PERGAMON

Journal of Steroid Biochemistry and Molecular Biology 68 (1999) 83–88

The Journal of Steroid Biochemistry & Molecular Biology

Hormonal interactions in human prostate tumor LNCaP cells

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Received 13 June 1998; received in revised form 7 October 1998; accepted 7 October 1998

Abstract

Melatonin, the hormone secreted by the pineal gland at night, has recently been found to attenuate growth and viability of benign human prostate epithelial cells. Estradiol suppressed these responses by effecting a protein kinase C mediated inactivation of melatonin receptors. In the present study, the effects of melatonin on growth and viability of the human androgen-sensitive prostatic tumor cell line-LNCaP and the influence of estradiol on these responses were explored.

Melatonin inhibited ^{3}H-thymidine incorporation into LNCaP cells at physiological concentrations. This response decayed within 24 h. The inactivation of the response slowed down in the presence of the protein kinase C inhibitor GF-109203X. Estradiol also inhibited ^{3}H-thymidine incorporation and its effects were additive to those of melatonin. Suppression of DNA content was observed in cells treated for 2 days with melatonin (0.1 nM); this suppression was maintained for longer periods in the presence than in the absence of estradiol. In addition, estradiol and melatonin slightly and additively decreased cell viability.

These results demonstrate for the first time a direct interaction of melatonin with androgen-sensitive prostate tumor cells leading to attenuation of cell growth. They also show that unlike in benign prostate epithelial cells, estrogen attenuates LNCaP cell growth and supports rather than inactivates melatonin's action.

Keywords: Melatonin; Prostate; Cancer; LNCaP; Growth; Viability; Androgen; Estradiol

1. Introduction

We have recently found that melatonin-the hormone secreted nocturnally from the pineal gland-transiently inhibits ^{3}H-thymidine incorporation in human benign prostate epithelial cells and attenuates their growth [1–3]. The transient response to melatonin was due to inactivation of its receptors via a protein kinase C-mediated pathway. Accordingly, the inactivation could be prevented by bisindolylmaleimide (GF 109203X) a specific inhibitor of protein kinase C [3]. Estradiol also acted to inactivate melatonin receptors in the benign cells via a protein kinase C mediated pathway [3,4].

Nocturnal melatonin production is diminished in a number of human malignancies (breast, liver, kidney upper respiratory tract and skin) including prostate carcinoma [5–7]. Administration of melatonin has recently been shown to reverse the clinical resistance of metastatic prostate cancer patients to the elimination of testicular androgens (that is, chemical castration using luteinizing hormone releasing hormone analogs) [8]. Accumulating evidence suggests a direct interaction of melatonin with human prostate cancer cells. Melatonin has been shown to inhibit the growth of R3327H Dunning prostatic adenocarcinoma [9]. On the other hand, melatonin enhanced growth of a transplantable androgen-insensitive prostatic adenocarcinoma in rats [10]. We have recently observed (unpublished) that melatonin can enhance growth of the androgen-insensitive prostate tumor PC3 cells.

In the present study we have thus explored the effects of melatonin on growth of the androgen-sensitive prostate cancer cell line-LNCaP and the effects of estrogen on these responses.

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0960-0760/99 $ - see front matter
PII: S0960-0760(98)00164-2

2. Materials and methods

2.1. Materials

RPMI-1640 medium (RPMI), RPMI-1640 medium without phenol red (RPMI-P), fetal calf serum (FCS), charcoal stripped FCS (FCSC), L-glutamine and antibiotics were obtained from Biological Industries (Beit Haemek, Israel). Melatonin, RNase, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), thymus calf DNA, bovine serum albumin (BSA) and 2′-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazole (Hoechst 33258), were obtained from Sigma (St Louis, MO). Methyl-^{3}H-thymidine was obtained from Rotem (Beer Sheva, Israel).

2.2. LNCaP cell culture

Human LNCaP prostate cell line was obtained from American Type Culture Collection, MA, USA. Cells were cultured in RPMI medium containing 10% FCS, 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and maintained at 37°C in humidified atmosphere with 5% CO_2. Cells (20–60 passages) were harvested by trypsin and replated (15,000 cells/well) in poly-L-lysine coated 24 well-dishes (that is, dishes treated for 24 h. with aqueous 50 μg/ml poly-L-lysine, and then washed with 3×1 ml double distilled water), in RPMI-P medium containing 2 mM glutamine, 5% FCSC, 10^{-10} M dihydrotestosterone (DHT), and vehicle (0.001% ethanol) or 10^{-8} M estradiol (E2). Medium was replaced every 2 days until the experiment. Protein was determined as described [11], using BSA as a protein standard. All data presented were normalized for protein content.

