Physiological Melatonin Inhibition of Human Breast Cancer Cell Growth in Vitro: Evidence for a Glutathione-mediated Pathway

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ABSTRACT

Melatonin, the chief hormone secreted by the pineal gland, has been previously shown to inhibit human breast cancer cell growth at the physiological concentration of 1 nM in vitro. In this study, using the estrogen receptor (ER)-positive human breast tumor cell line MCF-7, we have shown that 10 μM α-l-buthionine-[S,R]-sulfoximine (L-BSO), an inhibitor of γ-glutamylcysteine synthetase (the rate-limiting enzyme in glutathione synthesis), blocks the oncostatic action of 1 nM melatonin over a 5-day incubation, indicating that glutathione is required for melatonin action. The result was repeated with ZR75-1 cells, suggesting that the glutathione requirement is a general phenomenon among ER+ breast cancer cells. Addition of exogenous glutathione (1 μM) to L-BSO-treated groups restored the melatonin response in both cell lines. Further demonstration of the importance of glutathione was shown using the ER− breast tumor cell line HS578T, which is normally unresponsive to melatonin. Growth in this cell line was inhibited in the presence of 1 μM ethacrynic acid (an inhibitor of glutathione S-transferase) plus 1 nM melatonin, and this effect was blocked with 10 μM L-BSO. We also observed a steady decrease of intracellular glutathione in MCF-7 cells over a 5-day incubation, suggesting that these cells metabolize glutathione differently than do normal cells.

INTRODUCTION

Melatonin, the hormone produced and secreted by the pineal gland during the night in virtually all mammalian species including man, exerts oncostatic effects on a variety of neoplasms, most notably breast cancer (1–3). Pharmacological levels of melatonin are effective in inhibiting the growth of mammary cancers in rodent models of either spontaneous (4, 5), transplantable (6, 7), or carcinogen-induced breast cancer (8–14). The enhancement of chemically induced mammary carcinogenesis by either pinealectomy or constant light in rats eliminates the nocturnal melatonin surge (1, 9, 10, 13–16), suggesting that physiological levels of melatonin may also be important in inhibiting breast cancer growth. Furthermore, the nocturnal amplitude of melatonin secretion is blunted in breast cancer patients (17), including those with ER+–positive disease (18), suggesting that a link exists between physiologically relevant nocturnal concentrations of melatonin and breast cancer growth. In support of this hypothesis, we previously demonstrated that melatonin in the physiological range (10 pm to 1 nm) inhibits MCF-7 human breast cancer growth in vitro over a 5–7-day incubation period (19–22). Our initial finding of a direct inhibitory effect of melatonin, particularly at pharmacological concentrations, on MCF-7 cell proliferation in vitro has now been replicated in a number of laboratories (23–31). Two research groups, however, reported a cytostatic or cytotoxic action of melatonin on MCF-7 cell growth only at pharmacological levels (32, 33), whereas one of the groups cited above subsequently failed to find an effect of melatonin on MCF-7 cell growth at any concentration over a 12-day culture period (34). These discrepancies could be attributed to different clonal lines of MCF-7 cells (35) as well as to different culture conditions, particularly the serum used (36, 37), which are factors known to alter the responsiveness of these cells to hormones and chemotherapeutic agents including estrogen and tamoxifen (38).

In recent years, significant progress has been made toward elucidating the cellular and molecular mechanisms by which physiological melatonin inhibits MCF-7 cell growth in culture (3). For example, melatonin not only delays the progression of MCF-7 cells from G0/G1 to S-phase of the cell cycle (22, 26), but it also modulates both constitutive and estrogen-induced growth factor activity in these cells (39) and inhibits the mitogenic actions of estrogen (20), epidermal growth factor (39), and prolactin (40). Results showing that the oncostatic action of melatonin on breast cancer cells seems to be restricted to ER+ cells (20) led to studies showing that physiological melatonin suppresses ER expression (41) via an inhibition of the transcriptional regulation of ER mRNA in MCF-7 cells (42). Recent studies in MCF-7 cells also show that melatonin modulates the steady-state mRNA expression of a variety of other estrogen-regulated proteins such as p53 and the progesterone receptor (43). Furthermore, melatonin also modulates the expression of TGFB in MCF-7 cells such as TGF-α and TGF-β as well as some proto-oncogenes such as c-my c and c-fos (43).

