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ACTIVATION OF THE ORPHAN NUCLEAR RECEPTOR RORα COUNTERACTS THE PROLIFERATIVE EFFECT OF FATTY ACIDS ON PROSTATE CANCER CELLS: CRUCIAL ROLE OF 5-LIPOXYGENASE

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The incidence of prostate carcinoma is very low in Eastern countries, such as Japan, suggesting that life style conditions may play a crucial role in the development of this pathology. Dietary ω-6 polyunsaturated fatty acids, such as linoleic (LA) and arachidonic (AA) acids, have been shown to stimulate the proliferation of prostate cancer cells after being converted into 5-HETE by means of the 5-lipoxygenase (5-LOX) pathway. Blockade of 5-LOX activity has been proposed as an attractive target for the prevention of the mitogenic action of dietary fats on prostate cancer. The *5-LOX* gene has been shown to carry a response element for the orphan nuclear receptor RORα (for its RORα1 isoform in particular) in its promoter region. We attempt to clarify whether activation of RORα might modulate the expression of 5-LOX, thus interfering with the mitogenic activity of fatty acids in prostate cancer cells. We show that in androgen-independent DU 145 prostate cancer cells, LA, AA and their metabolite 5-HETE exert a strong stimulatory action on cell proliferation. This effect is completely counteracted by the simultaneous treatment of the cells with a non redox inhibitor of 5-LOX activity. We then demonstrate that: *i*) RORα, and specifically its RORα1 isoform, is expressed in DU 145 cells; *ii*) activation of RORα, by means of the thiazolidinedione derivative CGP 52608 (the synthetic RORα activator), significantly reduces 5-LOX expression, both at mRNA (as evaluated by comparative RT-PCR) and at protein (as investigated by Western blot analysis) level (this was confirmed by the reduced activity of 5-LOX in CGP 52608 treated cells); and *iii*) the treatment of DU 145 cells with CGP 52608 completely abrogated the proliferative action of both LA and AA. These results have been confirmed in another androgen-independent prostate cancer cell line (PC3). Our data indicate that, by decreasing the expression of 5-LOX, activation of RORα might interfere with the mitogenic activity of fatty acids on prostate cancer. We have shown previously that CGP 52608 reduces the proliferation and the metastatic behavior of DU 145 cells. These observations indicate that the orphan nuclear receptor RORα might be considered as a molecular target for the development of new chemopreventive or chemotherapeutic strategies for prostate carcinoma.
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Key words: *prostate cancer; RORα; 5-lipoxygenase; linoleic acid; arachidonic acid*

Prostate cancer incidence is particularly high in the United States and Europe and much lower in Japan and in other Asian countries.[1] In addition to circulating levels of androgens, environmental factors, such as dietary fats, have been suggested to be responsible for this difference.[2–4] Among polyunsaturated fatty acids, the ω-6 acids that are present in animal fat and in vegetable oils, have been shown to enhance the risk of prostate cancer occurrence. On the contrary, ω-3 acids, that can be found in high amounts in fish oils, seem to exert a protective action against the growth of the tumor.[5–7]

Linoleic acid (LA), a member of the ω-6 polyunsaturated fatty acid family, is converted to arachidonic acid (AA), which is then incorporated into the phospholipids of the cell membrane. Arachidonic acid is in turn metabolized to the series of eicosanoids after having been released from phospholipids by PLA2.[8,9] Eicosanoids have been reported to mediate the mitogenic activity of both LA and AA on different tumor cell lines.[5,10–14]

In prostate cancer, AA has been shown to stimulate cell proliferation mainly through its conversion to 5-HETE (5-hydroxyeicosatetraenoic acid), *via* the 5-lipoxygenase (5-LOX) pathway.[15–17] Moreover, 5-LOX has been reported recently to be overexpressed in prostate carcinoma.[18] Taken together, these observations led to the suggestion that 5-LOX might be considered as an attractive target for the development of new chemopreventive or chemotherapeutic strategies.

RORα is a transcription factor belonging to the family of orphan nuclear receptors, for which currently endogenous ligands are unknown.[19,20] The gene for the human RORα receptor encodes at least 4 distinct isoforms: RORα 1, 2, 3, and 4 (also named RZRα).[21,22] The 4 splicing variants share common DNA- and putative ligand-binding domains, but possess distinct amino-terminal domains.[22] RORα receptors bind preferentially to response elements (ROREs) composed of a typical hexameric core-binding site RGGTCA (R = A or G), and an A/T rich 5′-flanking sequence.[21,22] By binding to ROREs in the promoter region of target genes, RORα has been shown to be involved in the control of several physiological processes, such as cerebellar development,[23,24] muscle cell differentiation,[25] bone metabolism[26] and immune functions.[27]

A RORE sequence has also been identified in the promoter region of the human *5-LOX* gene. In particular, this RORE specifically binds RORα1 and RZRα, but not RORα2 and 3.[28] Our experiments attempt to verify whether in prostate cancer cells, the activation of RORα might interfere with the mitogenic action of fatty acids through the modulation of 5-LOX expression. We used the thiazolidinedione derivative CGP 52608, which has been shown previously to activate the nuclear receptor.[29,30]

MATERIAL AND METHODS

Materials

The thiazolidinedione derivative CGP 52608 (1-[3-allyl-4-oxothiazolidine-2-ylidene]-4-methyl-thisemicarbazone) was kindly donated by Dr. D. Evans (Novartis, Basel, Switzerland). This compound has been demonstrated previously to activate the orphan nuclear receptor RORα.[29,30] Linoleic acid (LA), arachidonic acid (AA), 5-hydroxyeicosatetraenoic acid (5[S]-HETE and 5[R]-HETE) and delipidized bovine serum albumin (BSA) were purchased from Sigma Chemical (St. Louis, MO). MK886, the inhibitor of 5-lipoxygenase activating protein (FLAP), was obtained from Merck Frosst (Quebec, Canada).

