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ONCOSTATIC ACTIVITY OF A THIAZOLIDINEDIONE DERIVATIVE ON HUMAN ANDROGEN-DEPENDENT PROSTATE CANCER CELLS

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Thiazolidinedione derivatives with potent antiarthritic activity, such as CGP 52608, have been suggested to exert their biological effects through the activation of the orphan nuclear receptor RORα. Since response elements for this receptor are present in the promoter region of cell cycle-related genes (*i.e.* , $p21^{WAF1/CIP1}$ and cyclin A), we reasoned that CGP 52608 might affect cell proliferation, cell cycle progression and the expression of cell cycle-related genes. This hypothesis has been verified in the human androgen-dependent prostate cancer cell line LNCaP. We found that the treatment of LNCaP cells with CGP 52608 brings about a significant and dose-dependent decrease of cell proliferation. Thiazolidinedione affected cell cycle distribution, inducing an accumulation of the cells in the G0/G1 phase and a decrease in the S phase. This effect was accompanied by an increased expression of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ and a decreased expression of cyclin A. These data indicate that, in human androgen-dependent LNCaP prostate cancer cells, the thiazolidinedione derivative CGP 52608 exerts a strong cytostatic activity, by reducing cell proliferation and by affecting cell cycle distribution through the modulation of the expression of cell cycle-related genes. These biological actions of CGP 52608 might be mediated by the activation of the orphan nuclear RORα receptor, which is expressed in LNCaP cells.

Key words: *thiazolidinedione; prostate cancer; cell cycle*

Thiazolidinedione derivatives, such as CGP 52608, have been shown to possess potent antiarthitic activity, as evaluated in experimental arthritis models.[1,2] CGP 52608 also possesses strong immunostimulatory properties, by stimulating interleukin and interferon production from human lymphocytes.[3]

The thiazolidinedione derivative has been suggested to exert its biological actions through the activation of the transcription factor RORα, belonging to the group of orphan nuclear receptors.[1,4]

Orphan nuclear receptors constitute a large subgroup of the superfamily of steroid/thyroid/vitamin D/retinoid receptors, which currently lack known endogenous ligands.[5,6] During the last few years, these receptors have drawn the attention of several scientists because of their deep involvement in the regulation of different physiological processes, such as homeostasis, development, cell growth and differentiation.[5–7] The gene for the human RORα receptor encodes at least four distinct isoforms (RORα-1, -2, -3 and -4) that share common DNA and putative ligand binding domains but possess distinct amino terminal domains.[8–10] Structure/function analysis of the RORα isoforms reveals that they preferentially bind as monomers to an RORα response element (RORE) composed of a 6 bp A/T-rich sequence immediately preceding a half-site core motif PuGGTCA on the promoter of target genes.[9,10] RORE sequences have been identified in the regulatory region of genes involved in the control of developmental and metabolic processes.[11–13] Response elements for RORα have also been shown to be present in the promoter region of cell cycle-related genes, such as those of the cyclin-dependent kinase (CDK) inhibitor[12] $p21^{WAF1/CIP1}$ and cyclin A (Dr. C. Carlberg, personal communication). This observation suggests that the nuclear receptor might be involved in the control of cell proliferation and differentiation and, therefore, might play a role in the mechanisms regulating tumor growth.

On the basis of these observations, we reasoned that CGP 52608, the RORα ligand and activator, might affect the proliferation of tumor cells, by modulating the expression of cell cycle-related genes. This hypothesis has been investigated in a human androgen-dependent prostate cancer cell line (LNCaP). It is actually well known that prostate cancer, which is an androgen-dependent pathology, at least in its early stages, is the most frequent malignancy and the second leading cause of male cancer deaths in the Western countries.[14,15] Understanding the molecular mechanisms regulating the proliferation of prostate cancer cells might help increase the therapeutical options for this pathology.