In spite of these important advances, no definitive signal transduction pathway has been shown to convey the oncostatic message of melatonin to the intracellular processes controlling the proliferation of MCF-7 or any other cancer cell types. For example, although melatonin (i.e., 2-α-cyclopentyl-1,2,3,4-tetrahydro-10β-iodomelatonin) binding sites have been reported to exist in a variety of tissues, including melanoma (44–46) and carcinogen-induced rat mammary tumors (47), little to no melatonin binding has been found in MCF-7 cell membranes (45), suggesting that the recently cloned, membrane-bound, high-affinity melatonin receptors (48) are not involved in the oncostatic action of melatonin in this cell line.

Increasing attention is now being devoted to the intracellular compartment, particularly the nucleus, as an important site for some of melatonin’s actions in some cells and tissues (49, 50). This is based on several converging lines of evidence including the demonstration of the potent antioxidant and free-radical scavenging capacity of melatonin (51), and its localization and binding in the nucleus (52) as well as its ability to serve as a ligand with the RZR/ROR family of orphan nuclear receptors (53). There is currently no evidence, however, that such pathways mediate the oncostatic effects of melatonin on cancer cells.

In studies relating to its antioxidant properties (51), pharmacological doses of melatonin administered to female rats have been shown to increase both the intramammary and intraperitoneal levels of glutathione and glutathione S-transferase (54). Additionally, melatonin stimulates glutathione peroxidase activity in brain tissue (51). These results suggest that glutathione, a tripeptide molecule that represents an important component of the cellular antioxidant system, may be involved in the mechanisms mediating some of the actions of melatonin. Glutathione plays a critical role in a metabolic pathway, using NADPH to provide the intracellular environment with a reducing milieu. Thus, it functions in reductive processes that are essential for protein metabolism, DNA synthesis, and enzyme regulation as well as drug and hormone metabolism by forming conjugates with these compounds either spontaneously or through a mechanism catalyzed by glutathione S-transferase. Via its redox cycling, glutathione is a potent antioxidant and thereby provides cells with a substantial

1 Received 11/12/96; accepted 3/24/97.

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3 The abbreviations used are: ER, estrogen receptor; L-BSO, l-buthionine-[S,R]-sulfoximine; TGF, transforming growth factor; PBS, fetal bovine serum.
degree of protection against oxidative stress, free radical damage, and other types of toxicity (55, 56). Additionally, through its complex metabolism, glutathione has an important impact on the therapeutic responsiveness or resistance of cancer cells to anticancer drugs. For example, suppression of intracellular glutathione synthesis with L-BSO, an irreversible inhibitor of the rate-limiting enzyme γ-glutamylcysteine synthetase, can make cancer cells either more or less sensitive to the cytotoxic effects of antineoplastic agents (57).

On the basis of the evidence that pharmacological levels of melatonin increase glutathione and glutathione-metabolizing enzymes in vivo (51, 54) and that L-BSO-induced glutathione depletion as well as ethacrynlic acid inhibition of glutathione S-transferase alters the sensitivity of cancer cells to anticancer drugs (57–59), we decided to test the hypothesis that the antiproliferative effect of physiological melatonin on MCF-7 cell growth in vitro is dependent on glutathione. We tested this postulate by examining the effects of L-BSO-induced glutathione depletion on the responsiveness of MCF-7 cells to physiological melatonin and supported these results by examining another ERα human breast cancer line, ZR75-1. We also tested whether HSS78T human breast cancer cells, which are ERα and are not normally affected significantly by melatonin (20), could be made responsive to melatonin when glutathione metabolism is altered by the addition of ethacrynlic acid.