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Cell culture

The human androgen-independent DU 145 and PC3 prostate cancer cell lines were obtained from American Type Culture Collection (Rockville, MD). Cells were routinely grown in RPMI-1640 medium (Seromed Biochrom, Berlin, Germany) supplemented with 5% fetal bovine serum (FBS) (Gibco, Paisley, Scotland, UK), glutamine (1 mM) and antibiotics (100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulphate) in a humidified atmosphere of 5% CO_2/95% air.

Cell proliferation studies

Effects of fatty acids and 5-HETEs on cell proliferation. To study the effects of fatty acids and 5-HETEs on cell proliferation, DU 145 cells were plated at a density of 2×10^4 cells/plate in RPMI-1640 supplemented with 5% FBS. Forty-eight hours after plating, the medium was changed to RPMI-1640 supplemented with 0.5% FBS and delipidized BSA (5 μM). Cells were then treated with LA (10^{-8}–10^{-5} M), AA (10^{-8}–10^{-6} M), 5(S)-HETE or 5(R)-HETE (5×10^{-8}–10^{-6} M) for 7 days. Fatty acids and 5-HETEs were pre-complexed with lipid-free BSA before addition to the medium. Final concentration of BSA was maintained at 5 μM throughout the experiments. Control cells were treated with the 0.5% FBS additioned medium containing BSA only. At the end of the treatment, cells were harvested and counted by hemocytometer.

To confirm the role of 5-LOX in the growth modulatory activity of fatty acids, DU 145 cells were treated, for 7 days, with AA (10^{-7} M), either in the absence or in the presence of the 5-LOX inhibitor MK886 (10^{-7}–10^{-5} M). Among the biochemical inhibitors of 5-LOX, this compound has been shown to act by interacting with FLAP and interfering with the presentation of AA to 5-LOX enzyme at the nuclear envelope membrane.[31]

Effects of RORα activation on the mitogenic activity of fatty acids. DU 145 cells were treated as described above, either with LA (10^{-6} M) or AA (10^{-7} M) in the absence or in the presence of CGP 52608 (10^{-7} M). Cells were harvested and counted after 7 days of treatment. All the cell proliferation experiments were repeated at least 3 times. Five plates/dose were included in each experiment.

RT-PCR for the RORα isoforms

The specific isoform of the RORα receptor that might be expressed in DU 145 cells was investigated by RT-PCR. After phenol-chloroform extraction, 1 μg of total RNA from cancer cells was used in a reverse transcription reaction. The same RNA has been used in all the RT-PCR reactions. cDNA synthesis was carried out using the Gene AMP kit (Perkin Elmer Cetus, Norwalk, CT), with an oligo$(dT)_{16}$ as a primer for the reverse transcriptase. Samples containing cDNAs were then amplified in a 100 μl solution containing PCR buffer (50 mM KCl, 10 mM Tris-HCl), 2 mM $MgCl_2$, 15 pmol of a pair of specific primers and 2.5 U Taq Polymerase. Thirty-five cycles of amplification were carried out in a programmable heat block (Perkin Elmer Cetus) (1-min denaturation at 94°C, 45-sec primer annealing at 60°C and 2-min primer extension at 72°C). The sense primers were: 5′-AAACATGGAGTCAGCTCCG-3′ for RORα1; 5′-CTCCAAATACTCCATCAGTGTATCC-3′ for RORα2; 5′-CAACTTGAGCACATAAACTGG-3′ for RORα3; 5′-TGTATTTTGTGATCGCAGAG-3′ for RORα4. For all the isoforms, the antisense primer used was: 5′-CATACAAGCTGTCTCTCTGC-3′.[28] The same RT-PCR conditions were used to amplify β-actin cDNA; in this case the primers used were 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ (sense) and 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′ (antisense).[32] After RT-PCR, the amplified DNA products were separated on a 1.5% agarose gel and stained with ethidium bromide.

Immunoprecipitation and western blot analysis for RORα1

To confirm the expression of the RORα1 isoform at the protein level, DU 145 cells were harvested and solubilized in RIPA buffer (50 mM Tris-HCl, pH 7.7), 150 mM Na_3VO_4, 50 mM NaF, 0.3 mM phenylmethylsulfonylfluoride and 5 mM iodoacetic acid. RORα has been immunoprecipitated with 10 μg/10 μl of a polyclonal antibody against the RORα1 receptor (sc-6062, Santa Cruz Biotechnology, Santa Cruz, CA) and electrophoresed on 10% polyacrylamide gel under reducing conditions. Precipitated proteins were transferred onto a nitrocellulose filter, which was then probed with the sc-6062 antibody for 2 hr at room temperature. Filters were then incubated for 1 hr at room temperature with an anti-goat IgG (1:10.000). Antibody bound to RORα1 was detected using the Supersignal detection system (Celbio, Pierce, Rockford, IL) after a 5–10-min exposure to a Hyperfilm X-ray film (Amersham Pharmacia Biotech, Milano, Italy) at room temperature.

