechanisms. In resting cells, low concentrations of AA are tightly maintained by basal levels of rapid catabolism, by membrane glycerolipid recycling<sup>(14)</sup> and by esterification<sup>(15)</sup>. On the other hand, a stimulus-induced release of AA by cytosolic phospholipase 2 $\alpha$  results in its rapid metabolism induced by cyclooxygenase-2, thereby limiting the intracellular AA pools<sup>(14)</sup>.

Once released from the membrane, AA and its metabolites are important signals that regulate a wide variety of cellular functions<sup>(16)</sup>. The range of biological processes in which AA and its metabolites participate is vast; they regulate the transcription of several families of genes including heat-shock-protein genes<sup>(17)</sup>, genes involved in cell-cycle control<sup>(18)</sup>, inflammation<sup>(19)</sup>, steroid biosynthesis<sup>(20)</sup> and proto-oncogenes<sup>(21,22)</sup>.

There are several other mechanisms probably implied in AA effects, not to be considered mutually exclusive, such as mechanisms implied in AA-induced apoptosis. In this regard, it has been reported that AA-induced apoptosis is transcriptionally dependent and involves the regulation of the key families of genes involved in cell survival and apoptosis.

**Abbreviations:** AA, arachidonic acid; DMEM, Dulbecco's modified Eagle's medium; ER, oestrogen receptor; ER $\alpha$ (+) and ER $\alpha$ (-), well differentiated and poorly differentiated ER $\alpha$  cells respectively; ERK, extracellular signal-regulated protein kinase; Tris, Tris(hydroxymethyl) amino methane.

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For example, an alternate downstream target of unesterified intracellular AA is ceramide signalling of apoptosis initiated via the TNF- $\alpha$  pathway<sup>(23)</sup>, since cancer cells treated with exogenous AA at concentrations that induce apoptosis accumulate ceramide<sup>(24)</sup>. It has been reported that exogenous AA and inhibitors of AA metabolism that lead to the accumulation of unesterified AA are cytotoxic to the colon cancer cell line<sup>(8)</sup>. Of interest is the observation that AA induced the suppression of the growth of different cancer cells through a mechanism that involves lipid peroxidation and PPAR activation<sup>(25)</sup>.

PPAR are transcription factors with a pivotal role in lipid metabolism and homeostasis<sup>(26)</sup>; they function by forming heterodimers with the retinoid X receptor<sup>(27,28)</sup>. There are three PPAR subtypes ( $\alpha$ ,  $\beta$  and  $\gamma$ ) which bind to different ligands, among them fatty acids and their metabolites, so regulating the expression of genes involved in lipid transport and metabolism within the cell. In mammals, PPAR $\alpha$  is most highly expressed in brown adipose tissue, followed by liver, kidney, and heart. Activation of rat liver PPAR $\alpha$  provides an anti-apoptotic mechanism<sup>(29)</sup>. PPAR $\beta$  is expressed in all tissues studied to date<sup>(30)</sup>, whereas PPAR $\gamma$  is highly expressed in adipose tissue, as well as in muscle, colon, and liver. The importance of PPAR activation in preventing epithelial carcinogenesis is evident in different tumour systems. Activation of PPAR $\gamma$  through troglitazone and other PPAR $\gamma$  activators causes the inhibition of proliferation and the induction of apoptosis, both *in vitro* and *in vivo*<sup>(31)</sup>. PPAR can mediate inflammation, and this effect is potentiated by elements from AA metabolic pathways. These include leukotriene B<sub>4</sub>, derived from the lipoxygenase pathway, and 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub>, related to the cyclooxygenase pathway. 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> is a PPAR $\gamma$  activator that has been shown to counteract the effects of the pro-inflammatory cytokines TNF $\alpha$ , IL-1, and IL-6<sup>(32)</sup>.

Eicosanoids and a range of fatty acids, such as the PUFA linoleic acid and AA, bind to PPAR at physiological concentrations and regulate gene transcription<sup>(33)</sup>. Furthermore, all three PPAR subtypes have been implicated in carcinogenesis<sup>(34)</sup> and it is known that PPAR signalling can influence cell survival and apoptosis<sup>(35)</sup>. The objective of the current study was to understand the potential mechanisms of action of AA modulation of breast cancer cell growth; for this purpose, we compared the influence of AA on proliferation, signal transduction and apoptosis in two breast cancer cell lines, the well differentiated oestrogen receptor (ER) $\alpha$ (+) MCF-7 cells and the poorly differentiated ER $\alpha$ (-) MDA-MB-231 cells. In particular we focused our attention on the interaction between the AA and PPAR pathways, an interaction that could provide an opportunity to develop drug combinations that maximize growth arrest and apoptosis in breast cancer cells.

## Materials and methods

### Antibodies and chemicals

MDA-MB-231 and MCF-7 breast cancer cell lines were a kind gift from Professor Sebastiano Andò, Department of Pharmacology, Faculty of Pharmacy, University of Calabria, Cosenza, Italy. AA, PPAR $\alpha$  antagonist (MK886), PPAR $\gamma$

antagonist (GW9662), culture media, mouse monoclonal antibody specific to  $\beta$ -actin and chemicals were purchased from Sigma Chemical (MO, USA). Cell culture plasticware was from TPP (Trasadingen, Switzerland). Rabbit polyclonal antibody specific for PPAR $\alpha$  (sc-9000), PPAR $\beta$  (sc-7197), PPAR $\gamma$  (sc-7196), Bak (sc-832), Bcl-2, caspase-3 (sc-7148), caspase-9p10 (sc-7885), caspase-8p20 (sc-7890) and for extracellular signal-regulated protein kinase (ERK)1 (sc-94), mouse monoclonal antibody specific for phospho-ERK1/2 (sc-7383), goat anti-rabbit (sc-2004) and anti-mouse (sc-2005) secondary antibodies were obtained from Santa Cruz Biotechnology (CA, USA). The enhanced chemiluminescence detection system was from Amersham Pharmacia Biotech (Uppsala, Sweden). The protein assay kit and iQ<sup>TM</sup> SYBR<sup>®</sup> Green Super-Mix Bio-Rad were from Hercules (CA, USA). The RNeasy Mini Kit<sup>®</sup> was from QIAGEN (GmbH, Germany); the cDNA Archive kit was from Applied Biosystems (Foster City, CA, USA).

### Cell culture

MDA-MB-231 and MCF-7 breast cancer cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% foetal bovine serum, 100 U penicillin/ml, 100  $\mu$ g streptomycin/ml and 25  $\mu$ g amphotericin B/ml. Cells were cultured at 37°C in a humidified incubator with 5% CO<sub>2</sub> and 95% air and regularly examined using an inverted microscope. For treatments, cells were seeded at a density of  $3 \times 10^4$  cells/cm<sup>2</sup> and cultured for 24 h to allow them to adhere to the substratum. The medium was then replaced with serum-free DMEM supplemented with 100 U penicillin/ml and 100  $\mu$ g streptomycin/ml, 25  $\mu$ g amphotericin B/ml, 2 mM glutamine, 1% ITS (insulin–transferrin–sodium selenite), 1% vitamin solution, 0.4% serum bovine albumin (fatty acid free) and AA. AA was dissolved in foetal bovine serum and the concentration of foetal bovine serum was adjusted so it was the same in all experiments and the final concentration was no stronger than 0.01% (v/v). Control groups received the same amount of foetal bovine serum. When GW9662 (PPAR $\gamma$  antagonist) and MK886 (PPAR $\alpha$  antagonist) were used, they were added to the medium 1 h before AA treatment. GW9662 is an irreversible PPAR $\gamma$  antagonist, identified in a competition-binding assay against the human ligand-binding domain; it binds PPAR $\gamma$  and covalently modifies a cysteine residue in the ligand binding site of PPAR $\gamma$ <sup>(36)</sup>. MK886 inhibits PPAR $\alpha$  by a non-competitive mechanism; it prevents the conformational change necessary for PPAR $\alpha$  ligand–receptor interaction<sup>(37)</sup>.

