Mechanisms by which energy restriction inhibits rat mammary carcinogenesis: in vivo effects of corticosterone on cell cycle machinery in mammary carcinomas

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Increased secretion of adrenal cortical steroids may account in part for its cancer inhibitory activity of energy restriction (ER). To test this hypothesis, a study was conducted to determine the effects of dietary administration of corticosterone on the post-initiation stage of mammary carcinogenesis. Eighty-four female Sprague–Dawley rats were injected with 50 mg 1-methyl-1-nitrosourea/kg body wt (i.p.) at 21 days of age. One week later, animals were randomly divided into three groups and fed control diet, or that diet to which was added 200 or 400 mg corticosterone/kg. Diets were fed for 5 weeks after which the experiment was terminated. With increasing dietary corticosterone, a dose-dependent reduction in the incidence (P = 0.03), multiplicity (P = 0.003) and size (P < 0.003) of mammary carcinomas was observed. Dietary administration of corticosterone also reduced plasma insulin-like growth factor-1 (IGF-1) and levels of IGF-1 receptor in mammary carcinomas (P < 0.01). In order to investigate molecular mechanisms underlying anticancer activity, the levels and activities of cell cycle components involved in the G1–S transition were investigated in mammary carcinomas that emerged in treated animals. Levels of cyclin D1, cyclin E, cyclin-dependent kinase (CDK)-2 and CDK-4 were reduced in carcinomas from corticosterone treated rats; whereas, levels of cyclin-dependent kinase inhibitors (CKI) Kip1/p27 and Cip1/p21 were elevated. Binding of these CKIs to both the cyclin D1–CDK-4 complex and the cyclin E–CDK-2 complex were increased and the kinase activities of these complexes were reduced with increasing dietary corticosterone. These effects were consistent with those observed in response to ER in vivo and corticosterone exposure in vitro. Whereas the effects of exogenously administered corticosterone and ER had many similarities, the lower efficacy of corticosterone versus ER in inhibiting the carcinogenic process imply that changes in cortical steroid metabolism alone are unlikely to explain the cancer inhibitory activity of ER.

Introduction

The 1-methyl-1-nitrosourea (MNU)3-induced model for breast cancer in the rat has been extensively used to study environmental factors that influence the carcinogenic process. One such factor that has been investigated is energy restriction (ER). In this model system, ER has been reported to reduce the incidence and multiplicity of chemically induced mammary carcinomas, to prolong cancers latency, and to limit the growth of the carcinomas that do occur (1,2). Comparable effects of ER also have been observed in other model systems [reviewed in refs (3,4)].

A goal of research in this area of investigation is to determine both the chemical effector(s) and the molecular target(s) by which ER inhibits the carcinogenic process. Because one of the effects that has been associated with ER is stimulation of adrenal cortical activity (5), a number of investigators have hypothesized that increased cortical steroids may account, at least in part, for the cancer inhibitory activity of ER (1.5–9). Moreover, as one consequence of ER is that cell proliferation is reduced in restricted animals (2,3), and this effect has been associated with a reduction in the ability of affected cells to progress through G1 to the S phase of the cell cycle (10), we have hypothesized that adrenal steroids might mediate their effects through the regulation of cell cycle machinery involved in the G1–S transition. More specifically, mammalian cells respond to the accumulative effects of mitogenic and anti-proliferative signals via the sequential accumulation of two G cyclins, cyclin D and cyclin E, and their binding to their respective catalytic partners, cyclin-dependent kinase (cdk)-4 and -6 and cdk-2, respectively (11). Cyclin D-dependent kinases initiate the phosphorylation of retinoblastoma (Rb) protein, a process that is completed by cyclin E–cdk-2. In turn, phosphorylation of Rb protein permits a cascade of events to occur that allow cells to enter S phase and to proceed through the remainder of the cell cycle independent of a need for additional extracellular mitogenic signaling. Glucocorticoids have been reported to modulate some of the events involved in the G1–S transition as well as to up-regulate the activity of members of Cip/Kip family of cyclin-dependent kinase inhibitors (CKI), which can alter the activity of the G cyclins and inhibit the ability of cells to enter S phase (12–15).

