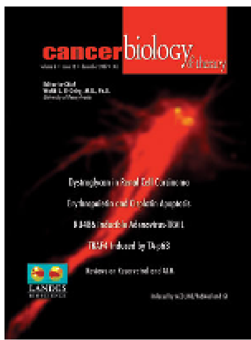

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Research Paper

Alteration of Glyoxalases Gene Expression in Response to Testosterone in LNCaP and PC3 Human Prostate Cancer Cells

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KEY WORDS

glyoxalase system, testosterone, prostate cancer, LNCaP, PC3, oxidative stress, cell proliferation, bioinformatics, estrogens

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ABSTRACT

Glyoxalase system, a ubiquitous detoxification pathway protecting against cellular damage caused by potent cytotoxic metabolites, is involved in the regulation of cellular growth. Aberrations in the expression of glyoxalase genes in several human cancers have been reported. Recently, we described a possible regulatory effect by estrogens on glyoxalase genes in human breast cancer cell lines. This result, along with those ones regarding changes in glyoxalases activity and expression in other human hormone-regulated cancers, such as prostate cancer, has prompted us to investigate whether also androgens, whose functional role in prostate cancer pathogenesis is well known, could modulate glyoxalases gene expression. Therefore, we treated LNCaP androgen-responsive and PC3 androgen-independent human prostate cancer cell lines with testosterone at the concentrations of 1 nM and 100 nM. After a two days treatment, glyoxalases mRNA levels as well as cell proliferation were evaluated by real-time RT-PCR analysis and [^{3}H]thymidine incorporation, respectively. Results pointed out that testosterone affects the expression of glyoxalase system genes and cell proliferation in a different manner in the two cell lines. The possibility that modulation of glyoxalase genes expression by testosterone is due to glyoxalases-mediated intracellular response mechanisms to the androgen-induced oxidative stress or to the presence of androgen response elements (ARE) in glyoxalase promoters are discussed. Knowledge regarding the regulation of glyoxalases by testosterone may provide insights into the importance of these enzymes in human prostate carcinomas in vivo.

ABBREVIATIONS

PCa, prostate cancer; GI, Glyoxalase I; GII, Glyoxalase II; MG, methylglyoxal; SLG, S-D-lactoylglutathione; MDA, malondialdehyde; AGEs, advanced glication end products; T, testosterone; ARs, androgen receptors; AREs, androgen response elements; L, letrozole; FBS, foetal bovine serum; E_2, 17β-estradiol; PBS, phosphate-buffered saline; ATCC, American Type Culture Collection; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; CSS, charcoal-stripped fetal bovin serum.

INTRODUCTION

Glyoxalase I (GI) together with glyoxalase II (GII) constitutes the glyoxalase system, a ubiquitous detoxification pathway protecting against cellular damage caused by potent cytotoxic metabolites, as methylglyoxal (MG). MG is converted to S-D-lactoylglutathione (SLG) by GI with reduced glutathione as a cofactor, and SLG in turn is hydrolyzed to D-lactate along with regeneration of reduced glutathione by GII.[1]

Methylglyoxal is a physiological substrate, derived from glycolysis, via degradation of triose phosphate intermediates, lipid peroxidation, threonine degradation, fragmentation of glycated proteins[2] and enzymatic isomerization from malondialdehyde (MDA).[3] As a highly reactive metabolite, MG has a strong ability to cross-link with protein amino groups to form stable products called advanced glycation end products (AGEs) and to attack guanine residues of DNA leading to DNA glycation.[4] The cytotoxicity of MG is due to its mutagenic and antiproliferative properties and to its ability to trigger apoptosis,[5,6] apparently via oxidative signalling.[7]

It has been demonstrated that also LSG is able to exert a cytostatic action on cell growth by modulating microtubule assembly.[8]

As known, the glyoxalase system is involved in the regulation of cellular growth.[9,10] Abnormal expression of this system has been demonstrated in a number of cellular disorders, including cancer.[11-15] In particular, altered expression and activities of GI and GII have been documented in tumor urogenital tissues[12-17] and in prostate tumor cell lines compared with corresponding normal tissues.[18] These alterations are considered to be crucial for sustaining tumor viability/survival under an altering microenvironment with tumor growth.

We recently described a possible regulatory role of estrogens on gene expression of glyoxalase system enzymes in MCF7 and BT20 human breast cancer cells.[19]

The observation of such a possible regulatory effect has prompted us to investigate whether other hormones, in addition to estrogens, could modulate glyoxalases gene expression. Besides, currently, little is known about factors pertinent to regulation of glyoxalase system.[19,20] In particular, we focused on the prostate gland for at least two reasons. Firstly, a distinguishing feature of prostate gland is an intimate association with androgens, essential molecules for its development, growth and functions. The androgen-dependence of prostate cancer (PCa) is also well documented.[21,22] Secondly, PCa is the leading cancer affecting men in the western world and we believe that knowledge regarding the regulation of glyoxalase by androgens in this tumor, may provide insights into the importance of these enzymes in human prostate carcinomas in vivo.