MATERIALS AND METHODS

Materials. Melatonin (lot 113H1083), tamoxifen (lot 101H0649), glutathione (lot 68F-0474), L-BSO, ethacrynlic acid, 5,5'-dithio-bis(2-nitrobenzoic acid), glutathione reductase IV (from baker’s yeast; lot 41H18954), β-NADPH, 5-sulfosalicylic acid, and human insulin were purchased form Sigma Chemical Co. (St. Louis, MO). MCF-7 human breast cancer cells were a generous gift from Dr. Steven Hill (Tulane University, New Orleans, LA). ZR75-1 and HSS78T human breast cancer cells were purchased from American Type Culture Collection (Rockville, MD). DMEM was purchased from Life Technologies, Inc. (Grand Island, NY). All media were supplemented with 10% FBS (Tissue Culture Biological, Tulare, CA), 1% penicillin (10,000 units/ml), and streptomycin (10,000 µg/ml; Life Technologies, Inc.).

Cell Culture. MCF-7 and ZR75-1 human breast cancer cells were cultured and maintained in complete DMEM supplemented with 10% FBS in Falcon T-175 culture flasks (Becton Dickenson, Oxnard, CA) at 37°C in a humid atmosphere containing 5% CO2 and 95% air as described previously (19, 20, 22). HSS78T cells were grown under the same conditions except high-glucose DMEM was used, and 10 µg/ml insulin were added. Stock flasks of exponentially growing cells were randomly selected, and the growth medium was removed followed by treatment with 0.2% EDTA in PBS (pH 7.3). After the cells had sloughed from the bottom of the flasks, 10 ml of complete medium were added, and the resulting cell suspension was removed and centrifuged at 60 × g for 5 min. After centrifugation, the supernatant was removed, and the resulting cell pellet was resuspended in 12 ml of complete medium, and a 1-ml sample was taken for hemocytometer counts. After the cell number was determined, the cells were adjusted to 3 × 10⁶ cells/ml with complete medium. One ml of this new suspension was added to each Falcon plastic cell culture dish (60 × 15 mm; Becton Dickenson; 4 dishes/treatment group) along with enough medium (3 ml) to facilitate cellular attachment. Four h after initial plating of the cells, the medium from each dish was removed and replaced by 5 ml of fresh complete medium containing hormone and/or drugs at the desired concentrations. The cells were returned to the incubator and allowed to grow for various time periods ranging from hours to days.

For the growth studies, cells were harvested on various days of incubation by treatment with 1 ml of PBS/EDTA and passed 3 times through a 25-gauge needle to obtain a single cell suspension. From this cellular suspension, a 1-ml sample was taken for glutathione analysis (see below), and the remainder of the cells were fixed with 50 µl of 25% glutaraldehyde for hemocytometer counts. Previous studies from our laboratory showed >95% cell viability with the trypan blue exclusion test.

Unless otherwise indicated, the incubations in most experiments were carried out for a total of 5 days, without medium changes, in the continuous presence of either physiological melatonin (1 nM), L-BSO (10 µM), glutathione (1 µM), or vehicle (0.0001% ethanol). In the time course study, cells were incubated with either melatonin (1 nM), L-BSO (10 µM), melatonin + L-BSO, or vehicle for either 1, 2, 3, 4, or 5 days, and cell counts and glutathione measurements were performed at each of these time points. Tamoxifen was used at a concentration of 1 µM. In the HSS78T experiment, ethacrynlic acid was used at a concentration of 1 µM.

Cellular Preparation and Analysis of Total (Reduced + Oxidized) Glutathione. From each culture dish a 1-ml cell suspension (see above) was removed and placed in a 1.5-ml tube. After the samples were centrifuged at 500 rpm for 5 min, the medium was removed and replaced with 100 µl of 10 mM HCl and 50 µl of 10% 5-sulfosalicylic acid. The cellular samples were lysed by repeated freezing in a dry ice 2-propanol bath and thawing at room temperature. Additional cellular preparation and glutathione analysis were performed according to the method of Anderson (60). The intracellular levels of glutathione are expressed as nmol/10⁶ cells.

Statistical Analysis. Cell number and glutathione levels are expressed as the mean ± SE. Differences among the means were determined with a one-way ANOVA followed by Student-Neuman-Keul’s post hoc test. Differences among various treatment groups were considered statistically significant at P < 0.05.