The objective of the experiments reported in this study was to determine the effects of an elevation of a specific adrenal steroid, corticosterone, in adrenal intact animals, and to compare the effects of this steroid on the carcinogenic response relative to the effects observed in energy restricted animals. We also determined if corticosterone has effects on cell cycle machinery involved in the G1–S transition in vitro similar to those that we have recently reported in vitro in a mammary tumor cell culture model (8). Given a recent report that ER exerts concomitant effects not only on cortical steroids but also on circulating levels of insulin growth factor (IGF-1) (16), and that the effects of corticosterone may be altered by IGFs (17), circulating levels of IGF-1 also were assessed.

Materials and methods

Chemicals

The following materials were purchased from commercial sources: anti-cyclin D1, anti-CDK-4, anti-CDK-2, anti-P21, anti-P27 antibodies (Neomarkers, 1225
Fremont, CA); Rb–GST fusion protein, protein A/G PLUS–agarose, anti-IGF-IR and anti-cyclin E antibodies, goat anti-mouse immunoglobulin- and goat anti-rabbit immunoglobulin-agarose horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, Santa Cruz, CA); histone H1 (Boehringer Mannheim, Indianapolis, IN); [γ-32P]ATP (sp. act. 3000 C/mmol; Amersham Pharmacia Biotech, Piscataway, NJ); enhanced chemiluminescence (ECL) detection system (Amersham Life Science, Arlington Heights, IL).

Carcinogen administration and dietary corticosterone
Eighty-four female Sprague–Dawley rats were obtained from Taconic Farms Germantown, NY at 20 days of age. At 21 days of age, animals were injected with 50 mg MNU/kg body wt (i.p.) as described previously (18). One week later, animals were randomly divided into three groups (28 rats/group) and fed one of the following diets for 5 weeks: modified AIN-93G (1) (control), or the AIN-93G diet supplemented with 200 or 400 mg corticosterone/kg. Rats were housed three per environment and room maintained at 22 ± 1°C and 50% relative humidity with a 12-h light/12-h dark cycle. All rats were weighed twice per week. The animal facility in which the rats were housed is AAALAC accredited. This experiment was reviewed and approved by the Institutional Animal Care and Use Committee.

Necropsy
The study was terminated after feeding the corticosterone-supplemented diets for 5 weeks according to the protocol published in (18). At necropsy, the abdominal-inguinal mammary glands from all animals were carefully excised and prepared as whole mounts as described previously (18). The whole mounts were processed for image analyses as described below.

Histopathological classification
All mammary pathologies identified in the whole mounts were processed for histopathological classification as reported previously (19).

Processing of mammary gland whole mounts for digital image analyses
Digital image analyses of mammary gland whole mounts were performed as described previously (9).

Assessment of IGF-I status
Blood samples were obtained during the necropsy procedure after the rats lost consciousness following inhalation of gaseous carbon dioxide. Blood was directly obtained from the retro-orbital sinus and gravity fed through heparin for 15 min in an Eppendorf centrifuge at 4°C (Brinkman Instruments, Westbury, NY). The lysate was collected by centrifugation for 1226 detection system. Signals were quantified by scanning the film with a ScanJet scanner (Hewlett Packard), and the intensity of the bands was analyzed using Image-Pro Plus software (Media Cybernetics).