As known, testosterone (T), the main circulating androgen, stimulates or inhibits target gene transcription, via nuclear androgen receptors (ARs)/androgen response elements (AREs) complexes. Coactivators and corepressors interact with ARs/AREs complexes and the general transcription apparatus to make T perform or enhance its biological function.[23]

On the other hand, T can be converted into estrogens by aromatase. The local production of estrogens in the prostate has been hypothesized to influence prostate growth and possible incidence of PCa, via their own receptors (ER).[24]

In order to verify a possible regulatory role of androgens on glyoxalases gene expression, we treated the human androgen-dependent LNCaP or androgen-independent PC3 prostate cancer cell lines with T for two days and evaluated GI and GII gene expression profiles (mRNA levels), as well as cell proliferation after the treatment. To ascertain that the observed biological effects were really due to T, we also performed experiments in combination with the aromatase inhibitor letrozole (L), that is capable of blocking the aromatase activity for the transformation of androgens into estrogens.

Finally, to confirm the possible influence of estrogens on both cell lines growth and glyoxalases gene expression modulation, suggested by T and L treatments, we exposed them to 17β-estradiol.

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MATERIALS AND METHODS

Materials. RPMI 1640 media, foetal bovine serum (FBS), L-glutamine, sodium pyruvate, non-essential amino acids, penicillin and streptomycin were purchased from Euroclone, UK. Testosterone (T), 17β-Estradiol (E_2) and phosphate-buffered saline (PBS) were from Sigma-Aldrich, USA. Letrozole (L) was a kind gift from Novartis, Basel, Switzerland. All the chemicals used in the present study were analytical grade reagents from various sources.

Cell culture and hormone/inhibitor treatments. LNCaP and PC3 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). LNCaP cells represent androgen- and estrogen-responsive human prostate cancer cells, expressing both the androgen receptor (AR) and the β isoform of the estrogen receptor (ERβ). PC3 cell line represents androgen-independent human prostatic cancer cells, expressing both the endogenous estrogen receptors α (ERα) and β (ERβ). Both cell lines were maintained routenly at 37°C in 5% CO_2 in RPMI 1640 supplemented with 10% heat inactivated (1 h at 56°C) FBS, 1X L-glutamine, 1 mM sodium pyruvate, 1X non-essential amino acids, 100 units/ml of penicillin and 0.1 mg/ml of streptomycin. LNCaP and PC3 cells were seeded into 6-well plates at a density of 10^5 cells per well and cultured in whole serum. Subsequently, to deplete endogenous steroids, the medium was changed with another containing 10% charcoal-stripped fetal bovin serum (CSS),[25] three days before treatments. Stock solutions of T, E_2 and L were prepared in ethanol. The same ethanol concentration (0.05%) was used in controls and ligand-treated samples. At subconfluence, cells were incubated with either vehicle (controls) and T or T and L in combination, vehicle (controls) and E_2 for two days at the concentrations shown in the appropriate figures. The used L concentrations were selected on the basis of our earlier observations from preliminary experiments that showed almost total aromatase inhibition associated with neither toxic effects on cells nor inhibitory action on their growth. Four independent cultures of PC3 were prepared and assayed in quadruplicate.

Cell proliferation studies. Cell number was determined by [^{3}H]thymidine incorporation assay.[26]

RNA isolation and real-time RT-PCR analysis. Total cellular RNA was isolated using TRIzol Reagent and following the manufacturer's instructions. cDNA was then synthesized from 1 μg of RNA using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) and random primers System (Gibco), according to the manufacturer's instructions. GI and GII mRNA expression was detected by Real-Time TaqMan PCR analysis on a LightCycler instrument (MX3005P System, Stratagene, La Jolla, CA). The sequences of oligonucleotide primers and TaqMan probes for GI, GII and β-actin (the housekeeping gene chosen for normalization) as well as the PCRs conditions used in this study are listed in Table 1. The amplification reactions were performed in quadruplicate for each sample. For comparative analysis of gene expression, data were obtained using the $\Delta\Delta C_t$ method.[27]

Bioinformatics analysis. Glyoxalases genomic sequences were identified from the National Centre for Biotechnology Information (NCBI) internet site (www.ncbi.nih.nim.gov), (GI: BC015934, GII: BC002627). The sequences of both genes were screened for androgen response elements (AREs) using the online PROMO program (www.lsi.upc.es/~alggen),[28,29] against a gene bank collecting nucleotide sequences that have been experimentally proved to be targets for androgens.

Statistical analysis. The statistical significance of differences between treated and untreated cells was assessed by analysis of variance. Experimental data were compared also by Student's t-test. All numerical data reported are expressed as the mean ± standard error (SE). Differences between groups were considered significant when $p < 0.05$.

Table 1 **Primers, Probes sequences for TaqMan RT-PCR Analysis and PCRs conditions**

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Probe (5′-3′)
GI	CTCTCCAGAAAAGCTACACTTTGAG (400 nM)	CGAGGGTCTGAATTGCCATTG (400 nM)	TGGGTCGCATCATCTTCAGTGCCC * (200 nM)
GII	AGAAAGCACGGGGTGAAACTG (200 nM)	TACACCTTCAGTCCCGACTCC (200 nM)	TGGGTCGCATCATCTTCAGTGCCC ** (200 nM)
βActin	CACTCTTCCAGCCTTCCTTCC (600 nM)	ACAGCACTGTGTTGGCGTAC (600 nM)	TGCGGATGTCCACGTCACACTTCA*** (200 nM)
Cycles	1 cycle: 95°C for 10 min	45 cycles: 95°C for 20 s 55°C for 1 min	

*Labeled with FAM fluorochrome; **Labeled with HEX fluorochrome; ***Labeled with TexasRed fluorochrome.