### Viability and growth rate determination

The exponentially growing cells were harvested with 0.25% trypsin–0.02% EDTA treatment and seeded in twelve-well culture plates. After overnight incubation to allow cell attachment, the medium was removed and replaced with fresh serum-free DMEM containing AA at a series of concentrations with or without 5  $\mu$ M GW9662 or MK886. Viability and cell number were determined using the trypan blue (0.5% in NaCl) exclusion assay. Briefly, treated cells were washed with PBS and trypsinized. Aliquots of cell suspension (100  $\mu$ l) were incubated with the same volume of trypan blue for 5 min. Finally, cells were transferred to the Bürker

chamber and counted by a light microscope. Dead cells were defined as those stained with the dye. Samples were measured in three replicates and each experiment was repeated at least three times.

#### Lactate dehydrogenase assay

Cells were seeded in twelve-well-culture plates and appropriately treated. After treatment, the cell supernatant was collected for the measurement of lactate dehydrogenase release. The lactate dehydrogenase activity was determined spectrophotometrically by an assay based on the oxidation of NADH and the rate of decrease in absorbance at 340 nm. The activity of lactate dehydrogenase was calculated as nanomoles of NADH consumed per ml per min. Samples were measured in three replicates and each experiment was repeated at least three times.

#### Analysis of nuclear morphology

Cells were plated on glass slides in twelve-well plates and appropriately treated. Changes in nuclear morphology were labelled by 4',6-diamidino-2-phenylindole and examined by fluorescent microscopy. The cells were fixed with 95 % ice-cold ethanol for 5 min and incubated with 4',6-diamidino-2-phenylindole (1 mg/ml in methanol) for 30 min at 37°C in the dark, then nuclear morphology was observed under a fluorescence microscope equipped with a UV light filter. Cells which exhibited reduced nuclear size, intense fluorescence, chromatin condensation and nuclear fragmentation were considered as apoptotic. Each experiment was repeated at least three times.

#### Protein extraction and Western blotting

Cells were seeded in 75 cm<sup>2</sup> plates and then appropriately treated. Collected cells were suspended in lysis buffer containing 20 mM Tris(hydroxymethyl) amino methane (Tris)-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.1 mM phenylmethyl-sulfonyl fluoride, 0.05 % aprotinin, 0.1 % Igepal and then incubated for 30 min at 4°C. The suspension was centrifuged for 25 min at 12 000 rpm, and the supernatant from this centrifugation was saved as total extracts. Protein contents in supernatants were measured using a commercially available assay (Biorad) with bovine serum albumin as a standard.

Equal amounts of proteins (60  $\mu$ g/well) were mixed with a solubilization buffer containing 250 mM Tris, pH 8.8, 4 % SDS, 16 % glycerol, 8 % 2-mercaptoethanol and 0.1 % bromophenol blue, and then fractionated by electrophoresis on SDS-PAGE. Proteins were transferred onto nitrocellulose for 2 h in a Biorad electroblotting device. Nitrocellulose matrices were blocked with 5 % milk in TBST (1 M Tris buffer, pH 7.4, 5 M NaCl, 0.1 % Tween-20) for 1 h at room temperature. For immunodetection, matrices were incubated overnight at 4°C with a primary antibody. The matrices were then detected by incubation for 1 h at room temperature with the corresponding horseradish peroxidase-conjugated secondary antibody. The immunoreactive bands were visualized using the enhanced chemiluminescence system. Band intensities were quantified by densitometry and the expression

of proteins was reported as a proportion of  $\beta$ -actin or ERK1 protein expression to control for any discrepancies in gel loading. Fold change *v.* control values were calculated by normalizing densitometric values obtained from the various proteins with those obtained for  $\beta$ -actin or ERK1 (VersaDoc Imaging System 3000, Biorad). Each experiment was repeated at least three times.

#### Evaluation of PPAR $\alpha$ expression by real-time PCR

Total RNA was extracted using RNeasy Mini Kit<sup>®</sup>. Real-time PCR was performed using single-stranded cDNA prepared from total RNA (1  $\mu$ g) with a High Capacity cDNA Archive kit. Forward and reverse primers were designed using Beacon Designer software (Bio-Rad, Hercules, CA) (see Table 1).

A PCR mixture (25  $\mu$ l), containing cDNA template equivalent to 80 ng total RNA, 5 pmole each of the forward and reverse primers and 2  $\times$  iQ<sup>™</sup> SYBR<sup>®</sup> Green SuperMix, were amplified using an iCycler PCR (Bio-Rad, Hercules, CA). Each sample was tested six times and the threshold cycle (Ct) values were the corresponding mean. The fold change was defined as the relative expression compared to that at time 0 (time of seeding cells), calculated as  $2^{-\Delta\Delta Ct}$ , where  $\Delta Ct = Ct_{\text{sample}} - Ct_{\text{GAPDH}}$  and  $\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \Delta Ct_{\text{time 0}}$ .

#### Statistical analysis

Differences between the means were analysed for significance using the one-way ANOVA test with the Bonferroni *post hoc* multiple comparisons used to assess the differences between independent groups. All values are expressed as means with standard deviation, and differences were considered significant at  $P < 0.05$ .

## Results

#### Cell viability

To assess the effect exerted on human breast cancer cell proliferation, MCF-7 and MDA-MB-231 cancer cells were treated with increasing concentrations (1–50  $\mu$ M) of AA. Cell viability was examined by the trypan blue assay, which provides a direct count of cells that exclude the trypan blue dye, including apoptotic cells. Twenty-four-hour AA treatment remarkably decreased the survival of both cell lines in

**Table 1.** Primer design (FW, forward primer and RV, reverse primer) for evaluation of PPAR $\alpha$  expression by real-time PCR

Gene access number	Sequence	Annealing (°C)	Cycles
GAPDH	FW: 5'-GTC GGA GTC AAC GGA TTT GG-3'	52	30
NM_002046	RV: 5'-GGG TGG AAT CAT ATT GGA ACA TG-3'		
PPAR $\alpha$	FW: 5'-GGA TGT CAC ACA ACG CGA TT-3'	52	35
NM_005036	RV: 5'-GCC AGA GAT TTG AGA TCT GCA GTT-3'		

a dose-dependent manner (Fig. 1), with a strong inhibitory effect against MDA-MB-231 cells (50% inhibition with 50  $\mu\text{M}$  AA). Thus, compared with the well-differentiated MCF-7 cells (Fig. 1 (A)), the poorly differentiated MDA-MB-231 cells (Fig. 1 (B)) appeared to be more susceptible to the AA growth inhibitory effect.