Kinase assay
CDK-2 and CDK-4 kinase activities were determined as described previously (20). Briefly, mammary carcinomas were homogenized in Rb lysis buffer [50 mM HEPES–KOH (pH 7.5), containing 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween 20, 10% glycerol, 80 mM β-glycerophosphate, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM phenylmethysulfonyl fluoride and 10 μg/ml leupeptin and aprotinin], and using anti-CDK-4 antibody (2 μg) and protein A/G PLUS–agarose beads (20 μl), CDK-4 were immunoprecipitated from 200 μg of protein lysate per sample as described above. Beads were washed three times with Rb lysis buffer and then once with Rb kinase assay buffer [50 mM HEPES–KOH (pH 7.5), containing 150 mM NaCl, 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10 mM MgCl2, and 1 mM DTT]. Phosphorylation of Rb was measured by incubating the beads with 40 μl of radiolabeled Rb kinase solution [0.25 μl (2 μg) of Rb–GST fusion protein, 0.5 μl of [γ-32P]ATP, 0.5 μl of 0.1 mM ATP, and 38.75 μl of Rb kinase buffer] for 30 min at 37°C. The reaction was stopped by boiling the samples in SDS sample buffer for 5 min. The samples were analyzed by 12% SDS–PAGE, and the gel was dried and subjected to autoradiography. Signals were quantified by scanning the film with a ScanJet scanner (Hewlett Packard), and the intensity of the bands was analyzed using Image-Pro Plus software (Media Cybernetics).

Statistical analyses
Differences among groups in cancer incidence and cancer latency were evaluated by logistic regression and life table analyses, respectively (21). Differences among groups in the number of mammary pathologies: intraductal proliferations, ductal carcinoma in situ and adenocarcinoma (AC) per rat were evaluated by multivariate analysis of variance using final body weight as a covariate in the analysis (22). Differences among groups in the volume of mammary ACs were evaluated by Kruskal–Wallis test (21). Epithelial and total area of mammary gland were evaluated by analysis of variance following log transformation to normalize the distribution of the data; post hoc comparisons were made using Tukey’s multiple-range test (21). Data derived from western blot analyses represent semi-quantitative estimates of the amount of a specific protein that is present in a cell extract. This fact was taken into account in the statistical evaluation of the data. The data displayed in the graphs are reported as means ± SEM of the ratio (experimental/control) of the actual scanning units derived from the densitometric analysis of each western blot. All values are the means of three independent experiments. For statistical analyses, the actual scanning density data derived from the analysis of the western blots using Image Pro Plus were first ranked. This approach is particularly suitable for semi-quantitative measurements that are collected as continuously distributed data. This approach has the advantage of taking into account both the fact that levels and/or activities of proteins in a molecular pathway may not vary independently of one another, as well as the issues that exist with multiple comparisons being made on a particular set of data. Systat version 10.0 was used to perform all statistical analyses.

Results

Body weight and mammary gland development
The effects of dietary corticosterone on body weight and on mammary gland size and mammary epithelial density are shown in Tables I and II, respectively. The feeding of corticosterone resulted in a small reduction in final body weight (2–8%), an effect that was unlikely to contribute significantly to the cancer inhibitory activity (23). In order to quantify and
evaluate the differences in the development of the mammary gland, the abdominal-inguinal mammary gland chains of all animals in this study were digitized and the digital images were analyzed as described in ref. (9). The area occupied by the mammary gland fat pad and the area occupied by mammary epithelium were slightly lower in corticosterone fed rats, but these effects were not statistically significant. Only the percentage of the mammary fat pad occupied by epithelium was observed to be modestly reduced ($P < 0.02$), although the effect was not corticosterone dose dependent.

Carcinogenic response

All palpable mammary tumors identified at necropsy and suspected mammary pathologies excised from mammary gland whole mount preparations were histologically classified. Only pathologies diagnosed as intraductal proliferations, ductal carcinomas in situ, or carcinomas were observed. Dietary corticosterone treatment had no effect on the occurrence of either intraductal proliferations or ductal carcinoma in situ (data not shown). On the other hand, the incidence and multiplicity of mammary carcinomas was reduced with increasing dietary corticosterone (Table I). The median size of carcinomas was $>50\%$ lower in corticosterone-treated groups, although this effect was not dose dependent. As illustrated in Figure 1, the time to the detection of the first palpable mammary carcinoma per rat and the occurrence of multiple carcinomas per rat was delayed dose dependently in rats given dietary corticosterone. Life table analyses indicated that dietary corticosterone significantly prolonged cancer latency ($P < 0.03$).