2.3. ^{3}H-Thymidine incorporation

Cells were grown for two days in the presence of 10^{-10} M DHT, and then for four additional days with 10^{-10} M DHT in the absence or presence of 10^{-8} M E2. The cells were incubated in culture medium in the absence or presence of melatonin (10^{-13}–10^{-6} M; obtained by serial dilutions in growth medium of an ethanolic 0.1 mM stock solution) or vehicle (ethanol at respective dilution; maximal concentration in culture not exceeding 1%) for 60 min. at 37°C in 5% CO_2 atmosphere, 100% humidity in the absence and presence of 0.5 mM GF 109203X. ^{3}H-thymidine (60 Ci/mmol, 1 μCi/well) was then added and the incubation was resumed for 60 min. Media were discarded, and the cells were fixed in methanol at −20°C for 15 min., rinsed with 2×2 ml PBS and then incubated 3×10 min with 2 ml ice-cold 10% trichloroacetic acid. Cell monolayers were solubilized in 0.4 ml 0.3 N NaOH solution containing 0.1% sodium dodecyl sulfate (SDS). The acid-precipitable material was counted for incorporated radioactivity in a β counter. Aliquots were retained for protein determination. In some experiments, cells were incubated in culture for various periods of time (1–72 h) in the absence or presence of 0.1–1 nM melatonin. ^{3}H-thymidine incorporation was then assessed as described above.

2.4. Cell viability

Cell viability was assessed by the MTT method (a water soluble tetrazolium dye, which is converted into an insoluble purple formazan by active mitochondrial dehydrogenases of living, cells [12]). Cells were incubated in the culture medium without or with melatonin (1 nM) for 1–72 h. MTT (30 μl of a 5 mg/ml solution in PBS) were then added and incubation resumed for 75–100 min. at 37°C. The medium was then removed and the cells solubilized in 200 μl dimethylsulfoxide. Absorbency of the converted dye was assessed from the ratio of absorbency at 490/650 nm wavelengths.

2.5. DNA determinations

Cells were washed with PBS and suspended in 0.5–0.7 ml of 0.2% SDS in ETN buffer (composed of 10 mM ethyleneglycoltetraacetate, 10 mM Tris HCl, pH 7.0). The cell suspension was incubated for 30 min. at 37°C to solubilize the cells. Aliquots (35–70 μl) of the solution were added to 1 ml of ETN buffer containing 1 μg/ml Hoechst 33258 and RNase (5 ng/ml) and incubated in the dark for 30 min. at room temperature. The fluorescence of the dye at 450 nm wavelength (excitation at 360 nm) was then assessed. Calf thymus DNA was used as a standard.

2.6. Statistical analyses

Results were compared by analysis of variance (ANOVA) followed by paired Student's *t*-tests. Significance was determined at $p < 0.05$.

3. Results

The effects of melatonin (1 h) on ^{3}H-thymidine incorporation into the LNCaP cells grown with DHT (0.1 nM) are presented in Fig. 1. Melatonin markedly inhibited thymidine incorporation in a dose dependent manner (Fig. 1a). The inhibition was however complex, extending over several orders of magnitudes, with 50% inhibition (IC50) between 0.1–1 nM.

The effects of incubation time with 0.1 nM melatonin on ^{3}H-thymidine incorporation by the cells is shown in Fig. 1b and c. Melatonin inhibited ^{3}H-thymidine incorporation at 1–24 h but its effect greatly