MATERIAL AND METHODS

Materials

The thiazolidinedione derivative CGP 52608 (1-[3-allyl-4-oxo-thiazolidine-2-ylidene]-4-methyl-thisemicarbazone) was kindly donated by Dr. I. Wiesenberg (Novartis, Basel, Switzerland). This compound has previously been demonstrated to bind and activate the orphan nuclear receptor RORα.[1,4]

Cell culture

The human prostate cancer LNCaP cell line was obtained from American Type Culture Collection (Rockville, MD). LNCaP cells were routinely grown in RPMI-1640 medium (Seromed Biochrom, Berlin, Germany) supplemented with 10% 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. In these culture conditions, the duplication time of LNCaP cells is 48 hr.

RT-PCR for RORα

To determine whether RORα receptors are expressed in prostate cancer cells, RNA extraction and RT reactions were performed as previously described. Total RNA (1 μg) was used in the reverse transcription (RT) reaction. Samples containing cDNAs were then amplified by polymerase chain reaction (PCR) in the presence of a pair of specific oligonucleotide primers. Thirty-five amplification cycles were performed in a programmable heat block (Perkin-Elmer Cetus, Norwalk, CT) (1 min of denaturation at 94°C, 45 sec primer annealing at 45°C and 2 min primer extension at 72°C). The primers were: 5′-AACGGCGAGACTTCCCCAACTG-3′ (sense, 637–658) and 5′-CAAAGGCACGGCACACTCTGA-TAA-3′ (antisense, 950–973).[16] After RT-PCR, the amplified DNA products were separated on a 1.5% agarose gel and stained with ethidium bromide. Southern blot was performed and blots

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were hybridized with a synthetic ^{32}P-labeled oligonucleotide probe: 5′-GGGAGCTGATGTGGCAATTG-3′ (803–822).

Proliferation studies

For proliferation studies, LNCaP cells were plated at a density of 1.4×10^3 cells/plate in RPMI-1640 medium supplemented with 10% FBS. Twenty-four hours after plating, cells were treated with CGP 52608 (10^{-11}–10^{-6} M) for 7 days. At the end of the treatment, cells were harvested and counted by hemocytometer.

FACS analysis

Fluorescence-activated cell sorting (FACS) analysis was performed as described.[17] LNCaP cells were plated at a density of 8×10^3 cells/cm^2 in 100 mm dishes in RPMI-1640 supplemented with 10% FBS. Twenty-four hours after plating, the medium was changed to RPMI-1640 without FBS. Twelve hours later, cells were treated either with vehicle or with CGP 52608 (either 10^{-9} or 10^{-7} M) for 48 hr. After the treatment, LNCaP cells were trypsinized, washed twice with ice-cold PBS, fixed by drop-wise addition of 70% ethanol and incubated at 4°C overnight with constant agitation. Thirty minutes before flow cytometric analysis, the cellular double-stranded nucleic acids were stained with propidium iodine (50 μg/ml). RNAse (100 U/ml) was included to degrade double-stranded RNA. Propidium iodine fluorescence was obtained using linear amplification with doublet discrimination. Five thousand forward scatter gated events were collected per sample. Data were analyzed by the Cellquest program (Becton-Dickinson, San Jose, CA).

RT-PCR for p21$^{WAF1/CIP1}$ and cyclin A

In preliminary experiments, RT-PCR was utilized to verify the expression of the mRNAs for p21$^{WAF1/CIP1}$, cyclin A and β-actin (as an internal standard) in LNCaP cells. After phenol-chloroform extraction, 1 μg of total RNA from LNCaP cells was used in a reverse transcription reaction. cDNA synthesis was performed using the Gene AMP kit (Perkin Elmer Cetus), 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 pmole of a pair of specific primers and 2.5 U Taq Polymerase. Primers were as follows: p21$^{WAF1/CIP1}$ sense 5′-GTGAGCGATGGAACTTCGA-3′ and antisense 5′-AATCTGTCATGCTGGTCTGC-3′;[18] cyclin A sense 5′-ATTAGTTTACCTGGACCCAG-3′ and antisense 5′-CACAAACTCTGCTACTTCTG-3′;[19] β-actin sense 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ and antisense 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′.[20]

PCR amplification was performed for 35 cycles (1 min denaturation at 94°C, 45 sec primer annealing at 60°C and 2 min primer extension at 72°C). cDNA products were separated on 1.5% agarose gel and stained with ethidium bromide. To confirm the specificity of the cDNAs obtained, RT-PCR products were Southern blotted and blots were hybridized with ^{32}P-labeled oligonucleotide probes, specific for the cDNA sequences of p21$^{WAF1/CIP1}$, cyclin A and β-actin, respectively.