Table I. Effect of dietary corticosterone on the carcinogenic response in the mammary gland

<table>
<thead>
<tr>
<th>Dietary corticosterone (mg/kg)</th>
<th>Number of rats</th>
<th>Carcinomas</th>
<th>Final body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Incidence % (No. TBA)</td>
<td>Average number per rat</td>
</tr>
<tr>
<td>0</td>
<td>28</td>
<td>96.4 (27)</td>
<td>3.89 ± 0.4</td>
</tr>
<tr>
<td>200</td>
<td>28</td>
<td>85.7 (24)</td>
<td>2.43 ± 0.4</td>
</tr>
<tr>
<td>400</td>
<td>28</td>
<td>75.0 (21)</td>
<td>1.98 ± 0.4</td>
</tr>
<tr>
<td>$P$-value</td>
<td></td>
<td>0.03$^a$</td>
<td>0.003$^b$</td>
</tr>
</tbody>
</table>

$^a$The effect of dietary corticosterone on cancer incidence was determined by logistic regression. The number in parentheses is number of tumor bearing animals (No. TBA).

$^b$The effect of dietary corticosterone on the average number of cancers per rat was determined by the multivariate analysis of variance using final body weight as a covariate.

$^c$The effect of dietary corticosterone on tumor volume was determined by the Kruskal–Wallis rank test; values in parentheses are the median volume of the carcinomas in that group. Note that the Kruskal–Wallis test determines whether inter-group median values are statistically different.

$^d$Determined by ANOVA; values in parentheses are the percentage of control (0 mg/kg dietary corticosterone). As body weights were different among groups, this factor was used as a covariate in those analyses that permitted its inclusion.

Table II. Epithelial and total areas of mammary gland of rat administrated with dietary corticosterone at 200 and 400 p.p.m.

<table>
<thead>
<tr>
<th>Control</th>
<th>200 p.p.m.</th>
<th>400 p.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial area (EA) (cm$^2$)</td>
<td>3.88 ± 0.17$^a$</td>
<td>3.49 ± 0.11$^a$</td>
</tr>
<tr>
<td>Total area (TA) (cm$^2$)</td>
<td>8.67 ± 0.39$^a$</td>
<td>8.61 ± 0.22$^a$</td>
</tr>
<tr>
<td>Ratio EA/TA (%)</td>
<td>45 ± 1$^a$</td>
<td>41 ± 1$^b$</td>
</tr>
</tbody>
</table>

Values are means ± SEM ($n = 28$). Data were log transformed and then evaluated by analysis of variance. Post hoc comparisons were made using Tukey’s multiple-range test. Values in a row with different superscript letters are significantly different ($P = 0.02$).
IGF-1 levels in plasma

The effects of dietary corticosterone administration on IGF-1 in plasma are shown in Figure 2. The levels of plasma IGF-1 were significantly reduced \((P < 0.01)\) by dietary corticosterone administration. In comparison with the control group, a 30 and 45% reduction was observed in rats fed 200 and 400 p.p.m. dietary corticosterone, respectively.

Cell cycle molecules in mammary carcinomas

Based on published \textit{in vivo} studies showing ER results in lower levels of cyclin D1 and increased levels of KIP1/P27 in rat mammary epithelial cells and tumors \((9,24)\), attention was focused on the expression of cyclin D1, CDK-4, cyclin E, CDK-2, KIP1/P27 and CIP1/P21. Effects of dietary corticosterone on IGF-1 receptor (IGF-1R) also were determined as circulating levels of IGF-1 were shown to be lower in corticosterone-treated rats (Figure 2). Western blot analyses were performed on mammary carcinomas the characteristics of which are summarized in Table IV. The results of those analyses are shown in Figure 3A and B. Protein levels of IGF-1R, cyclin D1, CDK-4, cyclin E and CDK-2 in mammary carcinomas of corticosterone administered rats were significantly lower in comparison with levels observed in carcinomas from control animals, and the reductions were dose dependent \((P < 0.01)\). Compared with the control rats, the reduction was 58 and 72% for IGF-1R, 28 and 36% for cyclin D1, 32 and 33% for CDK-4, 27 and 37% for cyclin E and 3 and 21% for CDK-2 in rats fed 200 and 400 p.p.m. dietary corticosterone, respectively.