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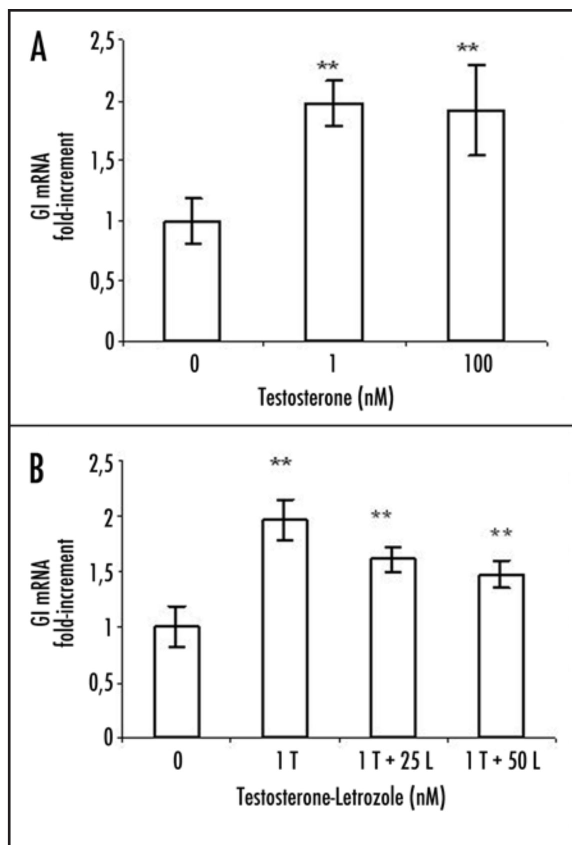

Figure 1. Effects of Testosterone (T) on glyoxalase I (GI) expression of the androgen-responsive human prostate cancer LNCaP cell line. Test agent was added either alone (A) or simultaneously with letrozole (L) (B), for two days in charcoal-stripped serum (CSS) medium. mRNA levels were measured by real-time RT-PCR analysis. Cell cultured without T and L served as negative controls. All the data are given as fold-increment related to negative controls. Each value is the mean ± SD of quadruplicate experiments. Significant differences from non-stimulated cells are denoted by two asterisks ($p < 0.001$).

RESULTS

Effect of testosterone (T), added singly or in combination with the aromatase inhibitor letrozole (L), on GI mRNA expression in LNCaP cells. To verify whether the T hormone regulation of GI takes place at a transcriptional level, the expression of GI mRNA was analyzed by real-time RT-PCR. The results of experiments carried out using T alone or together with L on GI mRNA levels, are shown in Figure 1A and B, respectively. Increasing concentrations of T (1 nM and 100 nM) strongly upregulated GI mRNA levels at both the used doses, maximum effect (about 2-fold) being at 1 nM (Fig. 1A). Experiments by combined treatment with L (1 nM T + 25 nM L and 1 nM T + 50 nM L), however, seem to indicate that induction of GI gene expression may be due to either T or E_2, with a more marked effect by T and a slight one by E_2 (Fig. 1B). Similar results were obtained when cells were treated with T at the concentration of 100 nM with 25 nM and 50 nM L, respectively (data not shown). At the used concentrations, L alone did not exert any significant effect on GI mRNA expression (data not shown).

Effect of testosterone (T), added singly or in combination with the aromatase inhibitor letrozole (L), on GII mRNA expression in LNCaP cells. Following a two-day T exposure (1 nM and 100 nM), a significant ($p < 0.001$) and consistent dose-dependent decrease (1.8- and 2.3-fold, respectively) in GII mRNA levels was observed (Fig. 2A). T and L combination experiments indicate that this result is likely due to estrogens, converted from T by aromatase, and that T, conversely, would increase GII gene expression (Fig. 2B). Similar results were obtained when cells were treated with T at the concentration of 100 nM with 25 nM and 50 nM L, respectively (data not shown). At the used concentrations, L alone did not exert any significant effect on GII mRNA expression (data not shown).

Effect of testosterone (T), added singly or in combination with the aromatase inhibitor letrozole (L), on cell proliferation in LNCaP cells. To investigate the effects of T on the growth of LNCaP cells, [^{3}H]thymidine incorporation was measured in LNCaP cells pre-cultured for three days in steroid-deprived medium. As shown in (Fig. 3A), after two-day exposure to different T concentrations (1 nM and 100 nM), without the non-steroidal aromatase inhibitor letrozole (L), the growth of LNCaP cells was significantly increased in the presence of all T doses, with a stimulation peak of over 6-fold above the control value with the 1 nM dose ($p < 0.001$). The results of separate experiments carried out in the presence of both T (1 nM) and L (25 nM and 50 nM), however, would suggest that the observed increase in LNCaP proliferation after T exposure, may be due to the steroid hormone estradiol (E_2), derived from aromatase conversion activity in cells, rather than directly to T. On the contrary, it appears that T would induce a significant ($p < 0.001$) inhibition of cell growth (Fig. 3B). Comparable results were obtained when cells were treated with T at the concentration of 100 nM with 25 nM and 50 nM L, respectively (data not shown). At the used concentrations, L alone did not exert any significant effect on cell proliferation (data not shown).