The growth-inhibitory effect of AA is accompanied by a cytotoxic side effect detected as the cell release of lactate dehydrogenase in the culture media (Fig. 2). In MCF-7 cells, the release is significantly increased at all the concentrations of AA used (Fig. 2 (A)), while in MDA-MB-231 cells the release is significantly augmented only with high concentrations of AA, with a maximal effect at 50  $\mu\text{M}$  dose (Fig. 2 (B)).

The most effective condition in inhibiting cell survival in both cell lines was obtained after 24 h treatment with 50  $\mu\text{M}$  AA, so we used this experimental protocol for further experiments.

#### Apoptosis induction

To assess whether the decrease of cell growth by AA treatment was due to the induction of apoptosis, we further evaluated the effects of AA on the expression of apoptosis-regulating proteins such as Bak, Bcl-2 (Fig. 3) and procaspase-3, -8 and -9 (Fig. 4).

In MCF-7 cells the inhibition of cell growth did not correlate with apoptosis induction, as AA caused a decrease in the content of the pro-apoptotic protein Bak concomitantly to an increase of the anti-apoptotic protein Bcl-2 (Fig. 3 (A)). On the contrary, a pro-apoptotic effect was detected in MDA-MB-231 (Fig. 3 (B)), in which AA induced a significant increase in the expression of Bak without any effect on the anti-apoptotic protein Bcl-2, which was expressed at basal condition. In addition, in MCF-7 cells AA did not modify the levels of procaspase-8 and -9 (Fig. 4 (A)); in this cell line procaspase-3 was undetectable, since the caspase-3 gene is not expressed<sup>(38)</sup>. In MDA-MB-231 (Fig. 4 (B)), AA induced a strong decrease of procaspase-3 and -9 levels, suggesting that the cleavage of the proteins to the active form (caspase-3 and -9) also occurred. The fact that in this cell line expression of procaspase-8 was not modified seems to exclude the activation of extrinsic pathways of apoptosis.

In MDA-MB-231 cells evidence of apoptosis was confirmed by the analysis of nuclear morphology by staining with specific DNA fluorochrome 4',6-diamidino-2-phenylindole,

which showed nuclear fragmentation and condensation above all after 24 h treatment with AA at 1  $\mu\text{M}$  concentration (Fig. 5 (B)).

#### Involvement of extracellular signal-regulated protein kinase–mitogen activated protein kinase pathway

In an attempt to explore the nature of the antiproliferative response exhibited by MCF-7 and MDA-MB-231 cells, we analyzed the AA effect on the ERK–mitogen-activated protein kinase signal transduction pathway, a large network of signalling molecules regulating cell growth and differentiation (Figs. 6 and 7).

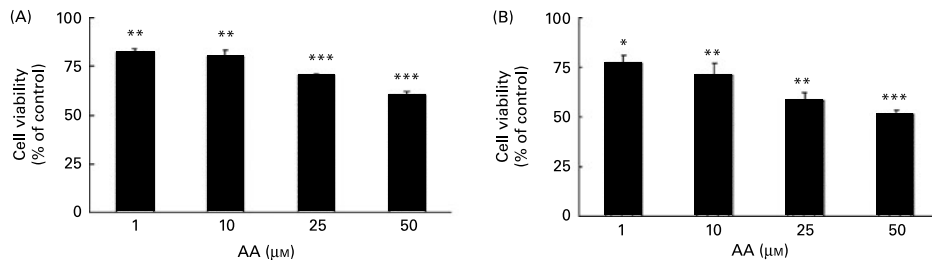
In MCF-7 cells 24 h treatment with AA caused a dose-dependent decrease of phospho-ERK expression (active form), with the highest magnitude of reduction at 50  $\mu\text{M}$  concentration (Fig. 6 (A)).

The analysis of the short-time effect (1, 4, 8 and 16 h) of 50  $\mu\text{M}$  AA on phospho-ERK level (Fig. 6 (B)) indicated a significant reduction of the ERK phosphorylation state starting from 8 h treatment. Also in MDA-MB-231 cells 24 h treatment with AA induced a strong decrease of phospho-ERK level, in this case the maximal downregulatory effect was observed at 25  $\mu\text{M}$  concentration (Fig. 7 (A)). The short-time analysis of the AA effect in this case indicated a reduction of phospho-ERK from 8 h from the start of treatment (Fig. 7 (B)).

#### PPAR expression

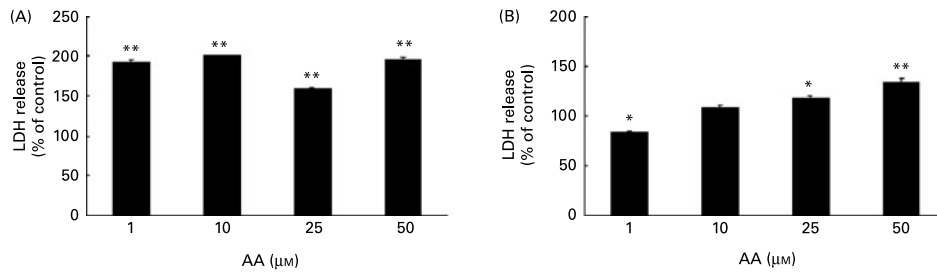
Effects on PPAR expression were further examined to study the AA influence on mechanisms involved in cell growth regulation. We examined changes in the protein contents of different PPAR isoforms by western blot analysis. As shown in Fig. 8, 24 h treatment with AA dose-dependently enhanced PPAR $\alpha$  expression in both cell lines. Also the PPAR $\gamma$  level was up-regulated by AA, which was more effective in MCF-7 cells. On the contrary, after AA treatment the PPAR $\beta$  level did not display substantial changes in MCF-7 cells and increased only slightly in MDA-MB-231 cells.

To evaluate whether AA could be directly responsible for the induction of PPAR $\alpha$  and  $\gamma$ , we also analysed its effects in the presence of a specific PPAR $\alpha$  (MK886) and PPAR $\gamma$  (GW9662) antagonist. Both antagonists were administered to MCF-7 and MDA-MB-231 cells at 5  $\mu\text{M}$  concentration, chosen on the basis of the existing literature<sup>(37,39)</sup>. As shown in Fig. 9 ((A) and (B)), in both cell lines pre-treatment (1 h)



**Fig. 1.** Arachidonic acid (AA)-related growth inhibition of MCF-7 (A) and MDA-MB-231 (B) breast cancer cells. Cells were incubated with different concentrations of AA (1–50  $\mu\text{M}$ ) for 24 h and viability was determined by the trypan blue exclusion test. Values are presented as the percentage of control, with standard deviation shown by vertical bars, from three independent experiments conducted in triplicate. Mean values were significantly different from controls: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .





**Fig. 2.** Cytotoxic activity of arachidonic acid (AA). MCF-7 (A) and MDA-MB-231 (B) breast cancer cells were treated with different concentrations of AA (1–50  $\mu$ M) for 24 h, then supernatants were collected for measurement of lactate dehydrogenase (LDH) amounts. LDH release is expressed as the percentages of nmoles of NADH consumed per ml per min. Values are means with standard deviation shown by vertical bars, from three independent experiments conducted in triplicate. Mean values were significantly different from controls: \* $P$ <0.05, \*\* $P$ <0.01.

with MK886 prevented an AA stimulatory effect on PPAR $\alpha$  expression; on the contrary, GW9662 was not able to influence AA effects on PPAR $\gamma$  expression (data not shown).