Discussion

The experiments reported in this study were formulated based on work from a number of laboratories hypothesizing that adrenal cortical steroids, tentatively corticosterone in the rat, are responsible at least in part for the inhibitory activity of ER against experimentally induced cancer, a concept that was originally advanced by Boutwell \textit{et al.} (1,5–9). The animal experiment described in the Materials and methods section was designed based on data reported in ref. (9) indicating that dietary administration of corticosterone could elevate plasma corticosterone concentrations to levels comparable with those observed in adrenal intact energy restricted animals. The highest level of dietary corticosterone results in plasma corticosterone concentrations similar to those observed in 40% energy restricted animals in which profound suppression of carcinogenesis is observed in the absence of deleterious effects \((1)\). As shown in Table I and Figure 1, all parameters by which the carcinogenic response in the mammary gland was assessed were inhibited in animals fed dietary corticosterone. However, as summarized in Table III, there were marked differences in the magnitude of protection against carcinogenesis afforded by dietary corticosterone versus ER with corticosterone being much less effective. Moreover, corticosterone treatment had

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|c|}
\hline
Treatment & Plasma corticosterone & Plasma IGF-1 & Cancer multiplicity & Carcinoma volume & Mammary gland density \\
 & fold excess of control & % of control & % of control & % of control & % of control \\
\hline
Dietary corticosterone & 2.83 & 54.9 & 50.9 & 41.2 & 87 \\
ER & 2.00 & 40.7 & 6.4 & 0.1 & 61 \\
\hline
\end{tabular}
\caption{Comparison of the effects of dietary corticosterone and ER}
\end{table}
no effect on the occurrence of pre-malignant mammary gland pathologies; whereas, ER was shown previously to block the progression of intraductal mammary proliferations to mammary carcinomas (1). Similarly, while mammary gland density was reduced by dietary corticosterone, the effect was much smaller than observed in response to ER (2). Collectively, these findings are consistent with a role of corticosterone in accounting for the cancer inhibitory activity of ER, but they

![Fig. 3. Western blot analyses of IGF-I receptor (IGF-IR), cyclin D1, Cdk-4, cyclin E, Cdk-2, P27, P21, Cdk-4-immunoprecipitated P27 or P21 (IP:Cdk-4 and WB P27 or P21), Cdk-2-immunoprecipitated P21 (IP:Cdk-2 and WB P21), Cdk-4 kinase activity and Cdk-2 kinase activity in mammary carcinomas of rats fed AIN-93G (control), or the AIN-93G diet supplemented with 200 or 400 mg corticosterone/kg as described in the Materials and methods. Representative expression of proteins or kinase activities in tumor lysates determined by immunoprecipitation (IP), kinase activity assay and/or western blotting (WB) as detailed in the Materials and methods are shown and the values per each group (means ± SEM, n = 4) are listed below the bands.](image-url)
Table IV. Summary of characteristics of the animals and carcinomas on which western blotting analyses were performed

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>200 p.p.m.</th>
<th>400 p.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body weight (g)</td>
<td>165 ± 7</td>
<td>165 ± 3</td>
<td>157 ± 9</td>
</tr>
<tr>
<td>Cancer latency (days)</td>
<td>31 ± 1</td>
<td>34 ± 2</td>
<td>31 ± 4</td>
</tr>
<tr>
<td>Carcinoma size (mm³)</td>
<td>961 ± 177</td>
<td>398 ± 37</td>
<td>258 ± 22</td>
</tr>
<tr>
<td>Carcinoma/rat</td>
<td>5.5 ± 1.0</td>
<td>4.3 ± 0.6</td>
<td>3.8 ± 0.8</td>
</tr>
</tbody>
</table>

Values are means ± SEM (n = 4).

strongly indicate that alterations in the levels of this steroid alone are unlikely to fully explain the effects of ER.