Effect of 17β-Estradiol (E2) on cell proliferation, GI and GII mRNA expression in LNCaP cells. To confirm the possible role

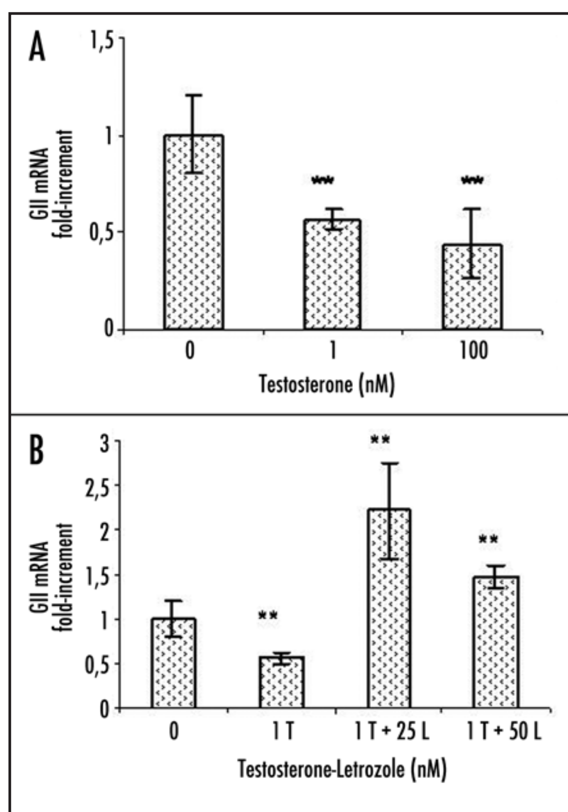

Figure 2. Effects of Testosterone (T) on glyoxalase II (GII) expression of the androgen-responsive human prostate cancer LNCaP cell line. Test agent was added either alone (A) or simultaneously with letrozole (L) (B), for two days in charcoal-stripped serum (CSS) medium. mRNA levels were measured by real-time RT-PCR analysis. All the data are given as fold-increment related to negative controls (represented by non-stimulated cells). Each value is the mean ± SD of quadruplicate experiments. **Significantly different from the negative control ($p < 0.001$).

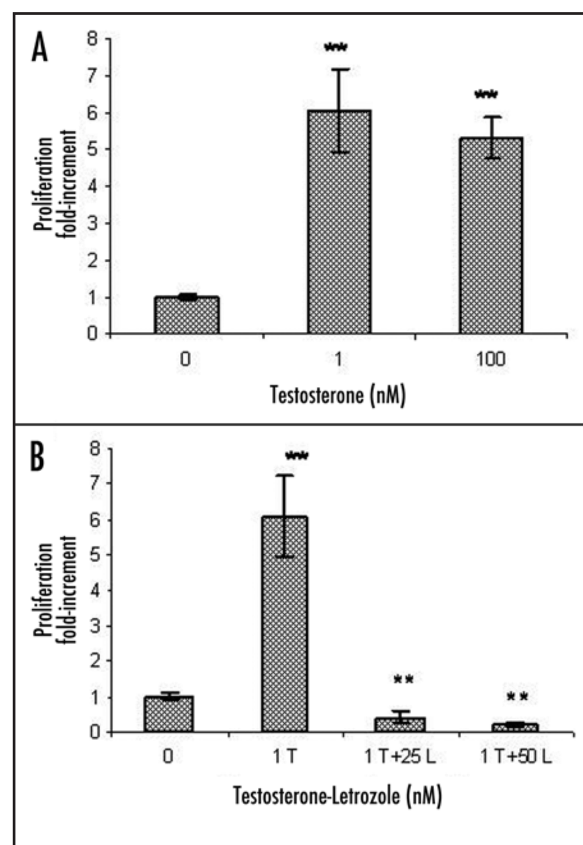

Figure 3. Effects of Testosterone (T) on proliferation of the androgen-responsive human prostate cancer LNCaP cell line. Cells were incubated with T alone (Panel A) or in combination with the aromatase inhibitor Letrozole (L) (Panel B), for two days in charcoal-stripped serum (CSS) medium. Proliferation was evaluated by [^{3}H]thymidine incorporation assay. Cell cultured without T and L served as negative controls. All the data are given as fold-increment related to negative controls. Each value is the mean ± SD of quadruplicate experiments. Significant differences from non-stimulated cells are denoted by two asterisks ($p < 0.001$).

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of estrogens in the regulation of cell proliferation, as well as in GI and GII mRNA expression in LNCaP cells, suggested by T and L treatments, we exposed LNCaP to E_2 (1 nM and 100 nM). Results indicate that E_2 would significantly ($p < 0.01$) stimulate LNCaP cell growth by upregulating GI gene transcription (in a combinatory effect with T) and downregulating GII expression (Fig. 4).