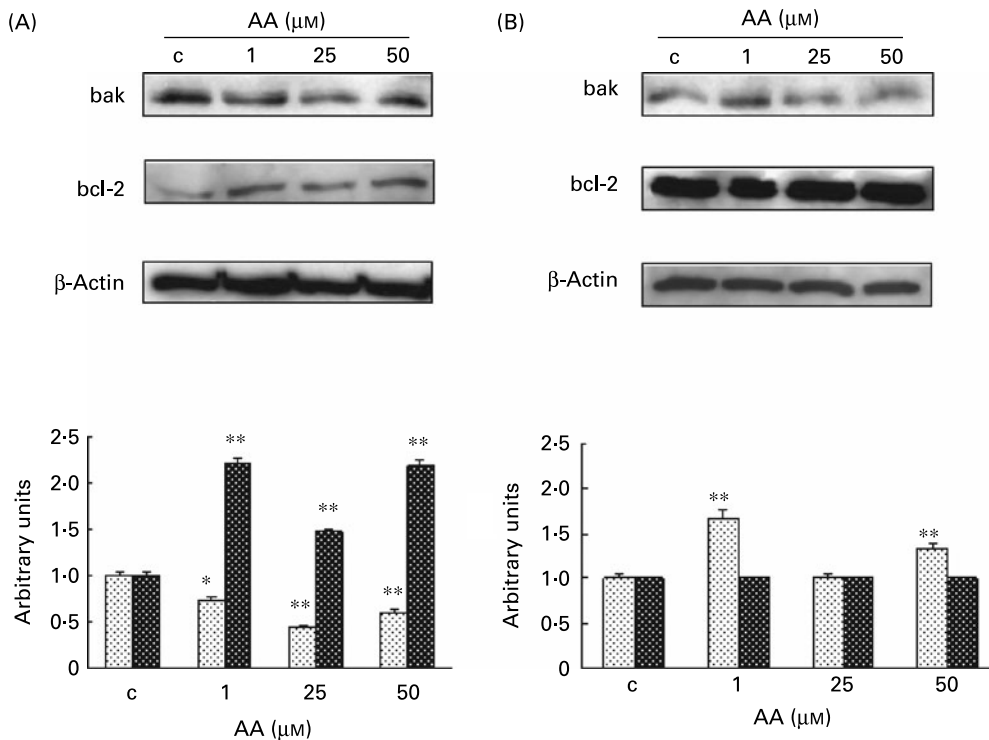
MCF-7 (Fig. 10 (A)) and MDA-MB-231 (Fig. 10 (B)) cells were cultured with AA, with or without 1 h pre-incubation with MK886, for 24 h and analysed by real-time PCR for subsequent changes in the PPAR $\alpha$  mRNA expression levels relative to control cells. In both cell lines PPAR $\alpha$  mRNA level increased after short time incubation with AA (data not shown) and declined below baseline level at 24 h, when PPAR $\alpha$  protein amount was higher than control cells. The pre-treatment of the cells with the antagonist, alone or in the presence of AA, caused in both cases the increase of PPAR $\alpha$  mRNA level.

The effect of the antagonist can probably be related to the fact that it binds to PPAR $\alpha$  protein, thus preventing the

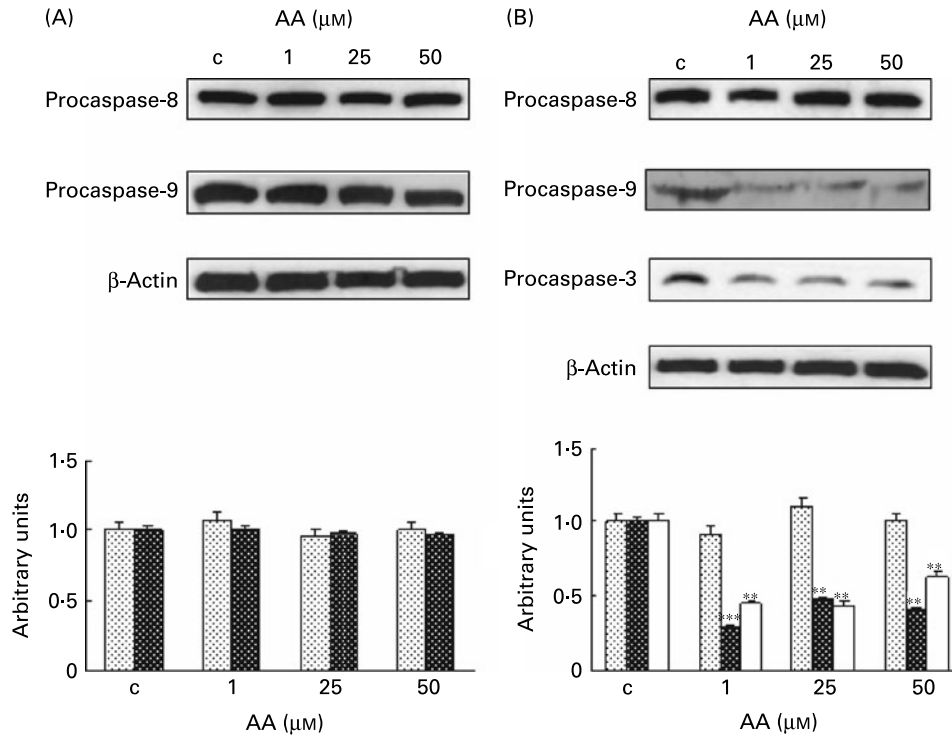
conformational change necessary for PPAR $\alpha$  ligand–receptor interaction<sup>(37)</sup>.

*Involvement of PPAR $\alpha$  in cell proliferation, extracellular signal-regulated protein kinase–mitogen activated protein kinase pathway and apoptosis*

To thoroughly evaluate whether AA-induced cell growth inhibition may be ascribed to PPAR $\alpha$  activation, the growth inhibitory effects of AA in MCF-7 and MDA-MB-231 cells were compared to those obtained in the presence of the specific antagonist MK886. As shown in Fig. 9 ((C) and (D)), in both cell lines the reduction of cell growth induced by AA was significantly prevented by MK886 administration, thus confirming the involvement of PPAR $\alpha$  in AA growth inhibitory activity.



**Fig. 3.** Effect of arachidonic acid (AA) on pro- and anti-apoptotic proteins. MCF-7 (A) and MDA-MB-231 (B) breast cancer cells were incubated with 1, 25 or 50  $\mu$ M AA for 24 h, and total cell lysates were separated on 10% SDS–PAGE gel, transferred to nitrocellulose membrane and probed with anti-bak (□) or anti-bcl-2 (■) antibody. Protein contents were normalized by probing the same membrane with anti- $\beta$ -actin antibody. The densitometric values of the bands are shown as means with standard deviation indicated by vertical bars, from three independent experiments. Mean values were significantly different from controls (c): \* $P$ <0.05, \*\* $P$ <0.01.

