Corticosterone Supplementation Reduced Selective Protein Kinase C Isoform Expression in the Epidermis of Adrenalectomized Mice[1]

Diane F. Birt,[2] Ellen Duysen, Weiqun Wang, and Ann Yaktine[3]

Department of Food Science and Human Nutrition, Iowa State University, Ames, Iowa 50011-1061 [D. F. B., W. W.]; Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, Nebraska 68198-6805 [D. F. B., E. D., A. Y.]; and Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, Nebraska 68198-4525 [D. F. B., A. Y.]

Abstract

Previous research in this laboratory demonstrated elevated plasma corticosterone and reduced protein kinase C (PKC) activity and selective isoform expression in the epidermis of dietary energy-restricted mice. Because PKC is implicated in skin carcinogenesis and because both energy restriction and glucocorticoid hormone inhibit skin carcinogenesis, the purpose of the present research was to determine whether the elevated glucocorticoid hormone in the energy-restricted mouse contributed to the changes in PKC protein expression. Two strategies were used to control corticosterone in adrenalectomized mice: (*a*) corticosterone-containing pellets were implanted in mice, and a dose response increase in corticosterone was observed with 5-, 10-, and 35-mg corticosterone implants with average peak values of 68 ± 22 ng/ml ($P < 0.01$); and (*b*) corticosterone was administered in the drinking water, and plasma corticosterone was elevated in a dose-dependent manner in mice killed at 6:00–6:30 p.m. ($P < 0.01$; peak values of 300–400 ng/ml). The expression of PKCα, PKCδ, and PKCϵ protein were not consistently altered by corticosterone with the two strategies. PKCη protein expression was elevated in the adrenalectomized mice administered 3 or 60 μg of corticosterone/ml in drinking water ($P < 0.01$). PKCζ protein expression was reduced by all doses of corticosterone in the implant or drinking water ($P < 0.05$), and a reduction of 41% was achieved with the mice administered 60 μg of corticosterone/ml in drinking water. In mice fed control or energy-restricted diet, with or without adrenalectomy, PKCζ protein was reduced in sham-operated, energy-restricted mice in comparison with control diet, sham-operated mice ($P < 0.02$), whereas PKCζ protein was not significantly different between adrenalectomized control and adrenalectomized, energy-restricted mice. These data indicate that administration of corticosterone in drinking water most closely mimicked the circulating corticosterone and epidermal PKC changes observed in dietary energy restriction. Elevated plasma glucocorticoid levels in the dietary energy-restricted mouse may contribute to the alteration of PKC protein levels in the epidermis.

Introduction

Previous research in the Birt laboratory demonstrated a selective reduction in the expression of PKC[4] α and ζ isoforms in the epidermis of dietary energy-restricted mice fed 40% of the energy intake of the control mice (1). Results also demonstrated an ~60% reduction in PKC activity in the epidermis of the energy-restricted mouse (2). Because PKC proteins have critical roles in cellular proliferation and differentiation and serve as a cellular receptor for the phorbol ester tumor promoters, we hypothesize that these alterations in PKC protein expression may be important in the inhibition of mouse skin carcinogenesis in the energy restricted mouse. However, because dietary energy restriction influences many events and probably not all of these events contribute to skin tumor prevention in the energy-restricted mouse, additional studies are needed to support this hypothesis. Research by Pashko and Schwartz (3) demonstrated the importance of the adrenal gland in the prevention of skin cancer promotion in the diet-restricted mouse. When the adrenal glands were removed, diet restriction failed to inhibit mouse skin carcinogenesis by 7,12-dimethylbenz(*a*)anthracene and TPA. Although this experiment did not demonstrate that GCH was responsible for the inhibition of cancer in the intact, diet-restricted mouse, data demonstrating cancer prevention by glucocorticoids (4) suggest that the elimination of corticosterone may have been a critical factor in the elimination of cancer prevention by diet restriction in the adrenalectomized mouse.

Results from several laboratories have demonstrated that diet-restricted mice (3) and rats (5) had elevated corticosterone secretion. Furthermore, results from our laboratory indicated a

Received 10/4/00; revised 3/8/01; accepted 3/14/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grants 95B126-REV and 97B039-REN from the American Institute for Cancer Research, Grant SIG-16 from the American Cancer Society, Grants R01 CA77451-01 and P30 CA36727 from the National Cancer Institute, and a grant from the United States Department of Agriculture-supported Center for Designing Foods to Improve Nutrition at Iowa State University. This is journal paper J-18674 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. Iowa 3360, and supported by the Hatch Act and State of Iowa funds.

[2] To whom requests for reprints should be addressed, at Department of Food Science and Human Nutrition, Iowa State University, 2312 Food Sciences Building, Ames, IA 50011-1061. Phone: (515) 294-3011; Fax: (515) 294-8181; E-mail: dbirt@iastate.edu.

[3] Present address: MGH Cancer Center, Molecular Oncology, Building 149, 13th Street, Charlestown, MA 02129.