RESULTS

Changes in Glutathione Levels during Growth of MCF-7 Cells. We measured intracellular glutathione levels in MCF-7 cells in vitro over a 5-day incubation period and found that there was a steady decrease in glutathione (measured as the total of reduced + oxidized glutathione) during this time (Fig. 1). From day 1 to day 3, there was a 21% decrease in glutathione concentration as the cells increased in number by over 3-fold. From day 3 to day 5, glutathione levels decreased by another 35%, whereas the cell number almost doubled. The decrease in glutathione was not due to depletion of the media, because replenishing the culture dishes with fresh media during the time course did not prevent this decrease (data not shown).
Effects of Melatonin on Growth and Glutathione Depletion in MCF-7 Cells. We tested whether the oncostatic effect of melatonin on MCF-7 cells slowed the rate of glutathione depletion. Previous studies (19) have shown that the melatonin effect on cell growth is observed at an optimal concentration of 1 nm (a physiological concentration). Thus a time course was done over a 5-day incubation period at this optimal melatonin concentration. Fig. 2a demonstrates that by day 2 of incubation, a 35% reduction in cell number was observed in the melatonin-treated group compared to that of the control group. By day 5, the cell number was 49% lower in the melatonin-treated group. This reduction in growth rate is comparable to what has been reported previously (19, 20, 22).

Differences in glutathione concentration between the control and melatonin groups were evident at day 1. By day 2, glutathione levels (Fig. 2b) in the melatonin-treated cells were 29% higher than those in control cells, whereas at day 5, the levels were 52% higher. Another time course done from time 0 to 24 h (day 1) showed no significant increase in glutathione in the melatonin group until the 24-h time point, which was about the time when the decrease was observed in cell number (data not shown). Although this increase in glutathione concentration in the melatonin-treated cells may have been due mostly to a slower growth rate, it led us to speculate that glutathione metabolism may be important for the effect of melatonin on cell growth.

Effects of the Inhibition of Glutathione Synthesis on the Melatonin Response in MCF-7 Cells. In the experiment cited above (Fig. 2), we also determined the effects of melatonin combined with L-BSO, an inhibitor of glutathione synthesis, on MCF-7 cell growth and glutathione concentration during the 5-day incubation period. Fig. 2a clearly demonstrates that the combination of L-BSO (10 μM) and melatonin blocked the melatonin effect on growth, whereas treatment with L-BSO alone had no effect on growth.

We also measured glutathione levels in these groups (Fig. 2b) to determine the extent of the decrease in glutathione synthesis caused by this concentration of L-BSO, a concentration that was much lower than that used in other studies (56, 58). By day 2, the L-BSO group had a 40% decrease in glutathione concentration compared to that of the control group, although the growth rate was unchanged. Consistent with Fig. 1, glutathione levels decreased during growth for all groups. The L-BSO effect on glutathione synthesis persisted throughout the incubation period because the relative difference in the levels between the control group and the L-BSO group was maintained through day 5. Similarly, the combination of L-BSO and melatonin maintained an approximately 25% decrease in glutathione relative to that of the control group throughout the incubation period.

Effects of the Inhibition of Glutathione Synthesis on the Tamoxifen Response in MCF-7 Cells. To determine whether the depletion of glutathione by L-BSO specifically inhibited melatonin action or inhibited oncostatic mechanisms in general, we assessed the influence of L-BSO on the oncostatic action of tamoxifen. Table 1 shows that treatment of MCF-7 cells with tamoxifen (1 μM) over 5 days resulted in a 49% decrease in the cell number. Exposure of cells to the combination of L-BSO (10 μM) and tamoxifen had no effect on the tamoxifen response. Intracellular glutathione was measured to confirm its depletion by L-BSO. Glutathione levels were approximately 50% lower in the L-BSO-treated groups.