Effect of testosterone (T), added singly or in combination with the aromatase inhibitor letrozole (L), on GI mRNA expression in PC3 cells. To verify whether the T hormone regulation of GI takes place at a transcriptional level, the expression of GI mRNA was analyzed by real-time RT-PCR. The results of experiments carried out using T alone or together with L on GI mRNA levels are shown in Figure 5A and B, respectively. Increasing concentrations of T (1 nM and 100 nM) significantly upregulated GI mRNA levels at both the used concentrations (Fig. 5A). Experiments by combined treatment with L (1 nM T + 25 nM L and 1 nM T + 50 nM L) seem to indicate that induction of GI gene expression is really due to T (Fig. 5B). Similar results were obtained when the cells were treated by 100 nM T with 25 nM and 50 nM L, respectively (data not shown). At the used concentrations, L alone did not give any significant effect on GI mRNA expression (data not shown).

Effect of testosterone (T), added singly or in combination with the aromatase inhibitor letrozole (L), on GII mRNA expression in PC3 cells. Following a two-day T exposure (1 nM and 100 nM), a significant ($p < 0.001$) increase in GII mRNA levels was observed at the lower dose of 1 nM T (Fig. 6A). Conversely, at the higher dose of 100 nM T, GII gene expression showed a significant decrease below the control (Fig. 6A). T and L combination experiments point out that the increased GII transcript amount is likely due to estrogens, converted from T by aromatase, and that T, conversely, would decrease GII gene expression (Fig. 6B). Comparable results were obtained when cells were treated with T at the concentration of 100 nM with 25 nM and 50 nM L, respectively (data not shown). At the used concentrations, L alone did not exert any significant effect on GII mRNA expression (data not shown).

Effect of testosterone (T), added singly or in combination with the aromatase inhibitor letrozole (L), on cell proliferation in PC3 cells. To investigate the effects of T on the growth of PC3 cells, [^{3}H]thymidine incorporation was measured in PC3 cells pre-cultured for three days in steroid-deprived medium. As shown in Figure 7A, after two-day exposure to different T concentrations (1–100 nM), without the aromatase inhibitor, letrozole (L), the growth of PC3 cells was significantly ($p < 0.001$) decreased in the presence of the minimum dose of T (1 nM), being almost halved above the control

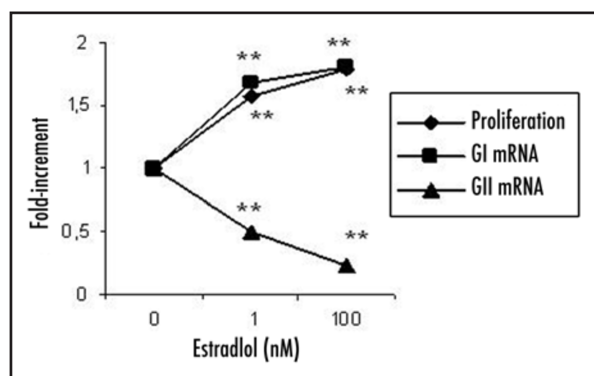

Figure 4. Effects of 17β-Estradiol (E_2) on cell proliferation, glyoxalase I (GI) and glyoxalase II (GII) gene expression in LNCaP cells. **Significantly different from the negative control, represented by non-stimulated cells ($p < 0.001$).

Table 2 **Predicted DNA sequences of androgen response elements (AREs) on GI and GII promoters and related similarity scores to the canonic ARE motif (5'-ggTACAnnnTgTTCT-3')**

Gene	Sequence Number	Sequence	Similarity Score
GI	(1)	5'- ttGgtCAggcTGgTCTc-3'	8/12
	(2)	5'- GGTAgAgaaTGTTCc-3'	11/12
	(3)	5'- GaAtCtgctTGTTCa-3'	8/12
	(4)	5'- gAGAcCAgccTGgcCaa-3'	8/12
	(5)	5'- AGAACAtcatcGcTCT-3'	10/12
	(6)	5'- ggaactaacagatcc-3'	9/12
	(7)	5'- agtactgcaagttat-3'	8/12
	(8)	5'- agGGTcacttcaGccCa-3'	5/12
	(9)	5'- GaGTCAactTGcTCT-3'	10/12
GII	(1)	5'- GGAtgAaacaGTTCC-3'	9/12
	(2)	5'- agcactccttcagca-3'	6/12
	(3)	5'- agcactgcttctact-3'	9/12
	(4)	5'- AGAcCAgctTGacCa-3'	8/12
	(5)	5'- ggcacttcctcatac-3'	7/12
	(6)	5'- AtgtCAtacTGacCa-3'	6/12

Putative AREs (6) and (7) for GI, and (2), (3), (5) for GII are positioned onto the DNA complementary strand and are the following: (6) 5'-GGAtCtgttaGTTCC-3', (7) 5'-AtAACttgcaGTaCT-3'; (2) 5'-AGcACtcctTcagCa -3', (3) 5'-AGTAgAagcaGTgCT -3' and (5) 5'-GtAtgAggaaGTgCC-3'. The nucleotides whose positions are maintained in AREs core motif are in uppercase letters.

value. Conversely, the maximum dose of T (100 nM) seems not to affect cell proliferation. The results of separate experiments carried out in the presence of T (1 nM) and L (25 nM and 50 nM), however, would suggest that the observed drop in PC3 proliferation after T exposure, may be due to the steroid hormone estradiol (E_2), derived from aromatase conversion activity in cells, rather than directly to T. On the contrary, it appears that T would not induce any significant change on cell growth (Fig. 7B). At the used concentrations, L alone did not exert any significant effect on cell proliferation (data not shown).