RT-PCR for 5-LOX expression

In preliminary experiments, RT-PCR was carried out to choose the adequate number of cycles for amplification of 5-LOX and β-actin (as an internal control) cDNAs. To this purpose, RNA (1 μg) from DU 145 cells was reverse transcribed and cDNAs were amplified by PCR as described above. The primers used for 5-LOX were: 5′-CACTGACGACTACATCTACC-3′ (sense) and 5′-GTTCCACTCCATCCATCGAT-3′ (antisense);[28] the primers for β-actin were the same as described above.[32] PCR reactions consisted of 1-min denaturation at 94°C, 30-sec annealing at 60°C and 2-min primer extension at 72°C. The number of cycles carried out were 30–45 for 5-LOX and 15–30 for β-actin cDNAs, respectively. After RT-PCR, the amplified cDNAs were separated on an agarose gel, stained with ethidium bromide and photographed under UV light.

To study the effects of RORα activation on the expression of 5-LOX, DU 145 cells were plated in 100 mm-dishes at a density of 8×10^5 cells/dish. Twenty-four hours after plating, the media were changed to experimental media (RPMI-1640 supplemented with 5% FBS) either in the absence or in the presence of CGP 52608 (10^{-7} M) for 6, 12, 24, 36, 48 and 60 hr. At the end of the treatment, cells were harvested and RNAs were extracted and submitted to RT-PCR at adequate cycles. Equal RT reaction solutions were utilized in PCR reaction in the presence of the specific primers for 5-LOX and for β-actin.

Western blot analysis of 5-LOX

To study the effects of RORα activation on the level of 5-LOX protein, DU 145 cells were plated at a density of $8x10^4$ cells/plate. Twenty-four hours later, the media were changed to experimental media and cells were treated daily with CGP 52608 (10^{-7} M) for 4 days (96 hr). At the end of the treatment, cells were harvested and protein fractions were prepared as described for the RORα protein. Proteins were electrophoresed and transferred onto a nitrocellulose filter. Filters were probed with the rabbit polyclonal 5-LOX antibody (LO32) (kindly provided by Dr. R.N. Young, Merk Frosst) for 2 hr at room temperature. Filters were then incubated for 1 hr at room temperature with an anti-rabbit IgG (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA). Antibody bound to 5-LOX was detected using the Supersignal detection system, as described for the RORα protein.

Evaluation of 5-LOX enzymatic activity

5-LOX activity was evaluated after the formation of 5(S)-HETE upon incubation of DU 145 cells with AA and the calcium ionophore A23187, after a daily treatment, for 4 days (96 hr), with CGP 52608 (10^{-7} M) or with its vehicle. Briefly, cells were washed and incubated with 1 ml of PBS containing 1 μM of AA and the calcium ionophore A23187 (2 μM) for 5 min at 37°C. After centrifugation at 12,000*g*, 4°C for 5 min, supernatants were loaded onto a solid phase extraction cartridge (1 ml OASIS, Waters, Milford, MA), preactivated with 1 ml methanol and 1 ml of water. Arachidonic acid metabolites were eluted using 1 ml of methanol/acetonitrile (50/50, v:v) and taken to dryness using a SpeedVac Rotary Evaporator (Savant, Farmingdale, NY). After reconstitution in 200 μl of HPLC mobile phase (see below) samples were

injected into a Beckman Liquid Chromatograph mod 110B (Beckman Analytical, Palo Alto, CA). Separation of mono-HETEs was carried out on Ultrasphere ODS column (4.6 × 250 mm, 5 μm, Beckman Analytical) eluted isocratically at 1 ml/min using a mobile phase of acetonitrile:water:acetic acid 60:40:0.01, pH 5 with ammonium hydroxide. UV absorbance was monitored at 236 nm with a Beckman diode-array UV Detector and full scans (220–300 nm) were acquired every 2 sec. Retention time of mono-HETEs was determined using synthetic 5(S)-HETE and 15(S)-HETE.

Experiments in PC3 cells

Experiments were carried out in PC3 cells, to confirm the effects of RORα activation on the proliferative action of fatty acids in a second experimental model of prostate cancer cells. In preliminary studies, PC3 cells were treated with CGP 52608 (10^{-11}–10^{-6} M), to confirm the antiproliferative effects of the thiazolidinedione derivative on androgen-independent prostate cancer cells, as previously reported for DU 145 cells.[33] PC3 cells were then treated with AA (10^{-8} M–5 $\times 10^{-6}$ M), harvested and counted after 7 days of treatment. Finally, PC3 cells were treated with AA (5 $\times$ 10^{-6} M), either in the absence or in the presence of CGP52608 (10^{-7} M) (as described for DU 145 cells) and counted after 7 days of treatment.