To choose the adequate number of cycles for amplification of p21$^{WAF1/CIP1}$, cyclin A and β-actin cDNAs, we performed RT-PCR on RNA extracted from LNCaP cells using different numbers of cycles from 15 to 30. 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 ligand-induced activation of RORα on the expression of cell cycle-related genes, LNCaP cells were plated in 100 mm dishes at a density of 15×10^3 cells/cm.^{2} Twenty-four hours after plating, cells were treated with CGP 52608 (either 10^{-9} or 10^{-7} M) for 3, 6, 12, 24 or 36 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 p21$^{WAF1/CIP1}$, cyclin A and β-actin. After RT-PCR, the relative intensities of the cDNA bands were quantitated by computer-assisted densitometric scanning using the Image program, and p21$^{WAF1/CIP1}$ and cyclin A cDNA levels were standardized to β-actin cDNAs at the corresponding time intervals.

Statistical analysis

Statistical comparisons of the data were performed using Dunnett's test[21] after one-way analysis of variance.

RESULTS

Expression of RORα in LNCaP cells

By RT-PCR experiments, we demonstrated that the orphan nuclear receptor RORα is expressed in LNCaP cells, since the predicted 337 bp cDNA fragment could be amplified and specifically hybridized with the ^{32}P-labeled oligonucleotide probe (Fig. 1, lane 1). The same fragment could be amplified in RNA from human lymphocytes (Fig. 1, lane 2, positive control). The fragment was not present in samples in which the reverse transcriptase was omitted (Fig. 1, lane 3).

Effects of CGP 52608 on LNCaP cell proliferation

Exponentially growing LNCaP cells were treated daily, for 7 days, with CGP 52608 (10^{-11}–10^{-6} M). The treatment resulted in a dose-dependent inhibition of tumor cell growth (Fig. 2), which was particularly significant on the 7th day of treatment. The thiazolidinedione derivative was significantly effective from 10^{-10} to 10^{-6} M, with a half-maximal response at approximately 5×10^{-9} M.

Effects of CGP 52608 on cell cycle distribution of LNCaP cells

Experiments have been performed to clarify whether CGP 52608 might affect LNCaP cell cycle distribution. To this purpose, synchronized LNCaP cells were submitted to FACS scan analysis. We have observed that the thiazolidinedione derivative, at both 10^{-9} and 10^{-7} M, significantly increases the number of the cells in G0/G1 phase while decreasing that of the cells in S phase (Table I). Lower doses of the compound were found to be ineffective (data not shown). These results indicate that CGP 52608 induces cell cycle withdrawal in LNCaP cells.

Effects of CGP 52608 on the expression of cell cycle-related genes in LNCaP cells

ROREs have been identified in the regulatory region of p21$^{WAF1/CIP1}$ and cyclin A, two cell cycle-related genes. These studies have been performed to verify whether CGP 52608 might affect the expression of these genes.

In preliminary experiments, we carried out RT-PCR in the presence of oligonucleotide primers for p21$^{WAF1/CIP1}$, for cyclin A and for β-actin (used as internal standard). cDNA products of 311,

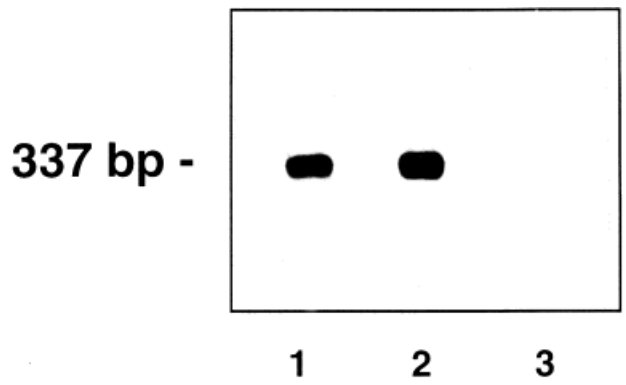

FIGURE 1 – RT-PCR analysis of RORα expression in LNCaP cells. The 337 bp amplified cDNA fragment specifically hybridized with the ^{32}P-labeled RORα oligonucleotide probe (lane1). Lane 2, human lymphocytes; lane 3, no reverse transcriptase.