Effect of 17β-Estradiol (E2) on cell proliferation, GI and GII mRNA expression in PC3 cells. To confirm the possible role of estrogens in the regulation of cell proliferation, as well as in GI and GII mRNA expression in PC3 cells, suggested by T and L treatments, we exposed PC3 to E_2 (1 nM and 100 nM). Results indicate that E_2 would significantly ($p < 0.01$) inhibit PC3 cell growth by downregulating GI gene transcription and upregulating GII expression (Fig. 8).

Bioinformatics analysis. In order to find out whether glyoxalase promoters contain sequences matching androgen response elements (AREs) frames, we used bioinformatics methods. At present, computational methods do not exclude glyoxalases among the predicted candidate genes responsive to androgen. In fact, analysing the sequences of both GI and GII genes by PROMO program,[28,29] nine and six DNA binding sequences for GI and GII, respectively, were predicted, similar to the canonic AREs sequences motif (5'-GGTAC AnnnTGTTCT-3'[30]). GI and GII promoter putative AREs, as well as their related similarity scores to the canonic AREs sequences motif, are reported in Figure 9, Figure 10 and Table 2, respectively.

DISCUSSION

Our results suggest that testosterone (T) affects the expression of glyoxalase system genes and proliferative activity in a different manner in LNCaP androgen-dependent and PC3 androgen-independent prostate cancer cell lines.

In particular, in LNCaP cells, T causes a decrease in cell growth by upregulating GI and GII mRNA levels. The upregulation of GI and GII by T may be the result of the presence in LNCaP cells of possible GI- and GII-mediated intracellular response mechanisms to androgen-induced oxidative stress.[31,32] As well known, oxidative stress leads to lipid peroxidation, which is an important pathway leading to the production of the intracellular toxic metabolite MG.[2] In addition, it has been described that androgens control lipid metabolism in human prostate cancer cells by inducing a marked accumulation of cytoplasmic lipid droplets,[33] likely giving a consequent enhanced lipid metabolism. Since MG also derives from this latter metabolic pathway,[2] enhanced androgen-stimulated lipid metabolism, likely leads to an overproduction of this cytotoxic metabolite. It is, therefore, possible that treatments with T may induce such an overproduction of MG, that the high levels of GI mRNA observed, potentially required and prearranged to produce an elevated amount of the enzyme removing the excess of MG, are not yet sufficient. Thus, a marked MG intracellular accumulation may explain the decrease in proliferation even in the presence of an upregulation of GI gene expression.

Moreover, it is known that MG can indirectly inhibit proliferation and induce apoptosis by rising the formation of advanced glycation end products (AGEs).[2,34] AGEs cell surface receptor (RAGE) is an immunoglobulin superfamily member, whose overexpression has been recently associated with prostate cancer (PCa) development either in vivo or in vitro.[35] Therefore, the overexpression of RAGE on LNCaP cells may potentiate the antiproliferative effect of free MG, via MG/AGE-RAGE complexes, thus contributing to the observed reduction in cell proliferation in the presence of GI mRNA levels likely non-sufficient to produce enough protein. Similarly, it is likely that the high expression levels of GII gene, occurring simultaneously with the high GI gene expression, may be the consequence of a major cell request of the corresponding GII enzyme essential to lower the

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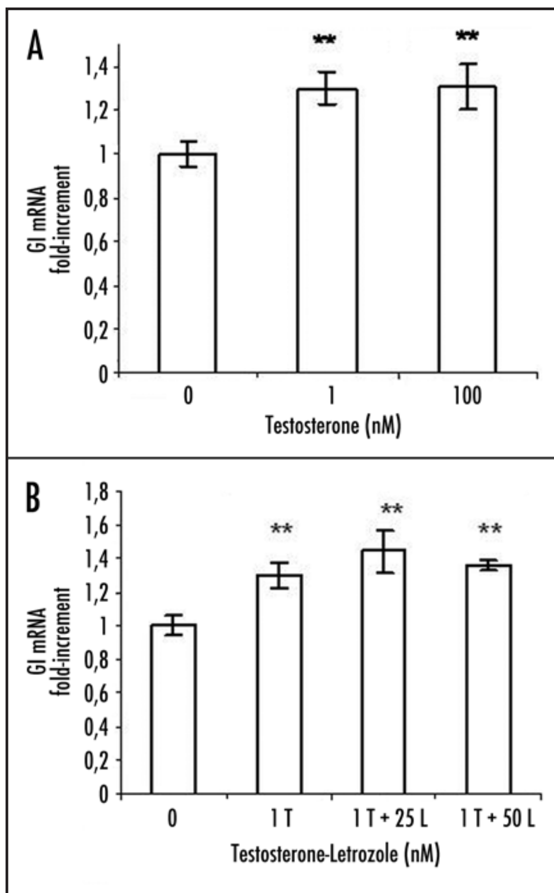

Figure 5. Effects of Testosterone (T) alone (A) or added simultaneously with letrozole (L) (B) on glyoxalase I (GI) expression in PC3 cells. Significant differences from non-stimulated cells are denoted by two asterisks ($p < 0.001$).