Effects of Exogenous Glutathione on the Melatonin Response in L-BSO-treated MCF-7 and ZR75-1 Cells. We also tested whether the addition of glutathione (1 μM) to the L-BSO/melatonin group could restore the melatonin effect on cell growth. Table 2 shows that
the exposure of cells to glutathione alone had no effect on the growth of either cell line. In contrast, when MCF-7 cells were coincubated with a combination of melatonin, L-BSO, and glutathione, cell growth was reduced to a rate similar to that of the melatonin-treated cells. The same treatment on ZR75-1 cells resulted in a 20% decrease in cell number compared to that of the control group and a 29% decrease compared to that of the L-BSO/melatonin group.

To further investigate the restoration of the melatonin response by glutathione in the presence of L-BSO, glutathione concentrations were measured in MCF-7 cells at day 5. Table 3 shows that treatment with glutathione alone resulted in intracellular glutathione levels that were 65% lower than controls. Interestingly, in the group treated with glutathione combined with melatonin and L-BSO, a treatment that restored the melatonin response on growth, the level of glutathione was only restored to that of the control group and was significantly lower than that of the melatonin group. Noteworthy, however, was the relative difference in glutathione concentration in the glutathione/L-BSO/melatonin group compared to that of the L-BSO/melatonin group; this difference was even greater than that seen between the control group and the melatonin group.

**Effects of Ethacrynic Acid on the Melatonin Response in HS578T Cells.** Because the above evidence suggested that glutathione was fundamental to the oncostatic action of melatonin, we decided to test whether cells that are normally much less responsive to melatonin could be made responsive if glutathione metabolism is altered. The ER− breast tumor line HS578T was tested in a growth experiment using ethacrynic acid (1 μM), which inhibits glutathione S-transferase, an enzyme that conjugates glutathione. Treatment with melatonin (1 nM) resulted in only a 16% decrease in cell number compared to the control group (Table 4). The combination of melatonin and ethacrynic acid, however, resulted in a decrease of 63% from the control group, whereas ethacrynic acid alone had no effect on cell growth.

We also tested whether L-BSO could negate the effect of ethacrynic acid plus melatonin. When L-BSO treatment (10 μM) was combined with melatonin and ethacrynic acid, cell growth was restored to the level of melatonin treatment alone. Treatment with L-BSO alone or with L-BSO plus ethacrynic acid had no effect on growth compared to the control group.

We then measured glutathione levels at day 5, shown in Table 4. Interestingly, ethacrynic acid treatment alone had no effect on these levels. Treatment with L-BSO alone or in combination with ethacrynic acid reduced glutathione by 48% compared to the control group. This indicates that L-BSO did indeed block glutathione synthesis in these cells.

**DISCUSSION**

Previous studies from our group as well as other investigators have demonstrated that both physiological and pharmacological concentrations of melatonin inhibit the growth of ER− human breast cancer cell lines in either monolayer or suspension culture (19–31). Although physiological levels of melatonin have been shown to regulate ER and progesterone receptor expression as well as the expression of a number of estrogen-regulated oncogenes, proteins and growth factors in MCF-7 cells (41–43), the precise cellular and molecular mechanism of the oncostatic effect of melatonin on ER− breast cancer cells has remained elusive. The results of the present investigation show that depletion of glutathione with L-BSO in MCF-7 cells completely blocks the antiproliferative effect of a physiological concentration of melatonin in vitro. The same result was observed with ZR75-1 cells, indicating that the glutathione requirement is not restricted only to MCF-7 cells but may be a more generalized phenomenon among ER− human breast cancer cell lines that are sensitive to melatonin.

The role of glutathione in melatonin oncostasis was further demonstrated when the L-BSO-induced blockade of melatonin action was prevented by the addition of exogenous glutathione to both MCF-7 and ZR75-1 cultures. Although glutathione does not readily reenter most cells due to its extracellular breakdown by γ-glutamyltranspeptidase (55, 56), our observation with exogenous glutathione suggests that enough glutathione escaped extracellular degradation and entered the cells to restore the melatonin response. Alternatively, γ-glutamylcysteine synthetase may not have been completely inhibited by the low dose of L-BSO used, and thus enough enzyme activity may have persisted to resynthesize glutathione from its constituent amino acids after its extracellular degra-