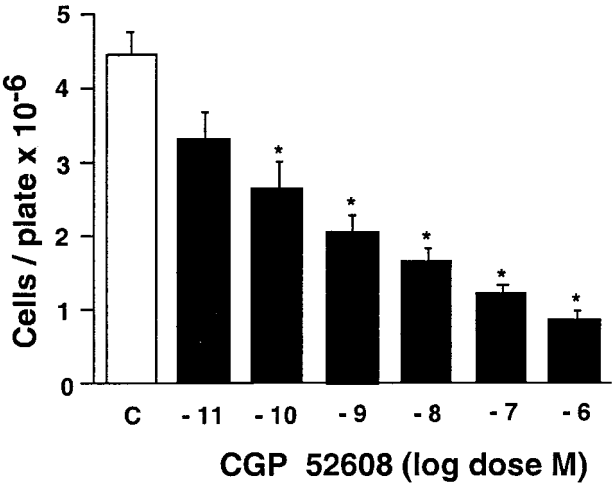

FIGURE 2 – Inhibition of LNCaP cell growth by CGP 52608. Each experimental group consisted of seven replicates. Each experiment was repeated three times with identical results. Data are expressed as the mean cell number/plate ± SEM. *, $p < 0.05$ *vs.* controls (C).

TABLE I – EFFECTS OF CGP 52608 ON CELL CYCLE DISTRIBUTION OF LNCaP CELLS

	Controls	CGP 52608 (10^{-9} M)	CGP 52608 (10^{-7} M)
G0/G1	53.7 ± 0.6	59.7 ± 2.4*	65.2 ± 1.2*
S	25.2 ± 1.2	15.1 ± 2.9*	12.2 ± 0.58*
G2/M	22.5 ± 1.5	27.4 ± 1.3	23.7 ± 1.2

*$p < 0.05$ vs. controls.

433 and 660 bp were obtained as expected according to the primers used, for the three genes, respectively (Fig. 3*a*). After Southern blotting, the cDNAs hybridized with ^{32}P-labeled oligonucleotide probes, confirming the specificity of the amplified products (Fig. 3*b*, lanes 1, 2 and 3).

RT-PCR experiments were then conducted to choose the adequate number of cycles for each gene. The intensity of each band increased in parallel with the increasing number of PCR cycles (data not shown) We decided the adequate number of PCR amplification cycles for $p21^{WAF1/CIP1}$ as 20 cycles, for cyclin A as 25 cycles and for β-actin as 20 cycles.

To study the effects of CGP 52608 on the expression of cell cycle-related genes, LNCaP cells were treated with the compound (either 10^{-9} or 10^{-7} M) for different time intervals, from 3 to 36 hr. At the end of the treatment, RNAs were extracted and RT-PCR was performed at adequate cycles. We found that, in controls cells, $p21^{WAF1/CIP1}$ was slightly expressed at all time intervals (Fig. 4*a*). CGP 52608, at both doses, increased the expression of $p21^{WAF1/CIP1}$ at 6 and 12 hr (Figs. 4*a*, 5*a*). When the data were submitted to a proper statistical evaluation, it appeared that this increased expression of the gene was highly significant. There were no significant effects at 3, 24 and 36 hr. In control cells, cyclin A mRNA levels remained at basal levels until 3 hr and then markedly increased (Fig. 4*b*). CGP 52608 induced a significant decrease of cyclin A mRNA levels at 6 hr when utilized at the dose of 10^{-9} M and at 6 and 12 hr when utilized at a higher concentration (10^{-7} M) (Figs. 4*b*, 5*b*). It is interesting that the highest dose had a longer effect, suggesting a more prolonged occupancy of the receptors. As expected, the levels of β-actin mRNA were not affected by the treatment at any time interval considered (Fig. 4*c*).