A second goal of this study was to determine whether recently reported in vitro effects of corticosterone on cell cycle regulatory molecules in a mammary tumor cell line are also observed in vivo (8). We judged it was feasible to test this hypothesis in mammary carcinomas as dietary corticosterone was associated with a decrease in tumor volume (Table I). As shown in Figure 3, levels of cyclin D–CDK-4 and cyclin E–CDK-2 kinase activities were dose dependent in mammary carcinomas obtained from corticosterone treated rats, and levels of P27 were increased. These effects are consistent with the smaller tumor volumes observed in corticosterone-treated rats. As similar effects on these cell cycle regulatory molecules have been demonstrated in vivo in response to ER (10), and in vitro in response to glucocorticoids (13–15), these findings are consistent with a corticosterone-related effect on the process of cell proliferation during mammary carcinogenesis.

As an alternative to the hypothesis that increased adrenal cortical steroid secretion alone accounts for the cancer inhibitory activity of ER, our laboratory has recently proposed that inhibition is due to concomitant effects of ER on the metabolism of glucocorticoids and insulin-like growth factors, specifically IGF-1 (16). Interestingly, as shown in Figure 2, increasing dietary corticosterone was associated with a dose dependent reduction in plasma levels of IGF-1 and in the down regulation of levels of IGF1R in mammary carcinomas (Figure 3). The fact that the magnitude of the reduction in plasma IGF-1 at the highest level of dietary corticosterone was still considerably less than observed in response to 40% ER (Table III) may provide a clue to understanding why corticosterone’s effects on the carcinogenic response were considerably less than that of ER. As reported in ref. (17), the biological activity of glucocorticoids in vivo are modulated by circulating levels of IGFs. Thus, in the absence of large changes in IGF metabolism, the effect of cortical steroids might be expected to be attenuated. An obvious question that arises from these observations is whether the effect of corticosterone on mammary carcinogenesis and cell cycle machinery regulating the G1–S transition are direct or are mediated by changes in IGF metabolism. Whereas the work reported in ref. (8) in an in vitro model indicates that effects on cell cycle machinery are direct, the in vivo literature does not provide much insight in this regard. While papers can be cited in which enhancement of mammary carcinogenesis has been demonstrated in response to adrenalectomy (25), and in which inhibition of mammary tumor development has been reported in response to synthetic cortical steroids (26), the potential involvement of the IGFs in contributing to these effects was not investigated. Similarly, reports of the effects of changes in IGF metabolism on the carcinogenic process [reviewed in refs (3,4)] also have failed to investigate concomitant effects on glucocorticoid metabolism. Given the integral linkages among glucocorticoids, insulin, and IGFs in the regulation of many aspects of cellular metabolism, we judge it important for future mechanistic studies of ER on the carcinogenic process to investigate cellular effectors and molecular targets in this broader context.

In conclusion, the experiments reported in this study demonstrate a clear-cut inhibitory effect of dietary corticosterone on the carcinogenic response in the mammary gland, an effect accompanied by down regulation of cell cycle regulatory molecules involved in the G1–S transition. Despite the striking parallels between the effects of exogenously administered corticosterone and ER on these parameters, the lower efficacy of corticosterone vs. ER in inhibiting the carcinogenic process, and concomitant changes in plasma IGF-1 and levels of IGF-1 receptor in mammary carcinomas, imply that changes in cortical steroid metabolism alone are unlikely to explain the cancer inhibitory activity of ER. The extent to which corticosterone exerts effects on the carcinogenic response and/or cell cycle regulation independent from effects mediated via IGF-1 or other growth factors or hormones requires further investigation.

Acknowledgement

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References


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