Statistical analysis

Data from cell proliferation studies were analyzed according to the Dunnett's test after one-way analysis of variance.[34]

RESULTS

Effects of fatty acids and 5-HETE on DU 145 cell proliferation

Figure 1*a* shows that LA significantly stimulates cell proliferation in the range of doses 5 $\times$ 10^{-7}–5 $\times$ 10^{-6} M. AA exerted its mitogenic action on DU 145 cells at the concentrations of 5 $\times$ 10^{-7} M and 10^{-7} M (Fig. 1*b*). Finally, 5(S)-HETE induced a significant increase of cell growth when used at the dose of 5 $\times$ 10^{-7} M (Fig 1*c*). 5(R)-HETE did not modify DU 145 cell proliferation at any dose used (data not shown). DU 145 cells were then treated with AA (10^{-7} M) either in the absence or in the presence of the 5-LOX inhibitor MK886 (10^{-7}–10^{-5} M). Figure 2 shows that MK886 counteracts the stimulatory action of AA on prostate cancer cells in a dose-dependent way, being significantly effective at 5 $\times$ 10^{-6} and 10^{-6} M. These results confirm the proliferative effect of fatty acids on prostate cancer cells and the crucial role played by 5-LOX in this activity.

Expression of the RORα1 isoform in DU 145 cells

The *5-LOX* gene carries a RORα1 response element on its promoter region.[28] We have shown previously that the RORα family is expressed in DU 145 prostate cancer cells.[33] Experiments have now been carried out to identify the specific isoform of the orphan nuclear receptor that might be present in these cells. By RT-PCR, we have observed that only one cDNA band, corresponding to the RORα1 cDNA, was present in DU 145 cells (Fig. 3*a*, *upper panel*, *lane 1*). No band corresponding to RORα2, 3, or 4 cDNAs could be obtained (Fig. 3*a*, *upper panel*, *lanes 2–4*). β-Actin cDNAs were amplified in all the 4 samples, indicating that RNA was not degraded (Fig. 3*a*, *lower panel*, *lanes 1–4*). To confirm the expression of RORα1 in prostate cancer cells, the receptor was first immunoprecipitated and then submitted to gel electrophoresis and to Western blot analysis. As shown in Figure 3*b*, a major protein band with molecular weight of about 80 kDa, recognizing the sc-6062 antibody (Santa Cruz Biotechnology), was present in DU 145 cells. These results indicate that RORα1 is the major isoform of the receptor to be expressed in these cells.

Effects of RORα activation on 5-LOX expression

Because the *5-LOX* gene carries a RORE in its promoter region, we reasoned that the activation of the nuclear receptor might affect the expression of the enzyme. We verified this hypothesis both at the mRNA (comparative RT-PCR analysis) and at the protein (Western blot analysis) level.

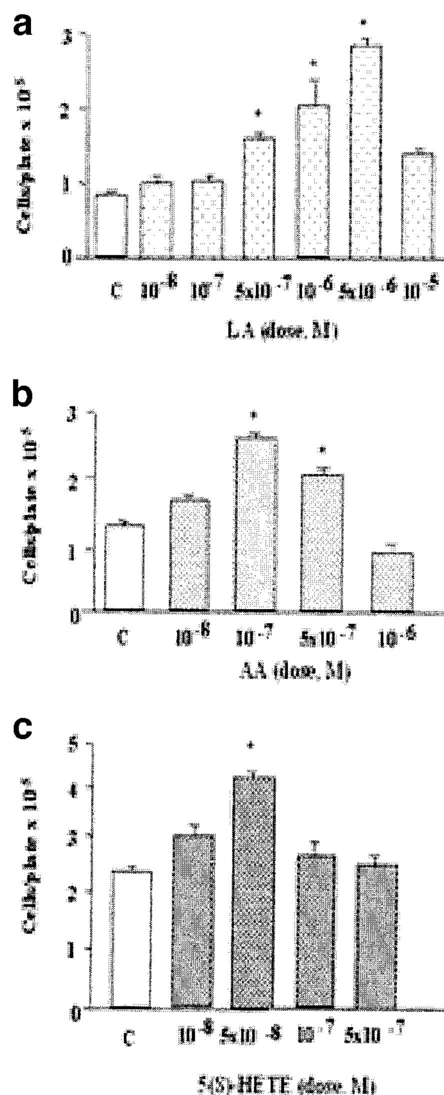

FIGURE 1 – Effects of linoleic acid (LA) (*a*), arachidonic acid (AA) (*b*), and 5(S)-hydroxyeicosatetraenoic acid, 5(S)-HETE (*c*) on DU 145 cell proliferation. Each experiment was repeated 3 times with identical results. Data are expressed as the mean cell number/plate ± SEM. *$p < 0.05$ *vs*. controls (C).

RT-PCR analysis. In preliminary experiments, we carried out RT-PCR analysis at various cycles to choose the adequate number of cycles for the detection of the mRNA expression of 5-LOX and of β-actin (as an internal standard) mRNA expression. The intensity of each band increased in parallel with the increasing number of PCR cycles (data not shown). We concluded that the adequate number of cycles was 35 for 5-LOX and 20 for β-actin.