[4] The abbreviations used are: PKC, protein kinase C; AIN, American Institute of Nutrition; ERK, extracellular response kinase; GCH, glucocorticoid hormone; GR, glucocorticoid receptor; MAPK, mitogen-activated protein kinase; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; GR, glucocorticoid receptor; AP-1, activator protein 1; adx, adrenalectomized.

Downloaded from http://aacrjournals.org/cebp/article-pdf/10/6/679/3257723/ce0601000679p.pdf by guest on 25 June 2024

10-fold elevation in corticosterone in samples collected from energy-restricted mice in the morning in comparison with control diet mice and less difference later in the day when values were high in control fed mice or late at night when values were low in both diet groups (6). The purpose of the present research was to determine whether the elevated GCH in the energy-restricted mouse mediated the changes in PKC protein expression. We used two strategies to control circulating corticosterone in the adrenalectomized mouse: corticosterone-containing implants were placed in the back of the neck in one experiment, and drinking water containing corticosterone was provided in a second experiment. In the experiment with corticosterone in the drinking water, we explored the impact of two variables: (*a*) the time of sacrifice; and (*b*) pretreatments with drinking water by gavage on plasma corticosterone concentrations. In addition, we conducted studies to show that adrenalectomy eliminated the energy restriction associated reduction in the expression of PKCζ.

Materials and Methods

Experimental Animals. Female Sencar mice were purchased from NIH (Frederick, MD) at 6 weeks of age and housed in temperature- and humidity-controlled rooms fitted with lights timed on a 12-h light/dark cycle. Water was provided *ad libitum,* and food was given freely, except in the dietary energy restriction experiment described below. All mice were housed singly to allow for accurate food intake records on *ad libitum*-fed animals and to control food intake of the restricted animals to the desired dietary level. Mice were weighed weekly throughout all experiments, and food and water consumption was recorded.

Experimental Diets. Dietary formulations were published previously (6) and were based on the standard diet recommended by the AIN (AIN-76; Refs. 7 and 8) with the micronutrient modification suggested in the AIN-93 report for mature rats (9). The control diet was modified from AIN-76/AIN-93 by: (*a*) removing the antioxidant, because we stored the diets refrigerated only for short periods of time; and (*b*) changing the carbohydrate from sucrose to a glucose-dextrin mixture, because this results in a more readily pelleted diet. The restricted diet (60% of *ad libitum* intake) was formulated by removing energy from carbohydrate and fat in a manner to keep the proportional relationship between these nutrients equivalent. These diets were enriched with protein, vitamins, minerals, and fiber to allow all restricted animals to consume the same amounts of these nutrients per day as the *ad libitum* controls. All experiments used the control diet fed *ad libitum* and the energy restriction study also used a diet with 60% of control energy intake, designated 40% energy restricted. Diets were pelleted by the cold pelleting method. Fresh diet was supplied to the mice daily at 4:00–5:00 p.m. to minimize changes in the normal circadian rhythm of plasma corticosterone seen in mice (10). All diets were stored at 4°C for not more than 3 weeks.

Adrenalectomy and Hormone Treatment Protocols for Mice. Sencar mice were bilaterally adrenalectomized at 7–8 weeks of age as described previously (11). The adrenals were removed via a dorsal approach using aseptic technique and sodium pentobarbital anesthesia. Saline (0.5 ml i.p.) was administered to the adrenalectomized mice to prevent dehydration during recovery from surgery. The adrenalectomized mice not receiving steroid replacement were given 0.9% NaCl as drinking fluid for the duration of the experiment. At the time of sacrifice, the site of operation was visually examined to verify complete removal of the adrenals. Sham adrenalectomy was conducted by the same procedure to expose the adrenals without their removal.

Corticosterone was administered by implantation of pellets containing 5, 10, or 35 mg of corticosterone under the skin at the back of the neck (Innovative Research of America, Sarasota, FL) or by providing 3, 30, or 60 μg/ml corticosterone in the drinking water. All drinking water in the studies with corticosterone in the drinking water contained 0.6% ethanol included as the corticosterone vehicle control. Mice used for the plasma corticosterone assay were taken to a room separate from other live animals and quickly decapitated upon removal from their cage to avoid a corticosterone-induced response due to handling mice with intact adrenals. Mice were killed between 7:30 a.m. and 8:30 a.m. in the implantation study and in the first drinking water study, because this was the time of day when mice were killed in our earlier studies on dietary intake on PKC expression (1). Furthermore, this is the time of the greatest difference in circulating corticosterone between control and energy restriction values in intact mice (6). In the second and third drinking water trials, mice were killed between 6:00 p.m. and 6:30 p.m., because this is the time when they would most consistently eat and drink. Thus, differences in plasma corticosterone in mice given different drinking water solutions (0–60 μg/ml corticosterone) would be most evident.