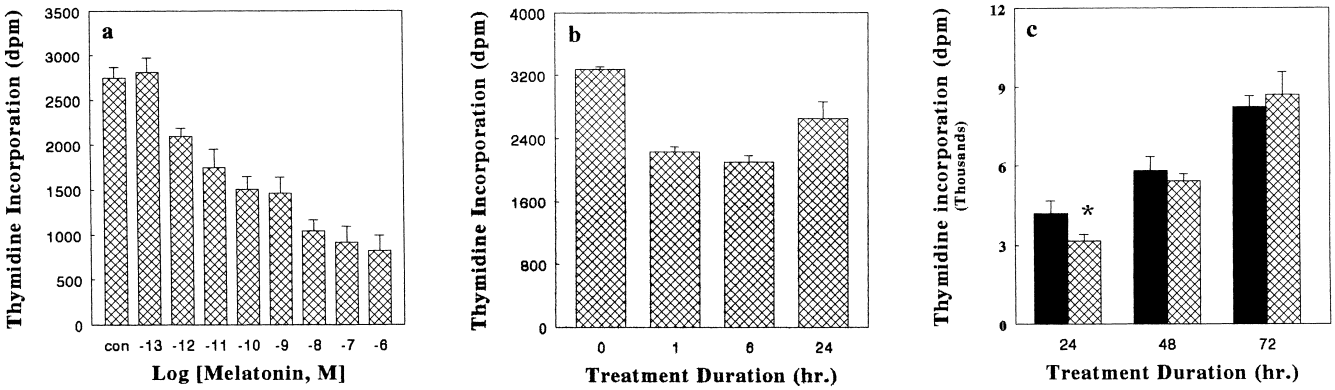

Fig. 1. Effects of melatonin on ^{3}H-thymidine incorporation in LNCaP cells. Cells grown for 6 days in the presence of 10^{-10} M DHT were incubated with: (a) melatonin (10^{-13}–10^{-6} M) for 60 min. (b) melatonin 0.1 nM for 1–24 h or (c) vehicle (black bars) or melatonin 0.1 nM (hatched bars) for 24–72 h The incorporation of ^{3}H-thymidine was then assessed. Results are mean±S.E.M. of 4 independent studies, run in quinta-plicates, and are expressed in dpm per plate. *$P<0.05$ compared with control cells cultured with vehicle.

decreased at longer incubation times. The decrease in ^{3}H-thymidine incorporation at $\leq$24 h was not due to a change in the DNA content of the culture since DNA content was still unaffected by melatonin at this time (see for example Fig. 4a).

The effects of melatonin (1 h) on ^{3}H-thymidine incorporation into the LNCaP cells grown with DHT (0.1 nM) and E2 (10 nM) are presented in Fig. 2. ^{3}H-Thymidine incorporation in cells grown in the presence of E2 was significantly lower (by 28%; $p<0.05$) than in its absence. Melatonin inhibited ^{3}H-thymidine incorporation in these cells in a dose dependent manner; incorporation decreased by 50% at 0.5 nM (Fig. 2a). At high concentrations (0.1;10 μM) the inhibition by melatonin was less pronounced.

The effects of incubation time with 0.1 nM melatonin on ^{3}H-thymidine incorporation by cells grown in the presence of E2 (10 nM) is shown in Fig. 2b and c. Melatonin inhibited ^{3}H-thymidine incorporation at 1 and 6 h. but its effect decreased afterwards. A significant inhibition by melatonin of ^{3}H-thymidine incorporation was observed at 48 h (Fig. 2c). However, this inhibition was not significant when the incorporation was normalized per DNA content of the cultures.

The effect of melatonin (0.1 nM) on ^{3}H-thymidine incorporation by the cells in the absence and presence of the PKC inhibitor- GF 109203X (0.5 mM) is shown in Fig. 3. GF-109203X slightly enhanced ^{3}H-thymidine incorporation in cells grown in the presence of E2, but not in its absence. Melatonin's effect on ^{3}H-thymidine incorporation diminished within 24 h of incubation. The inactivation of the response was significantly slower in the presence of GF 109203X.