To study the effects of RORα activation on the expression of 5-LOX, DU 145 cells were treated with the thiazolidinedione derivative CGP 52608 (10^{-7} M), the exogenous RORα ligand and activator,[29,30] and RNA was extracted at different time intervals (from 6–60 hr) for comparative RT-PCR. As shown in Figure 4, in control DU 145 cells, changing of the medium at the beginning of the experiments induces an increase of the expression of 5-LOX mRNA at 36, 48 and 60 hr. It is well known that FBS contains fatty acids and these have been shown to increase the expression of the

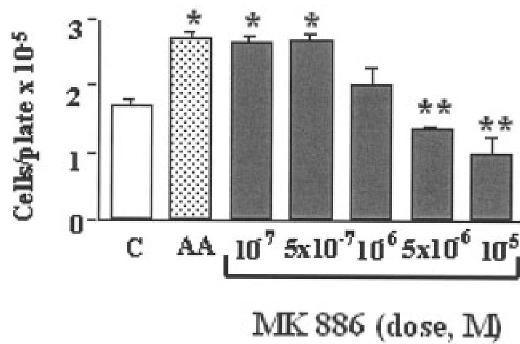

Figure 2 – Effects of the 5-LOX inhibitor MK 886 on the stimulatory activity of AA (10^{-7} M) on DU 145 cell proliferation. Each experiment was repeated 3 times with identical results. Data are expressed as the mean cell number/plate ± SEM. $*p < 0.05$ *vs.* controls (C); $**p < 0.05$ *vs. AA*.

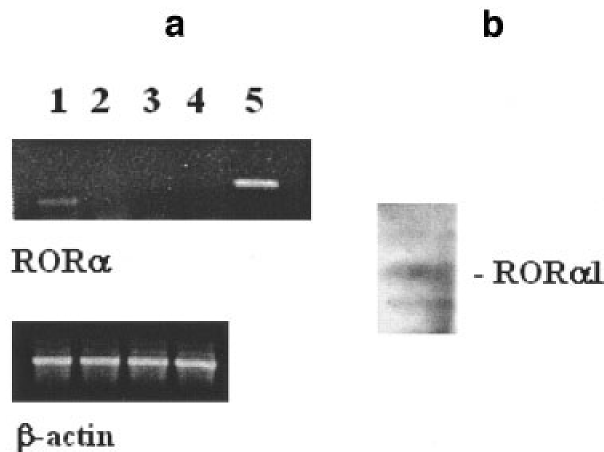

Figure 3 – Expression of the different RORα isoforms in DU 145 cells. (*a*) RT-PCR analysis of the different isoforms of RORα and of β-actin. (*Upper panel*) Ethidium bromide-stained gel of the amplified cDNAs obtained in the presence of oligonucleotide primers specific for RORα1 (*lane 1*), RORα2 (*lane 2*), RORα3 (*lane 3*), and RORα4 (*lane 4*). (*Lane 5*) RT-PCR control. (*Lower panel*) Ethidium bromide-stained gel of the amplified cDNAs obtained in the presence of oligonucleotide primers specific for β-actin. (*b*) Western blot analysis of RORα after immunoprecipitation using the sc-6062 antibody.

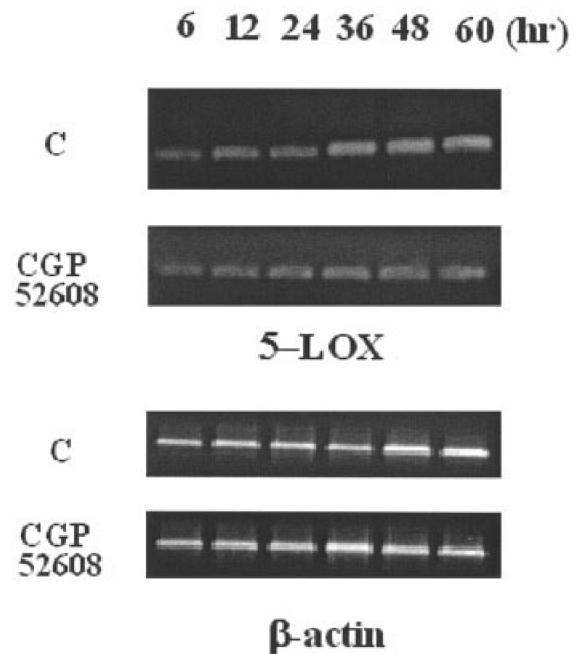

Figure 4 – Effects of CGP 52608 on 5-LOX mRNA expression in DU 145 cells. Cells were treated with CGP 52608 (10^{-7} M); at the indicated time intervals, total RNA was extracted and analyzed by RT-PCR at the appropriate cycles, as described in Material and Methods, for 5-LOX and β-actin. One representative of 3 experiments is reported.

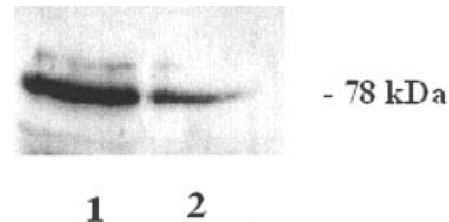

Figure 5 – Effects of CGP 52608 on 5-LOX protein expression in DU 145 cells. Cells were treated daily, for 4 days (96 hr), with CGP 52608 (10^{-7} M); protein extracts were then prepared and submitted to Western blot analysis, as described in Material and Methods. One representative of 3 experiments is reported. *Lane 1*, controls; *lane 2*, CGP 52608.

enzyme.[15–17] Treatment of the cells with CGP 52608 completely counteracted the increase at all the time intervals (Fig. 4). As expected, the level of expression of β-actin was not modified either in controls or in treated cells at any time interval considered (Fig. 4).