Experimental Design. Five experiments were conducted. The number of mice surviving to the end of each experiment is given with the results. The first experiment involved five groups of mice: group 1 was sham operated and implanted with a placebo pellet containing 0 mg corticosterone; the other four groups were adrenalectomized and implanted with a placebo pellet containing 0 mg of corticosterone or pellets containing 5, 10, or 35 mg of corticosterone and maintained for 5 weeks. Mice were killed at 7:30–8:30 a.m., and measurements were made on plasma corticosterone and epidermal PKCα, PKCδ, PKCϵ, and PKCζ protein levels. The second experiment similarly involved five treatment groups. One group was sham operated and given drinking water containing 0 μg of corticosterone; the other four groups were adrenalectomized and given drinking water containing 0, 3, 30, or 60 μg of corticosterone/ml and maintained for 12 weeks. Mice were killed at 7:30–8:30 a.m., and measurements were made on plasma corticosterone and epidermal PKCα, PKCδ, PKCϵ, PKCη, and PKCζ. The third and fourth experiments were designed like the second experiment, except that the mice in the third experiment were killed at 6:00–6:30 p.m., 6 weeks after surgery, and the fourth experiment excluded the adrenalectomized group given drinking water containing 3 μg of corticosterone/ml. Mice were killed at 6:00–6:30 p.m., 1 h after an oral bolus of 0.5 ml of their assigned drinking water, at 3 weeks after surgery. The fifth experiment consisted of four groups: sham-operated mice receiving control diet; sham-operated mice on the 40% energy-restricted diet; adrenalectomized mice receiving control diet; and adrenalectomized mice on the 40% energy-restricted diet. Mice were killed 15 weeks after the surgery at 7:30–8:30 a.m., and measurements were made on plasma corticosterone and epidermal PKCζ. The experiment was conducted with a 15-week sacrifice because our previous studies demonstrated that 10–15 weeks of dietary energy restriction was required to observe statistically significant alterations in PKCζ (1).

Corticosterone Measurement. Mice were decapitated, and blood was collected in glass heparinized centrifuge tubes, centrifuged, and stored at −20°C for future simultaneous RIA (12). After heat denaturation of the plasma to destroy corticosteroid-binding protein, antibody obtained from Endocrine Sciences

Downloaded from http://aacrjournals.org/cebp/article-pdf/10/6/679/3257723/ce0601000679p.pdf by guest on 25 June 2024

(Tarzana, CA) and ^{125}I-labeled corticosterone from ICN was used to assess the steroid level. Corrections were made for nonspecific binding.

PKC Western Blot Analysis. Mice were sacrificed by cervical dislocation, and dorsal skins were removed. Epidermal lysate was prepared from epidermal tissue immersed in a 55°C water bath for 30 s, followed by a 0°C water bath for 10 s. Epidermis was scraped from the dermal layer and homogenized in HBSS containing phenylmethylsulfonyl fluoride. Protein concentrations of extracts were determined by the Bradford DC assay (Bio-Rad, Hercules, CA), and equal amounts of protein extract were solubilized in 1× sample buffer to provide for loading equivalent amounts of total protein. Aliquots of protein were electrophoresed on 7.5% polyacrylamide gels. Gels were transferred onto nitrocellulose membrane, and membranes were blocked in 1% BSA, Tris-buffered saline, and 0.1% Tween 20 buffer and incubated with primary rabbit anti-peptide-PKCα antibody (Transduction Laboratories, Lexington, KY), anti-peptide PKCδ, PKCϵ, or PKCζ antibodies (Life Technologies, Inc., Gaithersburg, MD) or affinity-purified polyclonal PKCη antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The presence of the PKC isoform of interest was verified through peptide competition as described previously (1). Blots were washed and incubated with ^{125}I-labeled-goat-antirabbit IgG. Levels of immunoreactive protein were computed from the radioactivity in the appropriate bands using a PhosphorImager (Molecular Dynamics). Examples of western blots for PKC in mouse epidermis were published previously (1).

Statistics. PKC expression was log-transformed to normalize the variance for *t* test analysis and then converted to proportions for presentation. Corticosterone values, body weights, and food intake were statistically analyzed by *t* test.

Results

Body Weights and Diet Consumption. Body weights were not significantly influenced by the level of corticosterone in the implant or in the drinking water with an overall average ± SD of 31 ± 5 g at the end of the experiment for the mice given corticosterone in the drinking water. Survival was 82% in the implant and 87% in the drinking water administration studies. All of the premature deaths were among adrenalectomized groups, and 71% of these deaths occurred during the first 2 weeks after adrenalectomy. Diet consumption was not influenced by corticosterone administration in either protocol. However, water disappearance (consumption and loss) was increased in relation to the level of corticosterone administered in the drinking water. Overall daily water consumption and loss in ml was 13.5 ± 1.4 in sham-operated and adrenalectomized mice given 0 μg of corticosterone/ml; 14.1 ± 0.6 in adrenalectomized mice given 3 μg of corticosterone/ml; 16.4 ± 0.6 in adrenalectomized mice given 30 μg of corticosterone/ml ($P < 0.001$ in comparison with adrenalectomized mice given 0 μg corticosterone/ml); and 23.8 ± 0.9 in adrenalectomized mice given 60 μg of corticosterone/ml ($P < 0.0001$ in comparison with adrenalectomized mice given 0 μg of corticosterone/ml).