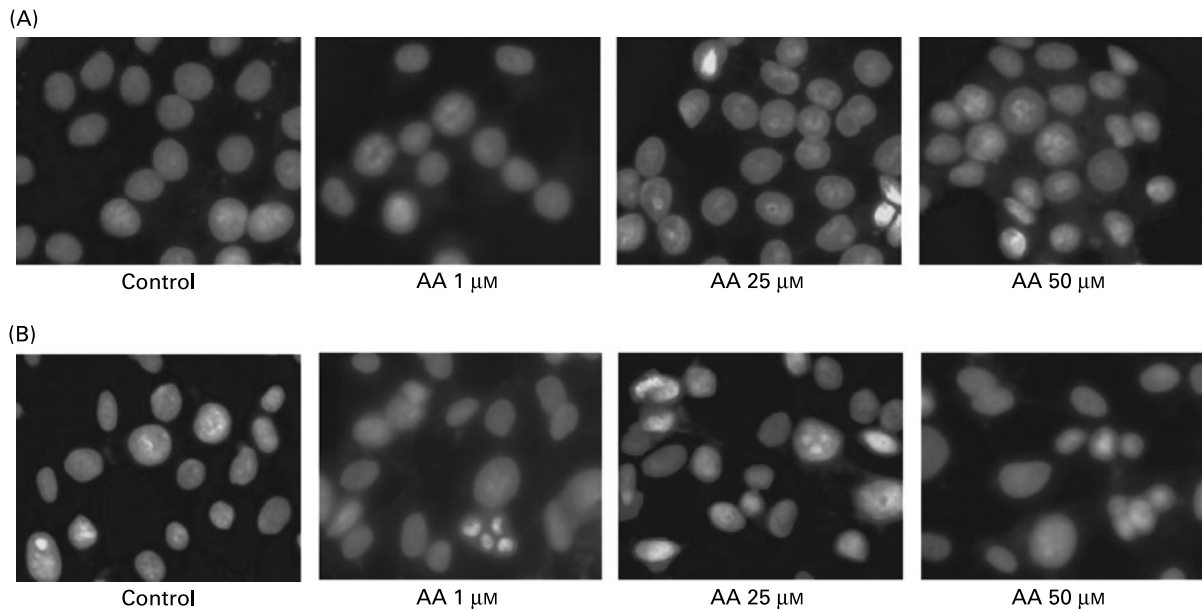


**Fig. 4.** Effect of arachidonic acid (AA) on procaspase-8, -9 and -3. MCF-7 (A) and MDA-MB-231 (B) breast cancer cells were incubated with 1, 25 or 50  $\mu\text{M}$  AA for 24 h, and total cell lysates were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membrane and probed with an anti-caspase-8p20 ( $\square$ ), anti-caspase-9p10 ( $\blacksquare$ ) and anti-caspase-3 antibody ( $\square$ ). Protein contents were normalized by probing the same membrane with an anti- $\beta$ -actin antibody. The densitometric values of the bands are shown as means with standard deviation indicated by vertical bars, from three independent experiments. Mean values were significantly different from controls (c): \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

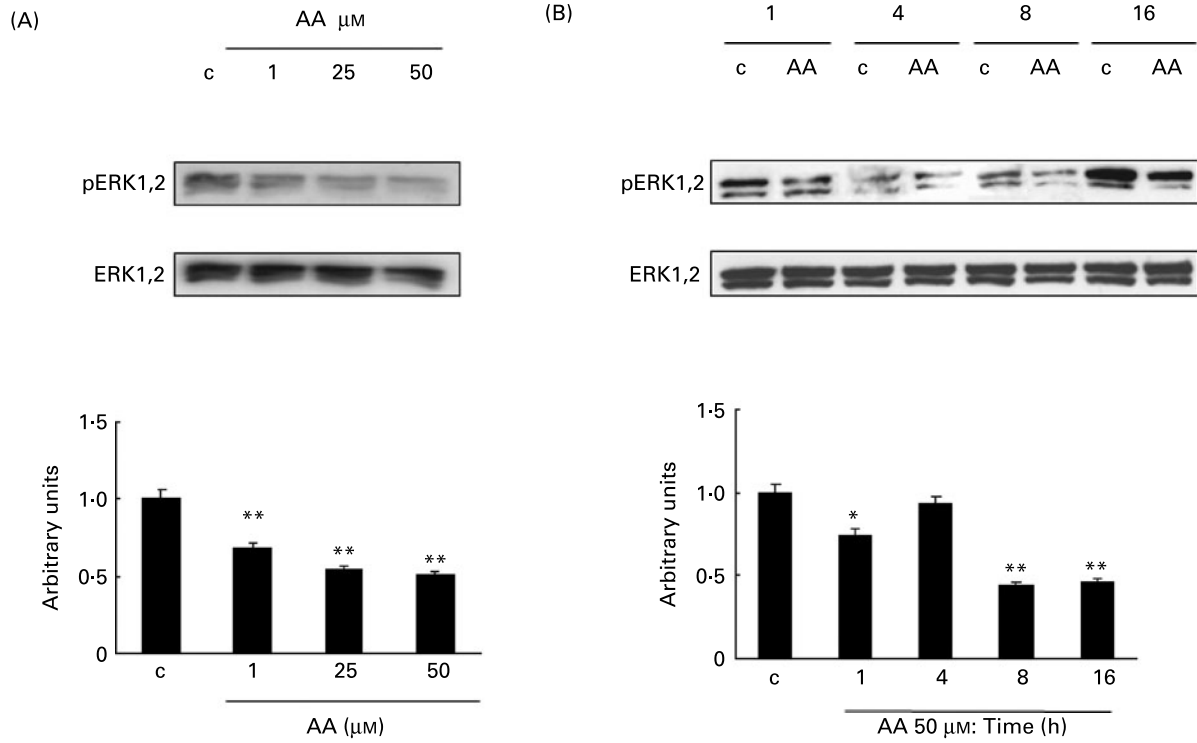
The pre-treatment of both MCF-7 (Fig. 11 (A)) and MDA-MB-231 (Fig. 11 (B)) cells with the antagonist was able to reduce the inhibitory effect of AA on the phosphorylation state of ERK1/2 (active form). In addition, in MDA-MB-231 cells the antagonist also reduced the pro-apoptotic effects of AA, as it strongly

down regulated Bak expression and augmented procaspase-3 levels (Fig. 11 (B)).

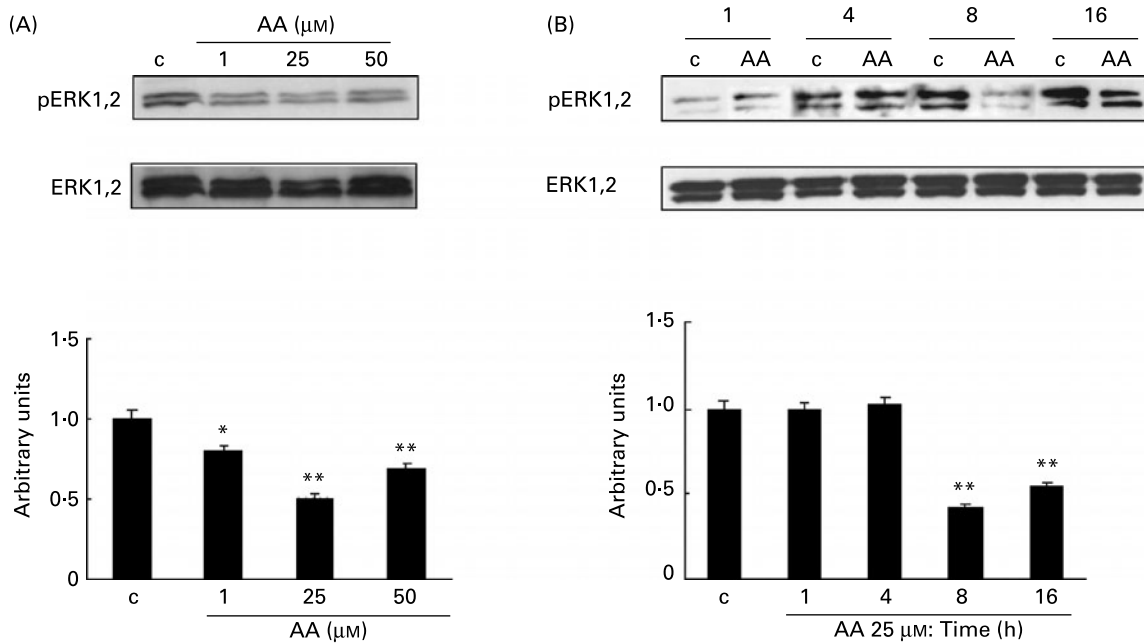
Taken together, these results indicate that PPAR $\alpha$  may be involved, at least in part, in the growth inhibitory activity of AA on MCF-7 and MDA-MB-231 cells; furthermore,