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**Table 2 Effects of L-BSO and exogenous glutathione on the melatonin response in ZR75-1 and MCF-7 cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ZR75-1 cell no. (day 5)</th>
<th>MCF-7 cell no. (day 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.80 ± 0.04 x 10⁶</td>
<td>2.98 ± 0.07 x 10⁶</td>
</tr>
<tr>
<td>Melatonin</td>
<td>0.55 ± 0.03 x 10⁶</td>
<td>1.06 ± 0.03 x 10⁶</td>
</tr>
<tr>
<td>L-BSO</td>
<td>0.79 ± 0.06 x 10⁶</td>
<td>2.85 ± 0.06 x 10⁶</td>
</tr>
<tr>
<td>Glutathione</td>
<td>0.86 ± 0.04 x 10⁶</td>
<td>2.92 ± 0.10 x 10⁶</td>
</tr>
<tr>
<td>Melatonin + L-BSO</td>
<td>0.96 ± 0.05 x 10⁶</td>
<td>2.87 ± 0.09 x 10⁶</td>
</tr>
<tr>
<td>Melatonin + L-BSO + glutathione</td>
<td>0.64 ± 0.04 x 10⁶</td>
<td>1.40 ± 0.12 x 10⁶</td>
</tr>
</tbody>
</table>

* a P < 0.05 versus control.
* b P < 0.05 versus melatonin.
* c P < 0.05 versus melatonin + L-BSO.

**Table 3 Effects of L-BSO and exogenous glutathione on intracellular glutathione concentration in MCF-7 cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glutathione (nmol/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.1 ± 1.9</td>
</tr>
<tr>
<td>Melatonin</td>
<td>45.8 ± 7.4*</td>
</tr>
<tr>
<td>L-BSO</td>
<td>1.7 ± 0.7</td>
</tr>
<tr>
<td>Glutathione</td>
<td>4.6 ± 1.2*</td>
</tr>
<tr>
<td>Melatonin + L-BSO</td>
<td>1.0 ± 0.6*</td>
</tr>
<tr>
<td>Melatonin + L-BSO + glutathione</td>
<td>16.0 ± 2.1*</td>
</tr>
</tbody>
</table>

* a P < 0.05 versus control.
* b P < 0.05 versus melatonin and versus melatonin + L-BSO + glutathione.
* c P < 0.05 versus melatonin.
vation. Interestingly, the addition of exogenous glutathione alone resulted in intracellular glutathione levels that were significantly lower than those of the controls, possibly due to a negative feedback on glutathione synthesis (55, 56). This result (Table 3), along with the measurement of intracellular glutathione levels in a melatonin/L-BSO/glutathione-treated group (a measurement that was not higher than that of the corresponding control group), indicates that the absolute amount of glutathione required for the melatonin response may not be important. With the dynamic state of glutathione synthesis observed in our studies, it is not surprising that absolute glutathione concentrations are not a good indication of whether a certain cellular growth response will be observed.

The requirement of glutathione in the melatonin response prompted us to examine whether this was a specific effect of melatonin or rather an effect that was secondary to an inhibition of cell growth. Our findings support a specific requirement because L-BSO failed to block the anti-proliferative effect of tamoxifen. This observation is consistent with the notion that melatonin and tamoxifen inhibit MCF-7 cell proliferation by fundamentally different mechanisms, in spite of the fact that they share some of the same antiestrogenic capabilities (20, 22).

The decrease in glutathione concentration over time, independent of any treatment with L-BSO or melatonin, was a significant discovery. Given the earlier finding that glutathione levels increase sharply during the first 4 days of growth of 3T3 fibroblast cells in vitro (61), it is quite likely that glutathione metabolism in breast cancer cells is much different from that in normal cells. The decrease in glutathione observed in our system is most likely due to its conjugation to other compounds, possibly tied to a glutathione S-conjugate export pump (62). The dynamics of glutathione metabolism reported in our study may have implications in cancer cell resistance in vivo over a specific time period. In many cases, high glutathione levels are associated with cellular resistance to toxic agents (57). Conversely, high glutathione levels are sometimes associated with increased toxicity (57), which is consistent with the glutathione requirement observed in our melatonin studies. Because glutathione levels may be changing during tumor growth and metabolism in vivo, the timing of any glutathione-related treatment could prove to be important. For example, previous in vitro studies from our laboratory have shown that the oncostatic action of melatonin is markedly reduced if MCF-7 cells are not exposed to melatonin within 12 h after plating.4