Plasma Corticosterone and PKC Expression in the Corticosterone Implant Study. Plasma corticosterone values in the corticosterone implant experiment are shown in Fig. 1. The sham-operated placebo mice had normal levels of circulating corticosterone for samples collected in the morning (6). Adrenalectomized control mice had no detectable plasma corticosterone. There was a dose-response increase in corticosterone with 5, 10, and 35 mg of corticosterone implants with elevations significantly greater than adrenalectomized, placebo control in the 10- and 35-mg implant groups (Fig. 1). The 5-mg implant restored values to the plasma corticosterone level in the sham-operated mice.

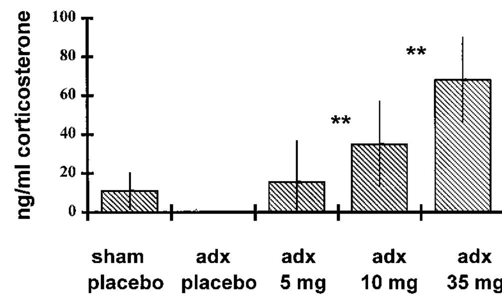

Fig. 1. Elevation of plasma corticosterone in adrenalectomized mice with corticosterone implants. Plasma was collected at 5 weeks after mice were sham operated and implanted with placebo or adrenalectomized (*adx*) and implanted with corticosterone-containing pellets at four doses (0, 5, 10, and 35 mg). Plasma corticosterone was determined by RIA. Each *column* (mean) represents data from 7 to 10 mice; *bars,* SD. **, significantly different from the adrenalectomized placebo control by two sample *t* test ($P < 0.01$). Our previous studies found peak plasma corticosterone in the range of 200–300 ng/ml in energy-restricted mice at 7:00 a.m.-4:00 p.m. (6).

PKCα, PKCδ, PKCϵ, and PKCζ were measured in the epidermis of sham-operated and adrenalectomized mice with different corticosterone implants. Values are shown as a percentage of the adrenalectomized, unsupplemented placebo control in Fig. 2. Adrenalectomy reduced PKCα protein expression, and the greatest reduction was in the adrenalectomized mice implanted with pellets containing a 5-mg pellet. PKCδ was reduced by all doses of corticosterone in comparison with the adrenalectomized mice implanted with the placebo pellets ($P < 0.05$), and there was no dose-response relationship with the concentration of corticosterone in the implant. PKCϵ was not significantly altered by adrenalectomy but was significantly reduced in the mice with 35-mg corticosterone implants. PKCζ was reduced by all doses of corticosterone implant, and the reduction was statistically significant with the 10- and 35-mg corticosterone implants in comparison with the adrenalectomized, placebo-treated control group ($P < 0.05$).

Plasma Corticosterone and PKC Expression in the Drinking Water Corticosterone Study. Plasma corticosterone was measured in mice killed at 7:30–8:30 a.m. and in mice killed from 6:00–6:30 p.m. in the experiments with corticosterone administered in the drinking water. The 7:30–8:30 p.m. measurement was done in the studies where PKC protein expression was assessed, and when no difference was observed with increases in drinking water corticosterone, we initiated an experiment to assess circulating corticosterone in mice at 6:00–6:30 p.m. This is a time when mice would be expected to be eating and drinking because of the lights just being turned off. Corticosterone values in mice killed at 7:30–8:30 a.m. averaged from 26 ± 8 to 43 ± 22 ng/ml with no differences between groups. Corticosterone in mice killed from 6:00–6:30 p.m. showed a dose-response increase in corticosterone with an increasing dose of corticosterone in the drinking water, as shown in Fig. 3*a* and *b*. In mice allowed free access to drinking water, the values were highly variable between animals, and the difference was not statistically significant (Fig. 3*a*). We suspected that the circulating corticosterone was responsive to the drinking pattern of the individual mouse and drinking behavior was not controlled in the experiment reported in Fig. 3*a*. We repeated this experiment but administered 0.5 ml by gavage of

Downloaded from http://aacrjournals.org/cebp/article-pdf/10/6/679/3257723/ce0601000679p.pdf by guest on 25 June 2024