*Western blot analysis.*The effects of RORα activation on the level of the 5-LOX protein were evaluated at 96 hr after the treatment of DU 145 cells with the thiazolidinedione derivative (10^{-7} M), by using a specific antibody against the enzyme. As shown in Figure 5, the treatment resulted in a decreased level of expression of the protein, when compared to that found in untreated controls. Treatment with CGP 52608 for shorter time intervals did not induce any significant variation in 5-LOX levels (data not shown). From these results, it seems that the decrease of 5-LOX levels at the protein level is more pronounced than that observed at the mRNA level. It must be underlined, however, that for the RT-PCR experiments the cells received only one treatment at the beginning of the observation period. Cells were treated daily for 4 days for the Western blot analysis.

Effects of RORα activation on 5-LOX enzymatic activity

Incubation of DU 145 cells with AA in the presence of the calcium ionophore A23187 resulted in the formation of 5(S)-HETE, as assessed by retention time using isocratic RP-HPLC separation (Fig. 6*a*,*b*) and UV spectral analysis (Fig. 6*b*, *inset*). Treatment of cells with CGP 52608 (10^{-7} M), the exogenous RORα ligand and activator, showed a marked decrease (68 ± 6%, mean ± SD) of the peak corresponding to 5(S)-HETE (Fig. 6*c*), supporting the hypothesis that 5-LOX mRNA, protein and activity are all downregulated by RORα activation.

Effects of RORα activation on the mitogenic activity of fatty acids

Because fatty acids are mitogenic on prostate cancer cells through their conversion into 5-HETE by 5-LOX activity, and because RORα activation significantly reduces 5-LOX expression in DU 145 cells, we reasoned that CGP 52608 might counteract the proliferative action of fatty acids on these cells. DU 145 cells were

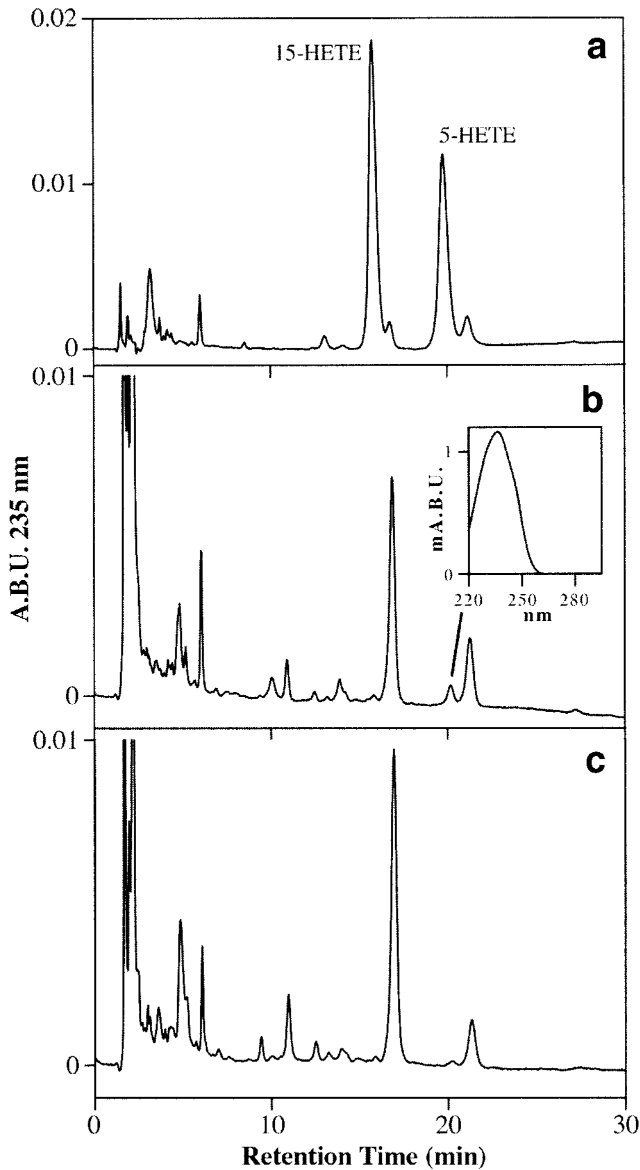

Figure 6 – Effects of CGP 52608 on 5-LOX enzymatic activity in DU 145 cells. Cells were treated daily, for 4 days (96 hr) with CGP 52608 (10^{-7} M); then, they were washed and incubated with AA (1 μM) and A23187 (2 μM) for 5 min at 37°C. Supernatants were analyzed by RP-HPLC as described in Material and Methods. (*a*) Chromatographic tracing of UV absorbance at 236 nm from standard 15(S)-HETE and 5(S)-HETE. (*b*) Chromatographic tracing of UV absorbance at 236 nm from control DU 145 cells; inset shows the full UV spectrum of chromatographic peak eluting at the retention time of standard 5(S)-HETE. (*c*) Chromatographic tracing of UV absorbance at 236 nm from DU 145 cells treated with CGP 52608 (10^{-7} M) for 96 hr.