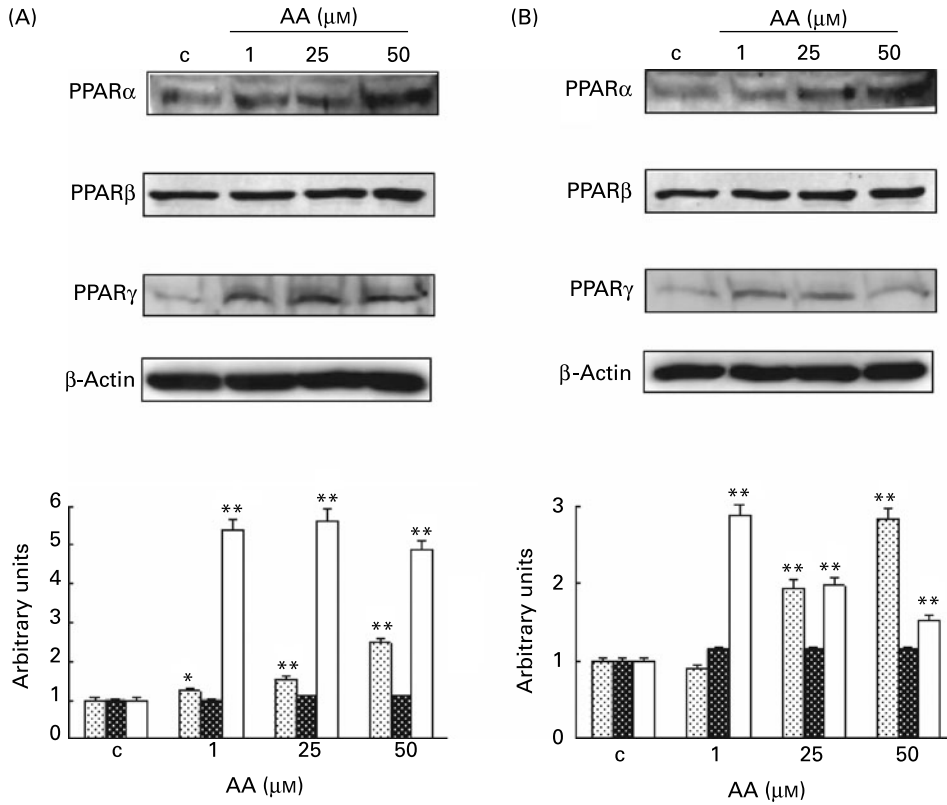
**Fig. 5.** Effect of arachidonic acid (AA) on nuclear morphology. MCF-7 (A) and MDA-MB-231 (B) breast cancer cells were treated with 1, 25 or 50  $\mu\text{M}$  AA for 24 h. To detect nuclei the cells were stained with 4',6-diamidino-2-phenylindole (1 mg/ml in methanol) and viewed under a fluorescence microscope equipped with a UV light filter.



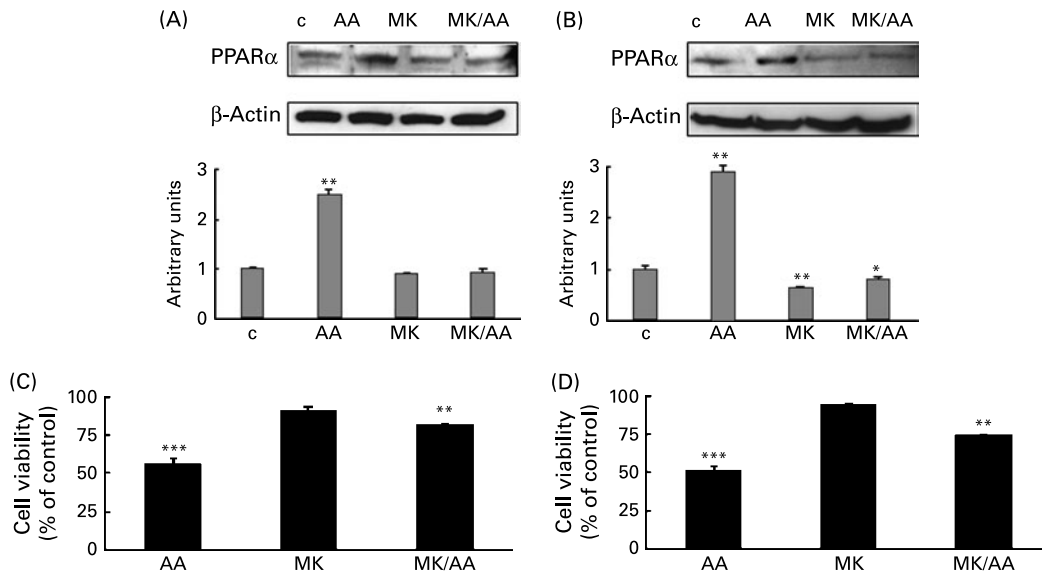
**Fig. 6.** Effect of arachidonic acid (AA) on extracellular signal-regulated protein kinase (ERK)1/2 phosphorylation state in MCF-7 breast cancer cells. MCF-7 cells were incubated with 1, 25 or 50  $\mu$ M AA for 24 h (A) or with 50  $\mu$ M AA for 1, 4, 8 or 16 h (B). After treatment, total cell lysates were obtained and probed with an anti-phosphoERK1/2 antibody. Protein contents were normalized by probing the same membrane with an anti-ERK1 antibody. The densitometric values of the bands are shown as means with standard deviation indicated by vertical bars, from three independent experiments. Mean values were significantly different from controls (c): \* $P < 0.05$ , \*\* $P < 0.01$ .



**Fig. 7.** Effect of arachidonic acid (AA) on the extracellular signal-regulated protein kinase (ERK)1/2 phosphorylation state in MDA-MB-231 breast cancer cells. MDA-MB-231 cells were incubated with 1, 25 or 50  $\mu$ M AA for 24 h (A) or with 25  $\mu$ M AA for 1, 4, 8 or 16 h (B). After treatment, total cell lysates were obtained and probed with an anti-phosphoERK1/2 antibody. Protein contents were normalized by probing the same membrane with an anti-ERK1 antibody. The densitometric values of the bands are shown as means with standard deviation indicated by vertical bars, from three independent experiments. Mean values were significantly different from controls (c): \* $P < 0.05$ , \*\* $P < 0.01$ .