The decrease of glutathione during growth also accounts for the elevated glutathione levels in the melatonin-treated cells. For example, the amount of glutathione in melatonin-treated cells during days 3–5 of incubation corresponds to the levels observed in control cells at the same density earlier in the growth curve. An initially elevated glutathione content in melatonin-treated cells on day 1 of culture, when cell density was identical to that of the control group, may have had a bearing on the apparent elevation of glutathione in melatonin-treated cells at the end of the culture period, when cell density was lower than that of the controls. Thus, melatonin-induced slowing of the growth rate seemed to blunt the normal decline in glutathione to create the impression that melatonin itself had actually raised glutathione levels by the end of the culture period, when in fact, the actual rates of the glutathione decline are very similar between control and melatonin-treated cells. We have also observed similar elevated levels of glutathione in MCF-7 cells treated with other growth inhibitors at the end of 5 days of incubation.

A striking result was obtained with HS578T cells. These cells, which are ERPositive, do not normally respond significantly to melatonin, but exposure to ethacrynic acid plus melatonin caused cell growth to be reduced to an extent similar to that typically observed in ERPositive cells. This finding again demonstrates that glutathione metabolism is somehow tied to the oncostatic action of melatonin, because altering an enzyme activity (glutathione S-transferase) central to this metabolism made these cells responsive to melatonin. The role of glutathione in this ERPositive system was further indicated by the blockade of the melatonin/ethacrynic acid effect with L-BSO-induced glutathione depletion. These results may have potential clinical implications because ERPositive breast tumors are usually resistant to standard treatments such as tamoxifen (63). Our results suggest that breast cancer cells could be made more sensitive to toxic agents if cellular ability to conjugate glutathione is hampered. These results may also change the prevailing ideas on how melatonin works in our in vitro system. Strong evidence supports the idea that melatonin action is linked to the presence of the ER in MCF-7 cells (20, 22, 41, 42). Our results with HS578T cells are not necessarily inconsistent with this idea because the mechanism of melatonin action may be operating through several different intracellular pathways. It is, however, inconsistent with an absolute requirement that the ER be present in order for melatonin to inhibit growth, particularly because melatonin inhibits the growth of other non-breast cancer cell lines devoid of the ER (1–3).

Depletion of glutathione may affect other cellular thiol, resulting in their oxidation to disulfides or the formation of thioesters. Glutathione depletion has been shown to cause a decrease in microtubule polymerization in cells that may relate to the oxidation of sulfhydryl groups (64). In contrast, physiological concentrations of melatonin are known to stabilize microtubules by inhibiting Ca2+/calmodulin depolymerization, which is itself a mitogenic signal transduction mechanism (65). Thus, adequate levels of glutathione may be required to maintain sulfhydryl groups of microtubule-associated proteins in a reduced state in order for melatonin to suppress Ca2+/calmodulin-mediated depolymerization of the cytoskeleton and thus cell proliferation. Indeed, other Ca2+/calmodulin antagonists have been demonstrated to inhibit MCF-7 cell proliferation (66). Furthermore, a recent report suggests that melatonin enhances the antioxidant activity of glutathione (67). Regardless of the exact nature of the interaction between melatonin and glutathione, it is clear that glutathione is involved in some fundamental way in the mechanism of melatonin action in human breast cancer cells.

As mentioned earlier, the activity of a number of cytotoxic drugs, including bleomycin, mephalan, and doxorubicin, is enhanced by L-BSO-induced glutathione depletion, whereas other cytotoxic agents such as necocarzinostatin and Taxol actually require reduced thiols for their antineoplastic activity (57, 58). It is possible that melatonin belongs to this latter category and requires a reducing intracellular milieu to exert its oncostatic action at physiological levels in human breast cancer cells.

ACKNOWLEDGMENTS

We thank Maria DeLima for assistance with figure designs.

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GLUTATHIONE REQUIREMENT IN MELATONIN ONCOSTASIS


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