The effects of melatonin on DNA content of cell

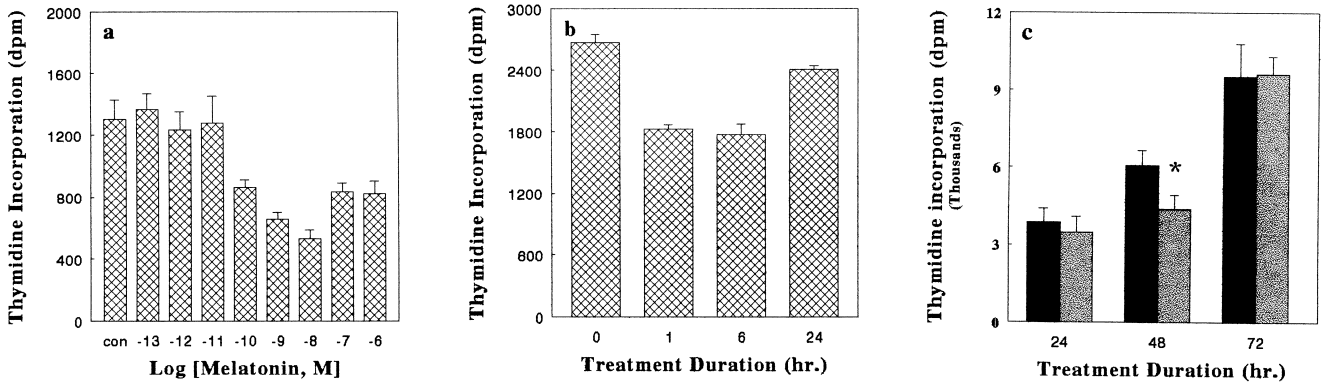

Fig. 2. Effects of melatonin on ^{3}H-thymidine incorporation in LNCaP cells. Cells grown for 2 days in the presence of 10^{-10} M DHT, and then for 4 days in the presence of 10^{-10} M DHT and 10^{-8} M E2, were incubated with: (a) melatonin (10^{-13}–10^{-6} M) for 60 min. (b) melatonin 0.1 nM for 1–24 h or (c) vehicle (black bars) or melatonin 0.1 nM (hatched bars) for 24–72 h The incorporation of ^{3}H-thymidine was then assessed. Results are mean±S.E.M. of 4 independent studies, run in quinta-plicates, and are expressed in dpm per plate. *$P<0.05$ compared with control cells cultured with vehicle.

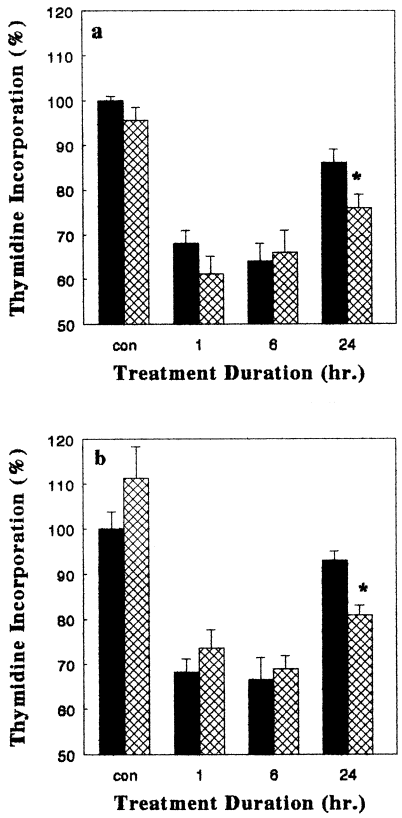

Fig. 3. Effects of GF 109203X on the melatonin-mediated inhibition of ^{3}H-thymidine incorporation in LNCaP cells. Cells grown for 6 days in the presence of 10^{-10} M DHT (a), or grown for 2 days in the presence of 10^{-10} M DHT, and then for 4 days in the presence of 10^{-10} M DHT and 10^{-8} M E2 (b), were incubated without (con) or with 0.1 nM melatonin in the absence (black bars) or presence (hatched bars) of GF 109203X (0.5 mM) for 1–24 h. ^{3}H-thymidine incorporation was then assessed. Results are mean±S.E.M. of 2 independent studies, run in quadruplicates and presented as % of control values (2650 in (a) and 1980 dpm in (b)). $*P<0.05$ compared with control cells without GF 109203X.

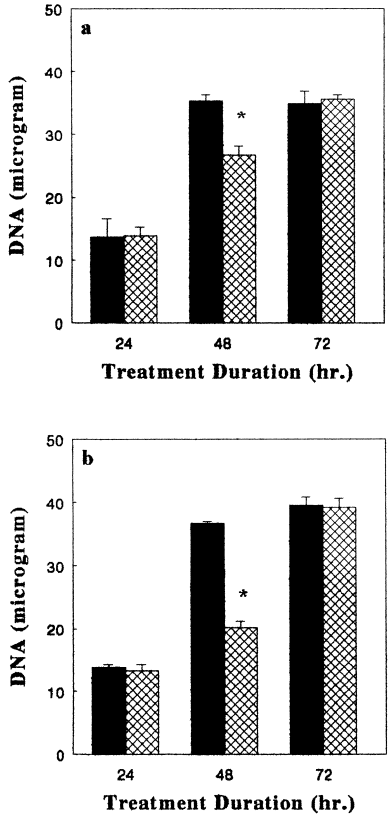

Fig. 4. Effects of melatonin on DNA content of LNCaP cultures. Cells grown for 6 days in the presence of 10^{-10} M DHT (a), or Cells grown for 2 days in the presence of 10^{-10} M DHT, and then for 4 days in the presence of 10^{-10} M DHT and 10^{-8} M E2 (b), were incubated in the absence (black bars) or presence (hatched bars) of: melatonin (0.1 nM) for 24–72 h DNA content was then assessed. Results are mean±S.E.M. of 3 independent studies, run in quinta-plicates. $*P<0.05$ compared with control cells cultured with vehicle.