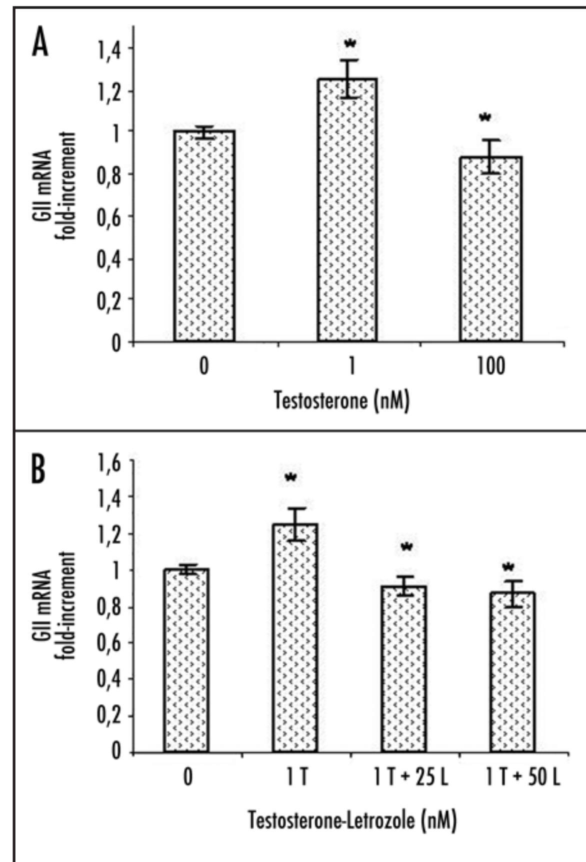

Figure 6. Effects of Testosterone (T) alone (A) or added simultaneously with letrozole (L) (B) on glyoxalase II (GII) expression in PC3 cells. *Significantly different from the negative control, represented by non-stimulated cells ($p < 0.01$).

GI enzyme-conversed product LSG, whose effects on growth arrest are well known.[8] However, also in this case, the enhanced GII expression may be still inadequate to synthetize the sufficient amount of enzyme required to remove the overproduced-LSG, thus explaining the LNCaP reduced cell growth after T exposure. Besides, it is known that, in general, GII is about ten-times less expressed than GI.[1] The observed inhibition of LNCaP growth by T seem to be in agreement with the existing literature.[36,37]

As to the androgen-indipendent PC3 cells, our results show that T treatment leads to an increase in GI mRNA levels and to a decrease in GII mRNA amount. Such results may be, once again, consequent to the GI- and GII-mediated intracellular response mechanisms to androgen-induced oxidative stress, as above described for LNCaP cells. However, in PC3 cells, GI and GII expression regulation by T, is paralleled by no changes in proliferativa activity. It is likely that the increased expression level of GI gene may prearrange the production of enough protein to remove the excess of MG, physiological inhibitor of cell growth, produced by T-induced oxidative stress. However, the low expression levels of GII, occurring simultaneously with the high GI gene expression, may lead to an accumulation of the intermediate product SLG, as a result of an increased production and a slackened hydrolysis. Such a condition could inhibit tumor growth.[8] It is likely that as a consequence of the two contemporaneous events, no alteration in cell proliferation was observed after T treatment.

An alternative hypothesis about the regulation of GI and GII gene expression by T in both cell lines, regards the possibility of a direct influence by T on glyoxalases genes.

Androgens are involved in the regulation of cell proliferation and differentiation in a variety of tissues. Androgen receptors (ARs) mediate hormone functions by binding to target gene selective DNA sequences, just named androgen response elements (AREs), whose consensus sequence is 5'-GGTACAnnnTGTTCT-3'.[30] Bioinformatics analysis does not exclude the presence in GI and GII genes of AREs very similar, but not identical, to the canonic AREs core motif. This is in accordance with the general assumption that most of the AREs identified to date do not exactly fit the consensus, suggesting that individual sequence variations may be important for the specificity of gene control. In particular, recently, DNA response elements mediating a preferential response to androgen stimulation have been described in some androgen-regulated genes[38,39] having selective AREs direct repeat features. Therefore, the presence of base deviations from the consensus in the predicted AREs of glyoxalases genes may lead to variations into the gene transcriptional activity, providing to glyoxalases a sensitivity, even slight, towards androgen receptors. If that is so, our study is the first suggesting the possible presence of regulatory sequences for androgens in the glyoxalase genes, providing insights into the function of these enzyme in hormone-regulated cancers. In fact, currently little is known about

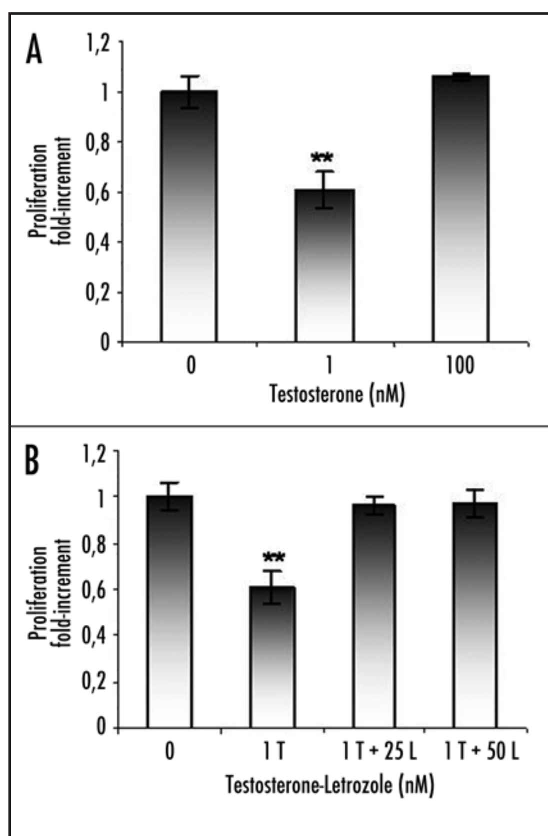

Figure 7. Effects of Testosterone alone (A) or in combination with the aromatase inhibitor Letrozole (B) on proliferation of PC3 cells. Significant differences from non-stimulated cells are denoted by two asterisks ($p < 0.001$).