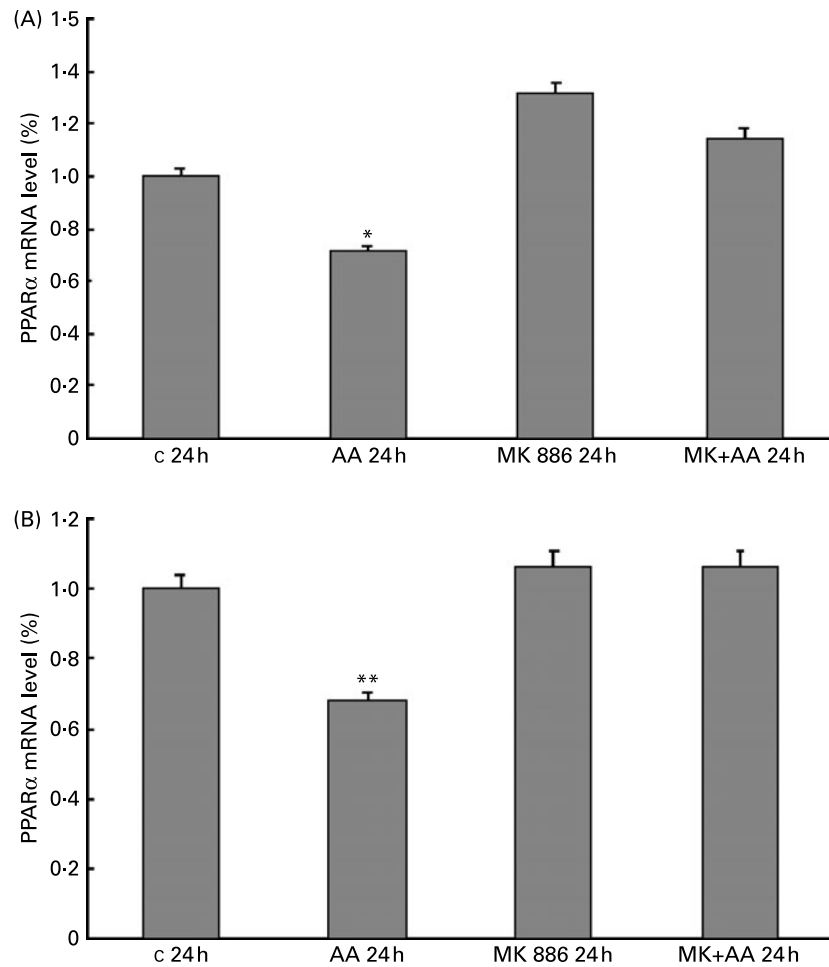


**Fig. 8.** Effect of arachidonic acid (AA) on PPAR expression. MCF-7 (A) and MDA-MB-231 (B) breast cancer cells were treated with 1, 25 or 50 μM AA for 24 h and total cell lysates were probed with an anti-PPARα (□), anti-PPARβ (■) and anti-PPARγ (□) antibody. Protein contents were normalized by probing the same membrane with an anti-β-actin antibody. The densitometric values of the bands are shown as means with standard deviation indicated by vertical bars, from three independent experiments. Mean values were significantly different from controls (c): \**P*<0.05, \*\**P*<0.01.



**Fig. 9.** Effect of arachidonic acid (AA) on cell growth and PPARα expression in the presence of MK886 (MK). MCF-7 (A, C) and MDA-MB-231 (B, D) breast cancer cells were treated for 24 h with 50 μM AA, 5 μM antagonist alone or 5 μM antagonist 1 h before 50 μM AA. Total cell lysates were probed with an anti-PPARα antibody and normalized by probing the same membrane with an anti-β-actin antibody. In (A) and (B) the densitometric values of the bands are shown as means with standard deviation indicated by vertical bars, from three independent experiments. Mean values were significantly different from controls (c): \**P*<0.05, \*\**P*<0.01. For MCF-7 (C) and MDA-MB-231 (D) cells were treated as described, and cell viability was determined by the trypan blue exclusion test. Values are presented as the percentage of control, as mean values with standard deviation shown by vertical bars, from three independent experiments conducted in triplicate. Mean values were significantly different from controls: \*\**P*<0.01, \*\*\**P*<0.001.





**Fig. 10.** Effect of arachidonic acid (AA) on PPAR $\alpha$  mRNA expression in the presence of MK886. MCF-7 and MDA-MB-231 breast cancer cells were treated for 24 h with 50  $\mu$ M AA, with or without 5  $\mu$ M MK886. PPAR $\alpha$  mRNA levels in MCF-7 (A) and MDA-MB-231 (B) cells were determined by real-time PCR. Each sample was tested six times and data are reported as variation calculated taking the values of control cells (C) as 1. Mean values were significantly different from controls: \* $P$ <0.05, \*\* $P$ <0.01.

in MDA-MB-231 cells, cross-talk between PPAR $\alpha$  induction and signalling pathways involved in the induction of apoptosis may occur.

### Discussion

Breast cancer is one of the most frequently diagnosed cancers and the second most common cause of cancer death in women. Epidemiological and experimental studies conducted over the past few decades suggest a protective role for *n*-3 PUFA against the development of breast and colon cancer<sup>(2,3)</sup>. It has been recently reported that also some *n*-6 PUFA, often believed to exert stimulatory effects on cancer cell growth and metastasis, can reduce the growth of different human breast and colon cancer cells<sup>(4)</sup>.

In this study, we investigated the effects of long-chain polyunsaturated 20:4*n*-6 AA on ER $\alpha$ (+) MCF-7 and ER $\alpha$ (-) MDA-MB-231 human breast cancer cell lines with the aim to elucidate the mechanisms by which AA regulates cell growth.

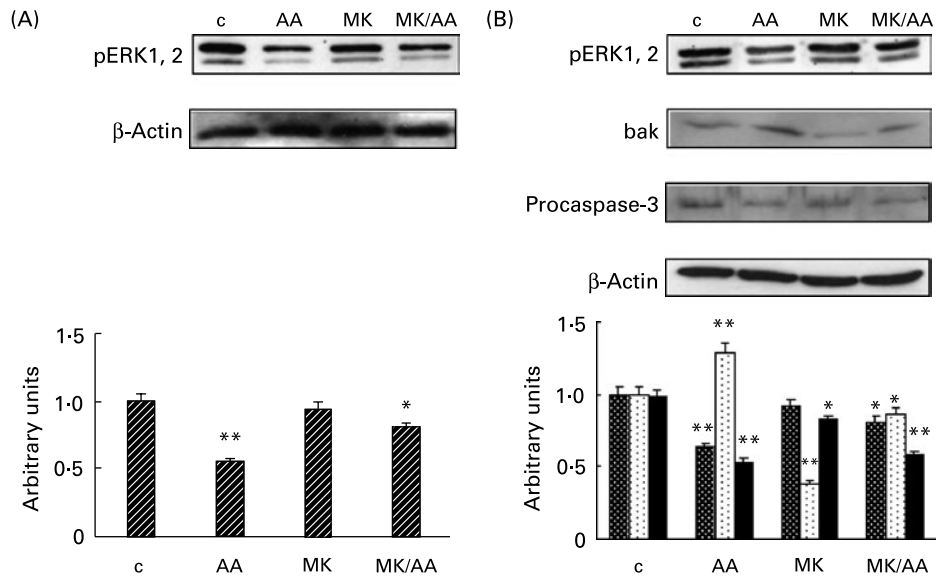
AA, like other fatty acids, represents a ligand of PPAR, transcription factors belonging to the nuclear hormone receptor super-family that includes receptors for steroids, thyroid

hormones, retinoic acid and vitamin D<sup>(28)</sup>. Increasingly studies have linked PPAR with mammary tumourigenesis<sup>(40)</sup> and, in particular, PPAR $\gamma$  activators have been trialled as a therapy for breast cancer<sup>(41)</sup>.