cultures grown in the absence and presence of E2 are shown in Fig. 4. The DNA content of cultures treated with melatonin (0.1 nM) for 48 h was significantly lower than in control cultures treated with vehicle regardless of the culture being grown with or without E2. This inhibition diminished at longer times (72 h) (Fig. 4a and b).

The effects of melatonin on viability of cell cultures grown in the absence and presence of E2 are shown in Fig. 5 and Fig. 6. The viability of cultures grown in the presence of E2 for 24–72 h was significantly lower than that in cultures grown with DHT alone whether calculated per culture (Fig. 5a and Fig. 6a) or normalized per culture DNA content (Fig. 5b and Fig. 6b). The viability of cultures treated with melatonin (0.1 nM) for 24– 72 h was significantly lower than in control cultures treated with vehicle, whether calculated per dish or normalized to DNA content (Fig. 5; Fig. 6).

4. Discussion

The results of the present studies indicate that melatonin attenuates growth of the androgen-sensitive prostate tumor cell line-LNCaP. As in benign prostate epithelial cells [2,3], this response was transient. ^{3}H-thymidine incorporation resumed within 24 h and DNA content was suppressed at 48 but not 72 h of melatonin treatment. As in the human benign prostate epithelial cells [3], the inactivation of the response in LNCaP cells was retarded by GF-109203X and may thus be due to a PKC-induced inactivation of melatonin receptors.

The involvement of PKC in agonist-induced desensitization of melatonin receptors, implies that melatonin activates PKC either directly or indirectly in the LNCaP cells. A melatonin-mediated activation of PKC is compatible with the recent findings that inhibition of PKC partially blocked melatonin responses in NIH 3T3 cells stabily transfected with the Mel-1a receptor expression vector [13]. However, GF-109203X did not

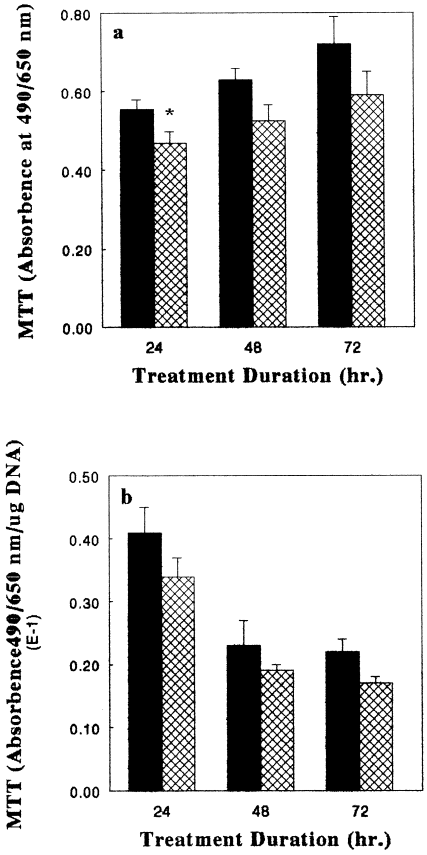

Fig. 5. Effects of melatonin on LNCaP cell viability. Cells grown for 6 days in the presence of 10^{-10} M DHT were incubated in the absence (black bars) or presence (hatched bars) of melatonin (0.1 nM) for 24–72 h Cell viability was then assessed by the MTT method. Results are mean±S.E.M. of 4 independent studies, run in quinta-plicates, and are expressed in absorbency per plate (a) or per μg DNA content of plate (b). $*P<0.05$ compared with control cells cultured with vehicle.