DISCUSSION

The results reported here show that the thiazolidinedione derivative CGP 52608 exerts an oncostatic action on the human androgen-dependent prostate cancer cell line LNCaP. Additional data have shown that the drug used induces arrest of the cells in the G0/G1 phase of the cell cycle. The activity of the compound on cell cycle progression is accompanied by a modification of the expression of cell cycle-related genes. The CDK inhibitor gene $p21^{WAF1/CIP1}$ is well known to counteract the activity of the cyclin D/CDK4 complex, which is crucial for the progression from G1 to S phase.[22] Cyclin A, which starts being synthesized at the end of the G1 phase, appears abundantly at the G1/S transition period and triggers the S phase.[22,23] Our data show that CGP 52608 increases the expression of $p21^{WAF1/CIP1}$, thus blocking G1/S progression, and decreases the expression of cyclin A, thus preventing the triggering of the S phase. Therefore, in LNCaP cells, CGP 52608 seems to induce growth arrest and cell cycle withdrawal, at least partially, through a modulation of the expression of genes that are deeply involved in the regulation of the cell cycle. Preliminary experiments performed in our laboratory indicate that CGP 52608 develops a similar antiproliferative action on androgen-independent DU 145 prostate tumor cell line, suggesting that, in prostate cancer, the antitumoral property of this compound might not be related to the androgen-receptor system.

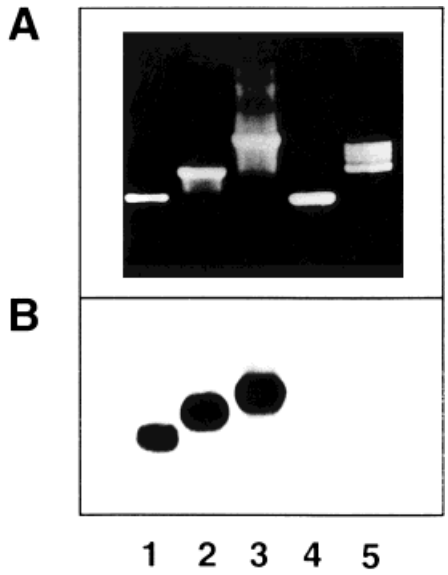

FIGURE 3 – RT-PCR analysis of the expression of $p21^{WAF1/CIP1}$, cyclin A and β-actin in LNCaP cells. (*a*) Ethidium bromide-stained agarose gel of the amplified cDNAs. (*b*) Autoradiography of the Southern blot obtained from the gel shown in (*a*) after hybridization with specific ^{32}P-labeled oligonucleotide probes. Lane 1, $p21^{WAF1/CIP1}$; lane 2, cyclin A; lane 3, β-actin; lane 4, RT-PCR control (308 bp); lane 5, DNA molecular weight marker IV (Roche Diagnostic, Monza, Italy).

The oncostatic activity of CGP 52608 on prostate cancer cells described here is in line with the observations reported by other authors. Karasek and coworkers[24] have shown that CGP 52608 inhibits the growth of the murine colon 38 adenocarcinoma, both in vitro and in vivo. More recently, this thiazolidinedione derivative has been reported to decrease the proliferation of an ovarian carcinoma cell line.[25]

The molecular mechanisms through which CGP 52608 exerts its antitumoral activity have not been fully clarified so far. However, as quoted in the introduction, this compound, and the specific class of thiazolidinediones to which it belongs, have been demonstrated to bind and activate the orphan nuclear receptor RORα,[1,4] independently of whether this receptor is or not the physiological melatonin receptor.[5]

The data reported here apparently support this hypothesis. RORα is expressed in LNCaP cells, and CGP 52608 modifies the