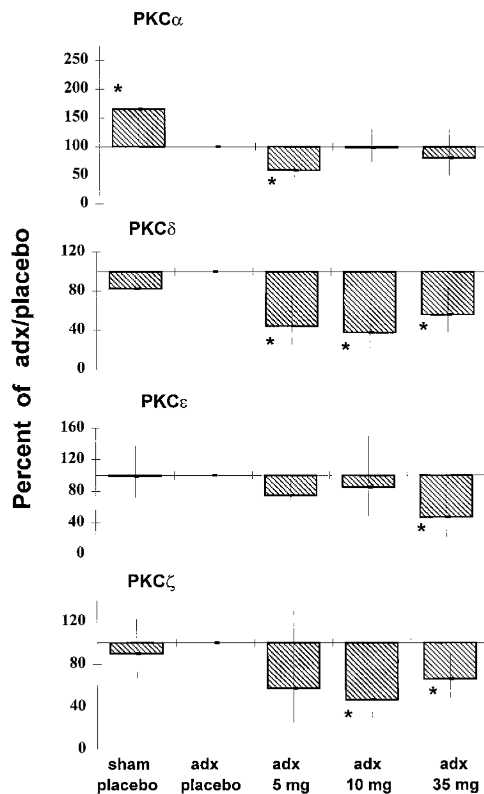

Fig. 2. The alteration of PKC isoform expression in adrenalectomized mice with corticosterone implants. Epidermis was collected at 5 weeks after mice were sham operated and implanted with placebo or adrenalectomized (*adx*) and implanted with corticosterone-containing pellets at four doses (0, 5, 10, and 35 mg). Relative protein expression of PKC isoforms was determined by Western blot analysis, and within each replicate the values for the treatment groups were presented as a percentage of the value for the adrenalectomized-placebo control group. Each *column* (mean) represents data from 8–13 mice; *bars,* 95% confidence interval. *, significantly different from the sham control by two sample *t* tests ($P < 0.05$).

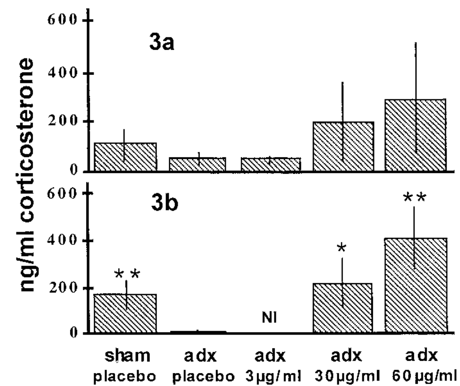

Fig. 3. Elevation of plasma corticosterone in adrenalectomized mice with corticosterone in the drinking water. Plasma was collected at the end of the experiment in mice that were sham operated and given unsupplemented drinking water or adrenalectomized (*adx*) and given corticosterone in the drinking water at four doses (0, 3, 30, and 60 μg/ml). *a,* data from mice provided corticosterone in the drinking water *ad libitum* for 5 weeks before they were killed at 1 h after the beginning of the dark cycle (6:00–6:30 p.m.). *b,* data from mice provided corticosterone in the drinking water *ad libitum* for 3 weeks and until 30 min after the beginning of the dark cycle on the day they were killed and then given 0.5 ml by gavage of their assigned water 1 h before they were killed (6:00–6:30 p.m.). Plasma corticosterone was determined by RIA. Each *column* (mean) represents data from three to five mice for *a* and from four to seven mice for *b*; *bars,* SD. *, significantly different from the adrenalectomized-placebo control by two sample *t* tests (*, $P < 0.05$; **, $P < 0.01$). *NI*, this group was not included in the experiment.

the assigned drinking water to each mouse (Fig. 3*b*). Mice were killed 1 h later, and plasma corticosterone was measured. This resulted in similar but more consistent data with a significant decrease in the adrenalectomized mice given drinking water with 0 μg of corticosterone/ml in comparison with sham-operated or intact controls (mean ± SD, 297 ± 206 ng corticosterone/ml; $n = 6$), and an increase in adrenalectomized mice given drinking water with 30 μg of corticosterone/ml ($P < 0.06$ in comparison with adrenalectomized mice given 0 μg of corticosterone/ml) or 60 μg corticosterone/ml ($P < 0.004$ in comparison with adrenalectomized mice given 0 μg corticosterone/ml).

PKCα, PKCδ, PKCϵ, PKCη, and PKCζ were measured in the epidermis of sham-operated and adrenalectomized mice given drinking water with different concentrations of corticosterone. Values are shown as a percentage of adrenalectomized, control placebo without corticosterone in the drinking water in Fig. 4. Adrenalectomy did not alter protein expression of any of the PKCs measured in this experiment because the values in the sham-operated mice did not differ from the values in unsupplemented adrenalectomized groups (Fig. 4). Furthermore, the mice that were maintained on 60 μg/ml corticosterone in the drinking water had significantly reduced PKCα protein expression ($P < 0.05$). The concentration of corticosterone in drinking water did not significantly influence PKCδ or PKCϵ protein expression. PKCη expression was significantly elevated in the adrenalectomized mice administered 3 or 60 μg corticosterone/ml ($P < 0.01$). PKCζ protein expression was reduced by all doses of corticosterone in the drinking water, and a reduction of 41% in comparison with the unsupplemented adrenalectomized group was achieved with the mice administered 60 μg corticosterone/ml.

Body Weight, Food Consumption, Plasma Corticosterone, and PKC Expression in Sham-operated and Adrenalectomized, Dietary Energy-restricted Mice. Dietary energy restriction resulted in reduced body weight as shown in Table 1, but the adrenalectomy did not alter body weight or food consumption (mean ± SE, 4.30 ± 0.01 g/day averaged over the experiment in the intact control diet group in comparison with 4.32 ± 0.05 g/day in the adrenalectomized control diet mice). Plasma corticosterone measured at 7:30–8:30 a.m. was elevated in the intact energy-restricted group (194 ± 55 ng/ml) in comparison with the intact control diet group (20 ± 5 ng/ml; $P < 0.01$). Furthermore, PKCζ protein was reduced 56% (95% confidence interval, 15–78%) in comparison with control sham-operated mice ($P < 0.02$), whereas PKCζ protein was not significantly different between adrenalectomized control and energy-restricted mice (Fig. 5). This result suggests that the elevated corticosterone may contribute to reduced PKCζ expression, but in view of the high variability between animals, other factors may also be involved.