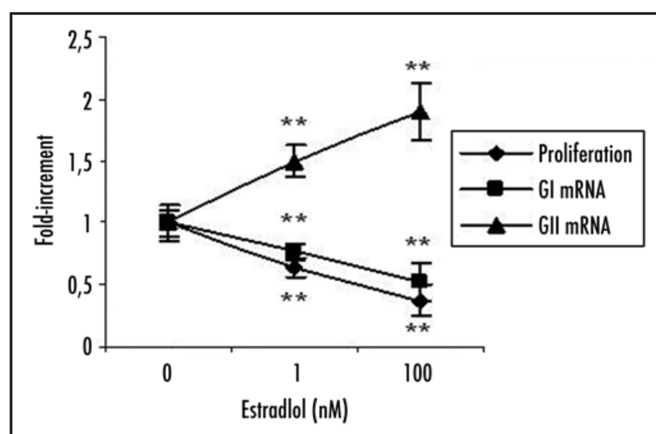

Figure 8. Effects of 17β-Estradiol (E_2) on cell proliferation, glyoxalase I (GI) and glyoxalase II (GII) gene expression in PC3 cells. **Significantly different from the negative control, represented by non-stimulated cells ($p < 0.001$).

factors pertinent to regulation of glyoxalase system and very little about hormonal ones. Only GI appears to be regulated by insulin and zinc via insulin response element (IRE) and a metal response element (MRE), respectively.[20] In addition, both GI and GII appear to be regulated by estrogens, as predicted by bioinformatics studies.[19] In general, however, the mechanisms by which androgens regulate gene expression, are fundamentally so complex, that the intervention of coactivator or corepressor proteins modulating ARs activation in the presence of ligand,[40] as well as ARs cross-talk processes with other cell signalling pathways,[41] might not be excluded. In fact, it has become widely recognized that diverse signalling pathways do not act independently, but intersect and interact at different levels, being subject to mutual cross-modulation.

The second part of our study, regarding the exposure of LNCaP and PC3 to E_2, shows that also estrogens may play an important role in LNCaP and PC3 proliferation control, via regulation of glyoxalases expression at a transcriptional level. In fact, in LNCaP cells, E_2 treatment stimulates LNCaP cell growth by upregulating GI gene transcription (in a combinatory effect with T) and downregulating GII expression. The observed stimulatory effect of estrogens on LNCaP proliferation would be in agreement with the results of Castagnetta et al.[42] Conversely, in PC3 cells, E_2 treatment leads to a decrease in cell growth, paralleled by a decrease in GI mRNA levels and a concomitant augmentation in GII mRNA levels. The observed potential inhibitory effect of E_2 on PC3 growth is in agreement with the observations of Carruba et al.[43] The different behaviour of LNCaP and PC3 cell lines, as to proliferation and GI and GII gene expression, to E_2 exposure may reflect the different expression pattern of estrogen receptors (ERs) isoforms: LNCaP cells express only ERβ, while PC3 cells have both ERα and ERβ. In fact, it has been described that, the two ER isoforms may interact with the same DNA response elements, exhibiting similar, but not identical, biological effects through specific intracellular signalling pathways.[44] Thus, the impact of ERs on estrogens biology is likely to occur as a consequence of (1) direct actions of ERβ, where it is responsible for regulating target gene transcription and (2) indirect activities, when ERβ modulates ERα action in tissues where they are coexpressed.[45-47]

In conclusion, our results show that T regulates glyoxalases gene expression in human AR-positive LNCaP and AR-negative PC3 cell lines, with a mechanism still to be more widely elucidated. However, the modulation by T seems to be different according to the enzyme and the cell line. This might reflect different potentials to develop either androgen susceptibility or antiandrogen resistance of individual tumor cells within a particular solid prostate tumor in vivo, by means of glyoxalase system modulation. In addition, on a clinical prospect, our findings appear to point out beneficial effects of both antiestrogens and aromatase inhibitors in the adjuvant therapy for human androgen-responsive prostate tumors. Moreover, our findings suggest the possible use at pharmacological doses of estrogens as well as the uselessness of aromatase inhibitors and/or antiestrogens, in the treatment of human androgen refractory PCa.

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Figure 9. Promoter sequence of the human glyoxalase I. The putative regulatory elements are shown in bold and numbered. The repeated sequences are underlined. In uppercase letters and highlighted are sequentially the 5'UTR and the transcription start site (ATG).

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Figure 10. Promoter sequence of the human glyoxalase II. In bold and numbered are the putative regulatory elements. The repeated sequences are underlined. In uppercase letters and highlighted are sequentially the 5'UTR and the transcription start site (ATG).

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