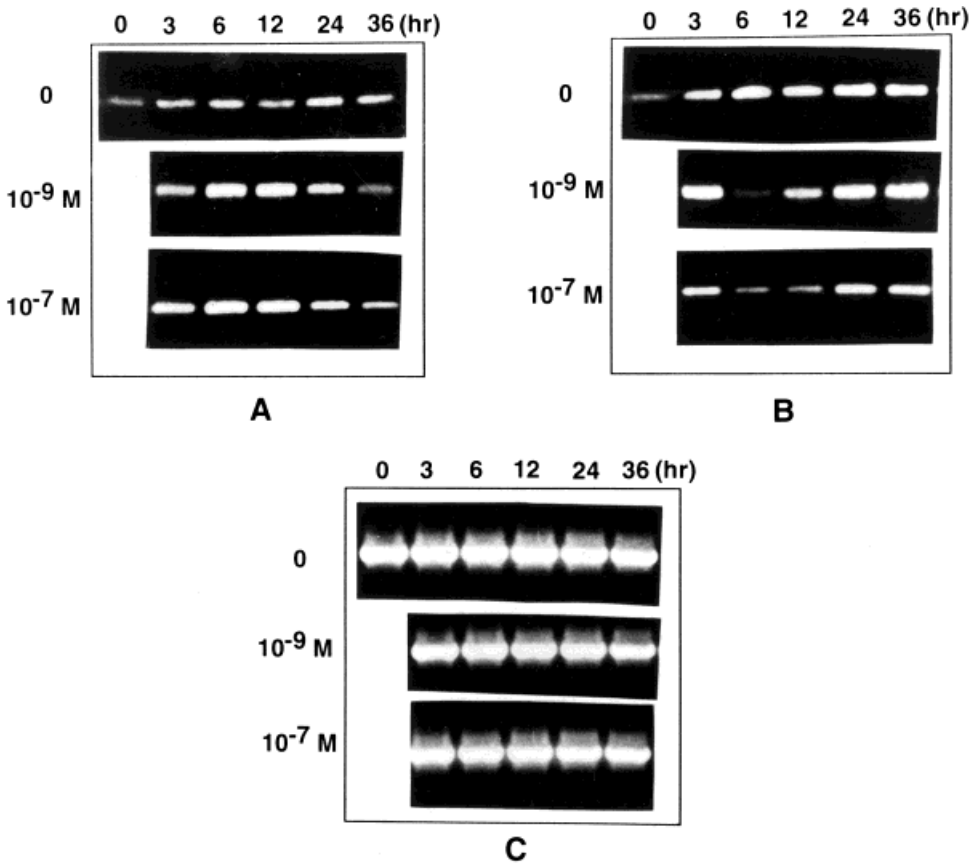

FIGURE 4 – Effects of CGP 52608 on cell cycle-related gene expression in LNCaP cells. Cells were treated with CGP 52608 (10^{-9} M, middle lane, and 10^{-7} M, lower lane) or with vehicle alone (upper lane). Total RNA was extracted at the indicated times and analyzed by RT-PCR at the appropriate cycles as described in Material and Methods for $p21^{WAF1/CIP1}$ (*a*), cyclin A (*b*) and β-actin (*c*). One representative of three experiments is reported.