PPAR $\gamma$  has an important role in cell growth regulation, differentiation and fat metabolism<sup>(28)</sup>. Recent data have shown that activation of PPAR $\gamma$  causes inhibition of cell proliferation and extensive lipid accumulation<sup>(41)</sup> in human breast cancer cells that are characterized by high levels of PPAR $\gamma$ .

In contrast to the growth-inhibitory effect of PPAR $\gamma$  on breast cancer cells<sup>(42)</sup>, the anti-tumour effect of PPAR $\alpha$  appears to be less commonly reported. One reason for this may be the perception that PPAR $\alpha$  agonists are carcinogenic in rodents<sup>(43)</sup> and increase proliferation in some breast cancer cells<sup>(44)</sup>. This tumourigenic effect, however, is controversial as PPAR $\alpha$  agonists have demonstrated antiproliferative efficacy *in vitro* against melanoma<sup>(45)</sup>, endometrial and breast cancer cell lines<sup>(46)</sup>.

In our experimental condition AA induces a strong increase in both PPAR $\alpha$  and PPAR $\gamma$  expression, while it does not interfere significantly with PPAR $\beta$  expression. The order of magnitude of this effect differs according to the cell line considered: we detected higher levels of PPAR $\alpha$  in the



**Fig. 11.** Effect of arachidonic acid (AA) on phospho-ERK1/2, Bak and procaspase-3 levels in the presence of MK886. MCF-7 (A) and MDA-MB-231 (B) breast cancer cells were incubated for 24 h with 50  $\mu\text{M}$  AA, 5  $\mu\text{M}$  antagonist alone or 5  $\mu\text{M}$  antagonist 1 h before 50  $\mu\text{M}$  AA and the total cell lysates were probed with an anti-phosphoERK1/2 (for MCF-7 and MDA-MB-231 cells (■)), anti-Bak (□) or anti-caspase-3 antibody (■) (for MDA-MB-231 cells). Protein contents were normalized by probing the same membrane with anti- $\beta$ -actin antibody. The densitometric values of the bands are shown as means with standard deviation indicated by vertical bars, from three independent experiments. Mean values were significantly different from controls (c): \* $P < 0.05$ , \*\* $P < 0.01$ .

ER $\alpha$  (–) MDA-MB-231 than in the ER $\alpha$  (+) MCF-7, while PPAR $\gamma$  was more strongly expressed above all in MCF-7 cells.

These results indicate the involvement of distinct PPAR pathways and are in accordance with studies showing a relationship between the relative levels of PPAR $\alpha$  and ER $\alpha$  in breast cancer cells, where high levels of ER $\alpha$  mRNA expression are associated with reduced levels of PPAR $\alpha$ <sup>(47)</sup>. It has been reported that induction of ER $\alpha$  expression in MDA-MB-231 cells reduces PPAR $\alpha$  levels whereas inhibition of ER $\alpha$  activity in ER $\alpha$  (+) MCF-7 cells increases PPAR $\alpha$  levels<sup>(44)</sup>. These observations suggest that the relationship between ER $\alpha$  and PPAR $\alpha$  is more than correlative and reflects the ability of ER $\alpha$  to dynamically regulate PPAR $\alpha$  activity and expression<sup>(47)</sup>. The precise relationship between PPAR $\alpha$  and ER $\alpha$  is not completely elucidated, but studies on PPAR $\alpha$  promoter indicate the presence of at least two nuclear receptor response elements<sup>(48)</sup> that could potentially be regulated by activated ER $\alpha$ .

Although we found that AA induces the expression of both PPAR isotypes in the two cell lines tested, the fact that the PPAR $\alpha$  antagonist completely prevents AA stimulatory effect on PPAR $\alpha$  expression, while the PPAR $\gamma$  antagonist is ineffective, most likely indicates a specific involvement of PPAR $\alpha$  in AA-induced growth inhibition.

PPAR $\alpha$  exerts a dynamic regulation in the mammary gland during pregnancy and lactation and potentially it is hormonally regulated. It is expressed in the mouse mammary gland with the maximum levels at 2 weeks of age and with declining levels during pregnancy and lactation<sup>(49)</sup>, but this potential role of PPAR $\alpha$  may extend beyond the normal physiological regulation of the mammary gland to tumorigenesis<sup>(43)</sup>. Literature data outline various mechanisms to explain the effect of PPAR $\alpha$  in cancer cells; in particular, PPAR $\alpha$  regulates networks involved in the control of cell

growth, cell cycle and apoptosis and its agonists probably act through the involvement of the mitogen-activated protein kinase pathway and eicosanoid production<sup>(43)</sup>. In line with these reports, our study showed that in both cell lines treatment with AA produces a reduction of the ERK phosphorylation state, a reduction that was prevented by pre-treatment with PPAR $\alpha$  antagonist MK886. In addition, MK886 was also capable of attenuating AA-mediated cell growth inhibition, while it was notable that, in both cases, pre-treatment with PPAR $\gamma$  antagonist was ineffective.

We showed that AA inhibits the growth of both cell lines in a dose-dependent manner, MDA-MB-231 cells being more sensitive than MCF-7 cells. The growth inhibitory effect of AA is not characterized by alterations in cell cycle progression (data not shown) but is accompanied by different contributions from apoptosis and necrosis. In MDA-MB-231 cells AA growth inhibitory activity is likely related to apoptosis induction. Here again, the PPAR $\alpha$  antagonist was able to prevent AA effects, thus suggesting that the modulation of PPAR $\alpha$  expression contributes to some degree to cell growth inhibition by AA.

Emerging evidence has shown that elevated intracellular AA can induce cell death via the mitochondrial-mediated apoptosis pathway<sup>(50)</sup>. In addition, AA displays pro-apoptotic characteristics such as the ability to be converted to pro-apoptotic eicosanoids<sup>(51,52)</sup> or to regulate the expression of genes involved in susceptibility and resistance to apoptosis<sup>(16)</sup>.

Collectively, AA shows a different behaviour with regard to cell growth inhibition or cell death induction in correlation with variations in PPAR isotype expression levels. Our results most likely indicate that a PPAR $\gamma$ -mediated pathway is not apparently involved in the growth inhibitory activity of AA, whereas a connection can be established with the PPAR $\alpha$  pathway, both in MCF-7 and MDA-MB-231 breast cancer cell lines. The distinct response to AA that we detected could be

related to the differences between MCF-7 and MDA-MB-231 cells in ER $\alpha$  levels. Of interest is the observation that the greatest increase of PPAR $\alpha$  protein occurred in ER $\alpha$  (–) MDA-MB-231 cells which underwent induction of apoptosis, in accordance with reports indicating that PPAR $\alpha$  may be correlated to the apoptotic programme execution<sup>(29)</sup>.

These features demonstrate that the 20:4n-6 PUFA, AA, can exert its growth inhibitory activity on breast cancer cells in a similar manner to n-3 PUFA, and support the hypothesis that it can be considered also as an anti-tumourigenic compound.

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