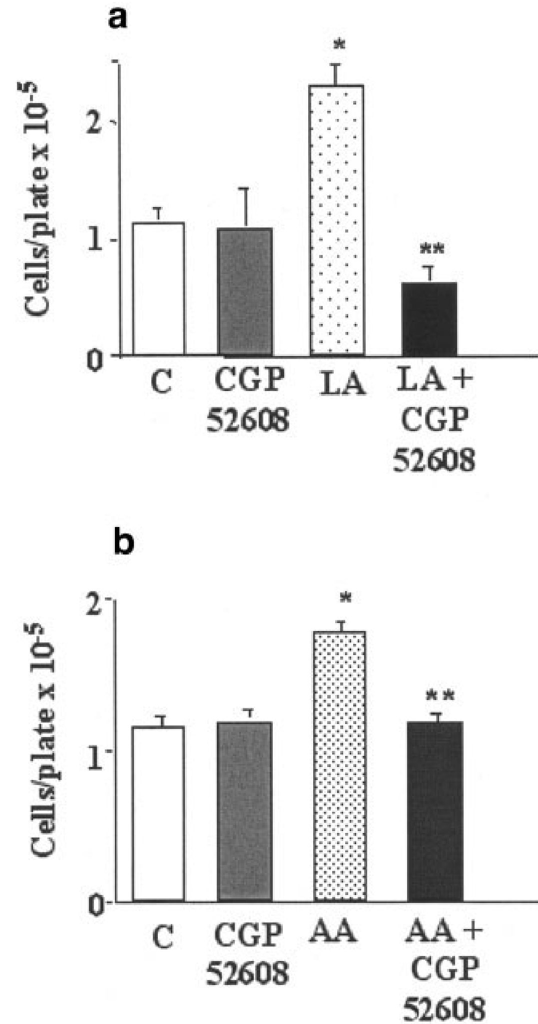

Figure 7 – Effects of CGP 52608 (10^{-7} M) on the stimulatory activity of LA (10^{-6} M) (*a*) and of AA (10^{-7} M) (*b*) on DU 145 cell proliferation. Each experiment was repeated 3 times with identical results. Data are expressed as the mean cell number/plate ± SEM. $*p < 0.05$ *vs.* controls (*c*); $**p < 0.05$ *vs.* LA (*a*) or *AA* (*b*).

treated with LA (10^{-6} M) or AA (10^{-7} M), either in the absence or in the presence of CGP 52608 (10^{-7} M). As expected, we found that CGP 52608 completely counteracts the stimulatory effect of both LA (Fig. 7*a*) and AA (Fig. 7*b*).

Experiments in PC3 cells

In preliminary experiments we have shown that the RORα1 isoform of the nuclear receptor is expressed in PC3 cells, both at mRNA and at protein levels (data not shown). The treatment of PC3 cells with CGP 52608 dose-dependently inhibited PC3 cell proliferation, as previously described by our group for DU 145 cells (Fig. 8*a*).[33] AA stimulated PC3 cell growth, although at a higher dose (5×10^{-6} M) than that required to increase DU 145 cell proliferation (Fig. 8*b*). This dose corresponds to that previously reported by other authors to be required for AA to maximally stimulate PC3 cell growth.[15]

Finally, PC3 cells were treated with AA (5×10^{-6} M), either in the absence or in the presence of CGP 52608 (10^{-7} M). The thiazolidinedione derivative completely counteracted the stimulatory effect of AA (Fig. 8*c*), confirming the results obtained in DU 145 cells.

DISCUSSION

Dietary fatty acids, and in particular ω-6 polyunsaturated fatty acids, are associated with an increased risk of the tumor of the prostate.[7] Our data supports this observation by showing that both LA and AA exert a significant proliferative effect on prostate cancer cells. This is in line with those data reported previously on PC3 cells, as well as on LNCaP androgen-dependent prostate cancer cells.[5,15] At variance with our data, Rose and Connolly[5] did not observe any effect of LA on DU 145 cell growth. At the

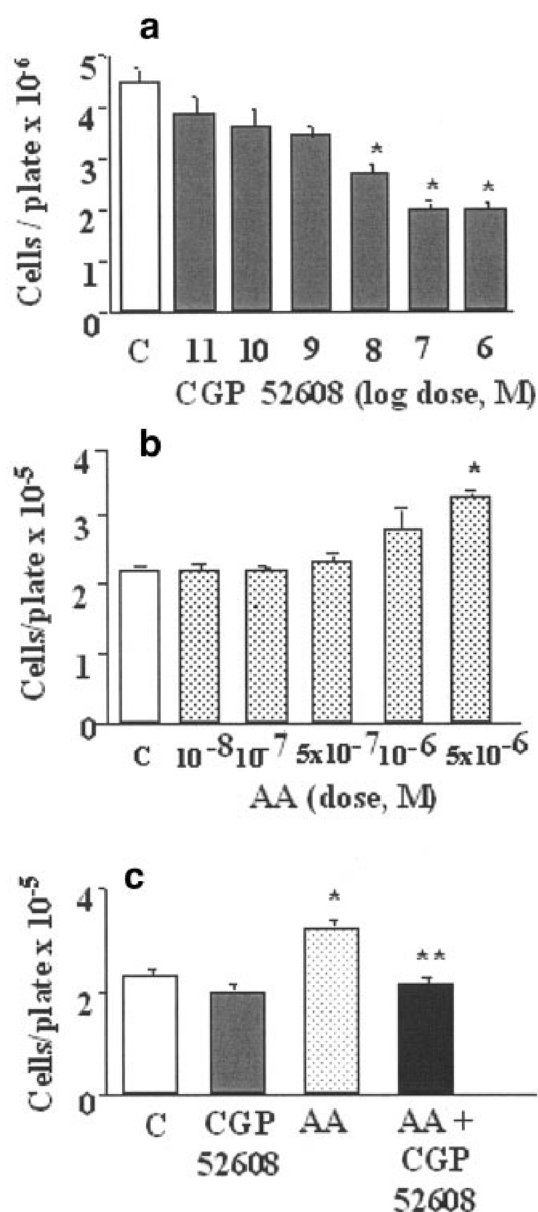

FIGURE 8 – Effects of CGP 52608 (10^{-11}–10^{-6} M) alone (*a*), of AA (10^{-8}–5×10^{-6} M) alone (*b*), and of CGP 52608 (10^{-7} M) either alone or in the presence of AA (5×10^{-6} M) (*c*) on PC3 cell proliferation. Each experiment was repeated 3 times with identical results. Data are expressed as the mean cell number/plate ± SEM. $*p < 0.05$ *vs.* controls (*c*); $**p < 0.05$ *vs. AA.*