Discussion

The results presented in this report provide evidence for corticosterone regulation of epidermal PKC protein expression in mice. Administration of corticosterone to adrenalectomized mice by an implanted pellet or in the drinking water reduced PKCζ in the epidermis in comparison with adrenalectomized mice without hormone replacement. Epidermal PKCα was re-

Downloaded from http://aacrjournals.org/cebp/article-pdf/10/6/679/3257723/ce0601000679p.pdf by guest on 25 June 2024

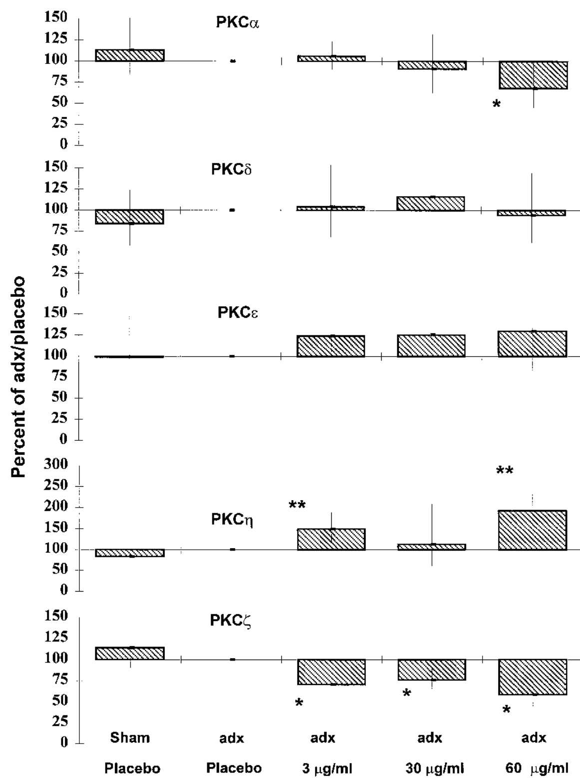

Fig. 4. The alteration of PKC isoform expression in adrenalectomized mice with corticosterone in the drinking water. Epidermis was collected at 12 weeks after mice were sham operated and given unsupplemented drinking water or adrenalectomized (*adx*) and given corticosterone in the drinking water at four doses (0, 3, 30, and 60 μg/ml). Relative protein expression of PKC isoforms was determined by Western blot analysis, and within each replicate the values for the treatment groups were presented as a percentage of the value for the sham control group. Each *column* (mean) represents data from four to six mice; *bars,* 95% confidence interval. *, significantly different from the adrenalectomized placebo control by two sample *t* tests (*, $P < 0.05$; **, $P < 0.01$).

Table 1 Body weights at the end of the experiment in control-fed and dietary energy-restricted mice that were sham operated or adrenalectomized

Sencar mice were adrenalectomized and fed the diets described in "Materials and Methods" for 15 weeks. Significant differences by two-sample *t* test, $a > b$ ($P < 0.001$) are shown.

Dietary and surgical treatment	No. of mice	Body weight (g ± SE)
Control diet/sham operated	10	33 ± 2 (*a*)
Control diet/adrenalectomized	10	31 ± 2 (*a*)
Energy restricted/sham operated	10	25 ± 3 (*b*)
Energy restricted/adrenalectomized	10	25 ± 3 (*b*)

duced, and epidermal PKCη was elevated in mice provided drinking water containing corticosterone. A role of GCH in the modulation of PKC was supported by studies from other laboratories, suggesting that GCH action in a number of other tissues may, at least in part, be mediated through PKC. For example, glucocorticoid-induced, sodium-dependent transport of P_i across opossum kidney cells required PKC (13). Furthermore, glucocorticoid-induced production of nitric oxide by murine microglial cells was shown to be mimicked by inhibition of PKC, and glucocorticoids were found to inhibit PKC in this system, suggesting that this GCH response was mediated by PKC down-regulation (14). These observations are consistent with glucocorticoid regulation of epidermal PKC protein levels.

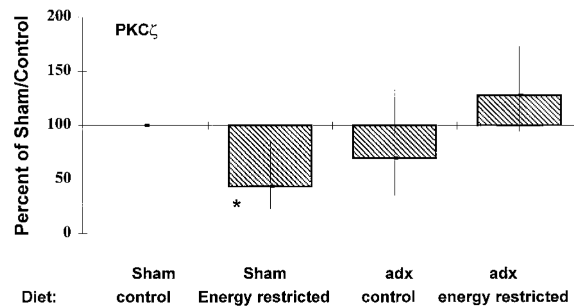

Fig. 5. Reduced PKCζ isoform expression in sham-operated, dietary energy-restricted mice but not in adrenalectomized mice. Epidermis was collected at 15 weeks in control-fed and dietary energy-restricted mice that were sham operated or adrenalectomized (*adx*). PKCζ protein was determined by Western blot analysis, and within each replicate the values for the treatment groups were presented as a percentage of the value for the sham control group. Sencar mice were adrenalectomized and fed the diets described in "Materials and Methods" for 15 weeks. Each *column* (mean) represents data from 10 to 12 mice; *bars,* 95% confidence interval. *, significantly different from the sham control by two sample *t* tests ($P < 0.05$).