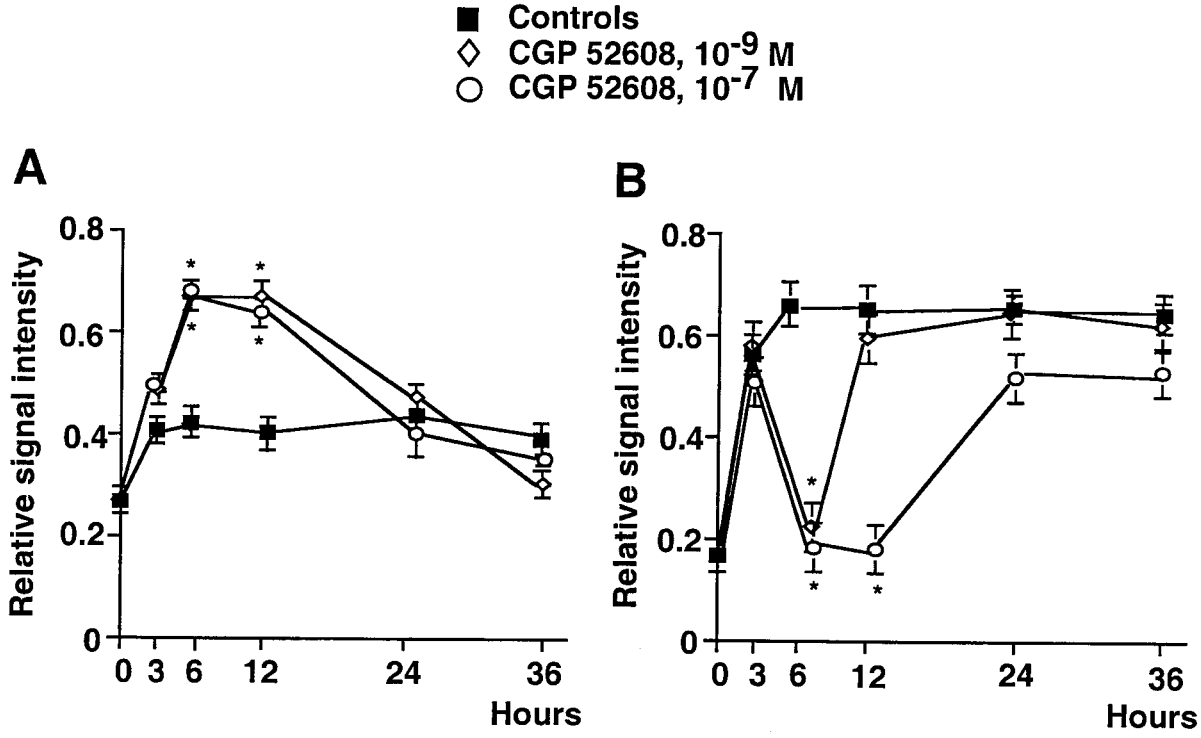

FIGURE 5 – Quantitative analysis of the effects of CGP 52608 on cell cycle-related gene expression in LNCaP cells. Relative intensities of the bands shown in Figure 4 were quantitated by computer-assisted densitometric scanning using the Image program, and $p21^{WAF1/CIP1}$ (*a*) and cyclin A (*b*) cDNA levels were standardized to β-actin cDNAs at the corresponding time intervals. *, $p < 0.05$ *vs.* controls at the corresponding time intervals.

expression of cell cycle-related genes, like $p21^{WAF1/CIP1}$ and cyclin A; these genes have been demonstrated to carry a RORα response element in their promoter region. Therefore, it is possible that modification of the expression of these genes induced by CGP 52608 might be mediated by the activation of RORα. In further support of this hypothesis, preliminary experiments performed in our laboratory indicate that, in prostate cancer cells, CGP 52608 represses the expression of 5-lypoxygenase (Moretti et al., unpublished observations), another gene carrying a RORE in its promoter region.[11,12] 5-Lypoxygenase is an enzyme that is deeply involved in the regulation of lipid metabolism and in allergic and inflammatory reactions.[26] Our unpublished experiments confirm previous reports showing that both the antiinflammatory activity and the immunostimulatory properties of CGP 52608 are mediated by RORα activation.[1–3] Taken together, these observations point to the suggestion that the biological actions of CGP 52608 might involve the activation of RORα.

In the last few years, another group of thiazolidinediones, such as trioglitazone, pioglitazone and BRL 49653, has attracted the interest of the scientific community. These compounds are well known for their ability to improve insulin resistance by enhancing insulin action in target tissues and, therefore, for representing the prototypes of novel therapeutic agents for the treatment of non-insulin-dependent diabetes mellitus.[27,28] The antidiabetic activity of these drugs has been shown to be mediated by a receptor that shares some properties of RORα, *e.g.*, PPARγ, another transcription factor belonging to the orphan nuclear receptor family.[28,29] Interestingly, PPARγ has been reported to be involved not only in metabolic processes, but also in the control of cell proliferation and differentiation, suggesting its possible role in the regulation of tumor growth.[30–33] These observations further confirm the notion that orphan nuclear receptors, like the two just mentioned, are deeply involved in the control of several physiological and/or pathological processes and underline the relevance of the identification of specific ligands, either endogenous or pharmacological, for these receptors.

In conclusion, the thiazolidinedione derivative CGP 52608 induces cell cycle arrest and altered expression of cell cycle-related genes in androgen-dependent LNCaP prostate cancer cells. Combinations between cytostatic and more classical cytotoxic agents are currently under study in clinical settings due to the encouraging results obtained in preclinical models.[34] CGP 52608 and related compounds might then be considered as possible agents with cytostatic activity. The issue deserves further investigation.

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