moment, the reason for this discrepancy is not clear; however it might be related to methodological differences (*e.g.*, cell culture conditions, length of the treatment, *etc.*) present in the 2 studies.

Our results, together with previous reports[15–17] also confirm that both LA and AA, to exert their mitogenic effect, need to be metabolized through the 5-lipoxygenase pathway. Therefore, modulation of 5-lipoxygenase expression or activity has been suggested as a possible target for the development of new chemopreventive or chemotherapeutic strategies for prostate cancer.[17]

We have stated that the promoter region of 5-LOX contains a response element for the orphan nuclear receptor RORα1,[28,35] suggesting that the expression of the enzyme might also be regulated through the activation of the nuclear receptor. We have shown that this specific isoform of the receptor is expressed in DU 145 cells, both at the mRNA and at the protein level. We have demonstrated that 5-LOX expression is induced by FBS additioned medium; it is actually well known that FBS contains fatty acids and these have been shown to increase the expression of the enzyme.[15–17] The activation of RORα significantly counteracted this overexpression, both at the mRNA and at protein level. In line with these results, the treatment with the thiazolidinedione derivative markedly decreased 5-LOX enzymatic activity, as shown by the decreased production of 5(S)-HETE observed in DU 145 cells exposed to exogenous AA in the presence of the calcium ionophore A23187. These data indicate that in the presence of serum, and therefore in the case of high supply of fatty acids, the activation of the orphan nuclear receptor RORα significantly decreases the overexpression of 5-LOX and, consequently, its activity.

On the basis of these observations, we reasoned that the thiazolidinedione derivative CGP 52608, through the reduction of the expression and of the activity of 5-LOX, might affect the proliferative effects of ω-6 polyunsaturated fatty acids on prostate cancer cells. We verified this hypothesis by showing that, in DU 145 cells, the mitogenic activity of both LA and AA can be completely counteracted by the simultaneous treatment of the cells with the thiazolidinedione derivative. These results have been further confirmed in another androgen-independent prostate cancer cell line (PC3).

So far, only inhibitors of 5-LOX activity or compounds that reduce the association of the enzyme with arachidonic acid and FLAP[31,36] have been proposed as effective agents to inhibit fatty acid-induced cancer cell proliferation.[5,11,15–17,37] Our data indicate that RORα1 activating compounds might be considered as possible antagonists of the mitogenic activity of fatty acids on prostate cancer because of their ability to inhibit the 5-lipoxygenase expression.

In previous studies, we have shown that the activation of the orphan nuclear receptor RORα reduces the proliferation of DU 145 cells, both *in vitro* and *in vivo*, through the modulation of the expression of cell cycle-related genes ($p21^{WAF1/CIP1}$, cyclin A).[34] Moreover, CGP 52608 also decreases the metastatic behavior of DU 145 cells by affecting the expression of cell adhesion molecules.[38] These observations indicate that the activation of this receptor might counteract cancer growth through complementary and additive mechanisms: antiproliferative, antimetastatic and antagonistic toward the mitogenic effect of dietary fatty acids. Therefore, RORα might be considered as a possible molecular target for the development of new, and hopefully successful, strategies for prostate cancer chemoprevention or chemotherapy. It is interesting to underline that thiazolidinedione derivatives, such as CGP 52608, by modulating RORα activity, have also been shown to possess strong antiinflammatory and immunostimulatory activities.[27,29,30]

In 1995, Wiesenberg *et al.*[35] indicated melatonin as the possible endogenous ligand for RORα; however, this observation remained controversial. More recently, while analyzing the crystal structure of the ligand binding domain of RORα, Kallen *et al.*[39] have identified cholesterol as the possible endogenous ligand for the nuclear receptor. If confirmed, this finding will certainly improve the understanding of the multiple biological functions of this transcription factor.

The molecular mechanisms through which RORα and specifically the RORα1 isoform, might regulate the expression of 5-LOX are still unknown. It has been shown that the nuclear receptor can interact with either coactivators or corepressors. It has been then proposed that RORα1 can assume 2 different conformations: an active state that promotes binding to coactivator complexes, and a repressive state that allows the interaction with corepressor complexes.[40] Very recently, it has been shown that RORα1 induces *Rev-erbα* gene expression.[41] Rev-erbα, in turn, is known to inter-

act with the nuclear receptor corepressor N-CoR.[42,43] It is then possible that in prostate cancer cells the activated RORα1 might also reclute specific corepressors to inhibit the expression of the 5-LOX gene.

Our data indicate that activation of the orphan nuclear receptor RORα1, which is expressed in prostate cancer cells, decreases the expression of 5-LOX enzyme thus counteracting the mitogenic action of ω-6 polyunsaturated fatty acids on these cells.

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