Early investigations of two-stage mouse skin carcinogenesis using croton oil as the promoter first demonstrated the ability of oral or topically applied glucocorticoids to inhibit mouse skin tumor promotion (15). Additional investigations demonstrated that synthetic GCH, such as dexamethasone and fluocinolone acetonide, were potent inhibitors of TPA-induced promotion of skin carcinogenesis (4). GCH inhibits inflammatory processes and limits the proliferative response of cells in wound healing and chronic destructive diseases. The relative potency of synthetic and physiological hormones as anti-inflammatory agents correlates with their potency in the inhibition of tumor promotion (16, 17).

Pashko and Schwartz (3) demonstrated the importance of an intact adrenal gland for dietary restriction inhibition of 7,12-dimethylbenz(*a*)anthracene-initiated, TPA-promoted skin tumorigenesis. Diet restriction was not effective in the inhibition of skin tumorigenesis in adrenalectomized mice, whereas a striking inhibition of papilloma development was observed in the sham-operated mice. Pashko and Schwartz (3) showed that plasma corticosterone concentrations were doubled in the diet-restricted, sham-operated mice in comparison with the *ad libitum*-fed, sham-operated animals. Similar observations were reported for other rodent studies (18), and in self-imposed underfed humans (anorexia nervosa patients), an elevated circulating cortisol was observed, but the dietary components responsible have not been identified (19).

Our laboratory demonstrated previously that dietary energy restriction inhibited skin tumor promotion in Sencar mice (20, 21). An elevation of plasma corticosterone (6) and a reduction in selected PKC isoforms accompanied this inhibition of cancer. In particular, PKCα and PKCζ were reduced in the epidermis of the dietary energy-restricted mouse by 30–60%, but PKCδ and PKCϵ were not consistently altered in the epidermis of the energy-restricted mouse (1). We did not measure PKCη in this earlier study, but data from a more recent unpublished experiment suggested that dietary energy restriction may elevate PKCη. PKCη was at 151% of control with a confidence interval from 80% of control to 288% of control ($n = 10$ for each group). The data reported here support our hypothesis that elevation of corticosterone in the energy-restricted mouse may

Downloaded from http://aacrjournals.org/cebp/article-pdf/10/6/679/3257723/ce0601000679p.pdf by guest on 25 June 2024

cause the reduction of specific isoforms of PKC. In particular, PKC isoforms that were shown previously to be inhibited by dietary energy restriction were reduced by corticosterone administered in the drinking water (PKCα and PKCζ) or by implantation of corticosterone pellet (PKCζ), while PKC isoforms that were previously found to not be altered by energy restriction (PKCδ and PKCϵ) were not consistently altered by corticosterone in adrenalectomized mice in these experiments. The relevance of the elevation in PKCη is not clear, but it is noteworthy that PKCη expression was shown previously to be associated with epidermal differentiation (22). It is particularly interesting that dietary energy restriction and glucocorticoid administration in the drinking water were associated with reductions in the expression of PKCα and PKCζ, isoforms that are linked to epithelial proliferation (23–25), whereas these treatments were associated with an elevation of PKCη, an isoform linked with epidermal differentiation. These associations suggest that the alterations in PKC isoform expression may play a role in the inhibition of skin carcinogenesis by dietary energy restriction or GCH administration.

A rapidly growing body of literature has focused on the events that are downstream from PKC and that may be important in skin carcinogenesis. Several isoforms of PKC have been implicated in phosphorylation of Raf-1 (26, 27), a kinase that is also activated by ras in the MAPK pathway. The MAPK pathway leads to the induction of the transcription factor AP-1 through the ERK1/2 (28). Our laboratory demonstrated that dietary energy restriction inhibited signaling down the MAPK pathway and the induction by TPA of ERK1/2 (29) and of the AP-1 transcription factor and its constituent protein c-jun.[5]

Steroid hormones regulate gene expression through activation of their receptors, which can then bind to the hormone-responsive sequences in DNA or interact with transcription factors to enhance or inhibit gene expression (30). An observation of particular relevance to mouse skin tumorigenesis was the report that AP-1, a transcription regulatory protein that is induced by TPA and that activates TPA-responsive genes, forms a complex with the GR (31–33). This complex does not bind DNA or binds DNA but is not effective as a transcription factor (34), and thus activated GR inhibits the induction of gene expression by TPA.

Phorbol esters, UV radiation, tumor necrosis factor-α, and serum growth factors stimulate different signal transduction pathways, all of which ultimately converge onto the transcription factor AP-1, causing its activation (17). Jonat *et al.* (17) have demonstrated that GCH inhibits basal and induced transcription of an AP-1-inducible gene, collagenase, by interfering with AP-1. The mechanism of interference is novel in that it depends on the presence of GR, but it does not require protein synthesis (17). Studies in the Birt laboratory suggested that GR was not activated in the epidermis of the energy-restricted mouse (6). Thus, the observed inhibition in AP-1 induction in energy-restricted mice may not be attributable to interference by activated GR. Alternatively, dietary energy restriction elevation of corticosterone may reduce PKC activity and TPA-induced signaling down the MAPK pathways and thus block the induction of the AP-1 transcription factor.