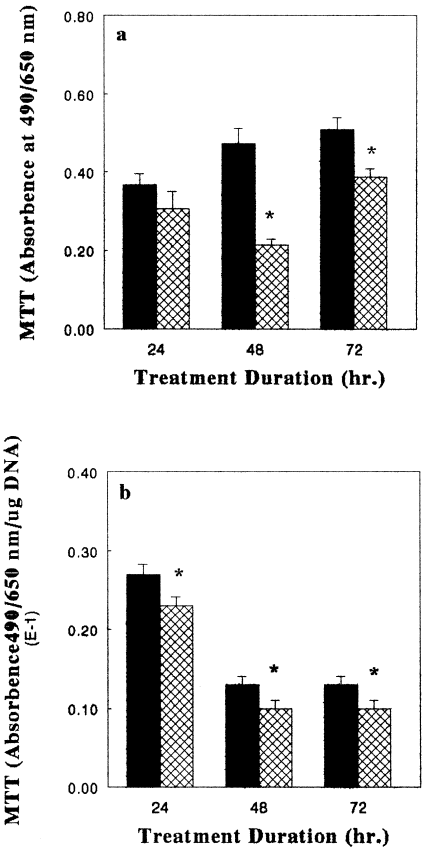

Fig. 6. Effects of melatonin on LNCaP cell viability. Cells grown for 2 days in the presence of 10^{-10} M DHT and then for 4 days in the presence of 10^{-10} M DHT and 10^{-8} M E2, were incubated in the absence (black bars) or presence (hatched bars) of melatonin (0.1 nM) for 24–72 h Cell viability was then assessed by the MTT method. Results are mean±S.E.M. of 4 independent studies, run in quinta-plicates, and are expressed in absorbency per plate (a) or per μg DNA content of plate (b). $*P<0.05$ compared with control cells cultured with vehicle.

prevent the suppression by melatonin of ^{3}H-thymidine incorporation in the LNCaP cells. This indicates that as in the benign prostate cells [3], this response is most probably not mediated by PKC.

Interactions between melatonin and estradiol have been documented in a number of systems. In the female rat hypothalamus, the inhibitory effects of melatonin on dopamine release diminished in ovariectomized female rats and were reinstated by estradiol supplementation [14]. Melatonin has been shown to suppress estrogen receptor expression in brain and breast cancer MCF7 cells [15,16]. In human benign prostate epithelial cells E2 inactivates melatonin receptors thereby preventing the inhibitory action of melatonin on thymidine incorporation [4]. Immunoblot analyses have recently indicated the presence of estradiol-alpha receptor protein in the benign prostate epithelial cells but not LNCaP cells (unpublished). The fact that E2 did not suppress melatonin activity in the LNCaP cells is compatible with the notion that some component(s) of the pathway involved in E2 action is absent in these cells.

The effects of E2 on growth of LNCaP cells are equivocal. High doses of estrogen had been used in prostate cancer therapy in past years; such treatment reduces the amount of circulating androgens to the castration levels by inhibition of the release of luteinizing hormone releasing hormone [17]. In some experimental tumors, the effect of high dose estrogen exceeded that of castration and extenuated the effect of orchidectomy [18,19]. The additional effects might be mediated through estrogen receptors present in the tumors. The presence of type II estrogen receptors in LNCaP cells has been demonstrated [20]. Our observation that E2 reduced ^{3}H-thymidine incorporation and viability in LNCaP cells is compatible with an inhibitory action of E2 on the growth of prostate cancer cells.

In contrast, other studies have shown that E2 enhanced growth of LNCaP cells at low (10 nM) and inhibited it at high (100 nM) concentrations [21]. Under the experimental conditions used by us (10 nM E2) it could be expected that E2 would enhance rather than suppress LNCaP cell growth. These bi-directional effects of E2 on LNCaP cell growth were sensitive to culture conditions and thus attributed to growth factor interferences [21]. This apparent discrepancy between the latter observations and ours may perhaps stem from the presence of DHT and other differences in culture conditions.

While the presence of estrogen receptors in LNCaP cells has been reported [20] some evidence suggested E2 -receptor independent responses in these cells. LNCaP cells contain a mutated androgen receptor, which is capable to respond, besides androgens, to progesterone, estrogen and adrenal steroids [22]. The inhibition by estradiol of LNCaP cell growth and viability might be due to competition with DHT on the mutated androgen receptor, thus preventing the growth promoting effects of the androgen. In addition, E2 has been found to provoke a rapid enhancement of calcium influx in LNCaP cells [23]. This influx was insensitive to blockade by flutamide (antiandrogen) as well as tamoxifen (antiestrogen). This suggests that some E2 responses might be independent of either androgen- or estrogen receptors [23].

Taken together, the results presented here indicate a role for melatonin in the hormonal modulation of LNCaP cell growth. They also imply that in these cells estradiol supports rather than inactivates, the growth suppressing action of melatonin.

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