It is not possible to completely mimic the plasma corticosterone fluctuations seen in energy-restricted mice, where peak values are about 200–300 ng/ml and are observed at 7:00 a.m–4:00 p.m., and the nadir was observed at 11:00 p.m. (6). However, providing corticosterone in the drinking water resulted in a circadian rhythm with the peak values in the evening and the nadir in the morning, as was reported by others for rats and mice (35, 36). Mice given implants would not have been expected to have a circadian variation, because this delivery system is designed for constant delivery. Supplementation of glucocorticoid in the drinking water was better at mimicking dietary energy restriction, because the fluctuation in daily water intake allowed a circadian variation and also provided for higher peak values to be achieved (about 300–400 ng/ml) than were seen in the plasma of the mice with implanted corticosterone-containing pellets (~70 ng/ml). These higher plasma corticosterone values were in the range of plasma corticosterone values that were reported previously in dietary energy-restricted mice (6). Furthermore, drinking water administration of corticosterone resulted in changes in epidermal PKC expression that more closely mimicked the alterations in epidermal PKC expression in dietary energy-restricted mice (1). There were striking differences between the impact of drinking water corticosterone and implants on PKCα, where the adrenalectomy caused a reduction in PKCα expression in mice that received implants but not in the experiments with drinking water supplementation of corticosterone. In addition, PKCδ and PKCϵ were reduced by the administration of glucocorticoid in an implant, but they were not influenced by glucocorticoids in the drinking water. Although the results with these two protocols may have been impacted by the fact that the mice in the drinking water protocol were under treatment longer, these observations have lead us to administer corticosterone in the drinking water in ongoing experiments designed to assess the role of GCH in the inhibition of skin cancer by dietary energy restriction.

[5] J. Przybyszewski, A. Yaktire, E. Duysen, D. Blackwood, W. Wang, A. Au, and D. F. Birt, unpublished observations.

References

1. Birt, D. F., Copenhaver, J., Pelling, J. C., and Anderson, J. Dietary energy restriction and fat modulation of protein kinase C isoenzymes and phorbol ester binding in Sencar mouse epidermis. Carcinogenesis (Lond.), *15:* 2727–2732, 1994.

2. Kris, E. S., Choe, M., Luthra, R., Conway, H., Barnett, T., Yaktine, A., and Birt, D. F. Protein kinase C activity is reduced in epidermal cells from energy-restricted SENCAR mice. J. Nutr., *124:* 485–492, 1994.

3. Pashko, L. L., and Schwartz, A. G. Reversal of food restriction-induced inhibition of mouse skin tumor promotion by adrenalectomy. Carcinogenesis (Lond.), *13:* 1925–1928, 1992.

4. Slaga, T. J., Lichti, U., Hennings, H., Elgjo, K., and Yuspa, S. H. Effects of tumor promoters and steroidal anti-inflammatory agents on skin of newborn mice *in vivo* and *in vitro*. J. Natl. Cancer Inst., *60:* 425–431, 1978.

5. Morimoto, Y., Arisue, K., and Yamamura, Y. Relationship between circadian rhythm of food intake and that of plasma corticosterone and effect of food restriction on circadian adrenocortical rhythm in the rat. Neuroendocrinology, *23:* 212–222, 1977.

6. Yaktine, A., Vaughn, R., Blackwood, D., Duysen, E., and Birt, D. F. Dietary energy restriction in the SENCAR mouse: elevation of glucocorticoid hormone levels but no change in distribution of glucocorticoid receptor in epidermal cells. Mol. Carcinog., *21:* 62–69, 1998.

7. American Institute of Nutrition report of the American Institute of Nutrition Ad Hoc Committee on Standards for Nutritional Studies. J. Nutr., *107:* 1340–1348, 1977.

8. American Institute of Nutrition Second Report of the Ad Hoc Committee on Standards for Nutritional Studies. J. Nutr., *110:* 1726, 1980.

9. Reeves, P. G., Nielsen, F. H., and Fahey, G. C., Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition Ad Hoc Writing Committee on the Reformulation of the AIN-76A Rodent Diet. J. Nutr., *123:* 1939–1951, 1993.

10. Kato, H., Saito, M., and Suda, M. Effect of starvation on the circadian adrenocortical rhythm in rats. Endocrinology, *106:* 918–921, 1980.

11. Hofert, J. F., Goldstein, S., and Phillips, L. S. Glucocorticoid effects on IGF-I/somatomedin-C and somatomedin inhibitor in streptozotocin diabetic rats. Metab. Clin. Exp., *38:* 594–600, 1989.

Downloaded from http://aacrjournals.org/cebp/article-pdf/10/6/679/3257723/ce0601000679p.pdf by guest on 25 June 2024

12. Unterman, T. G., Jemte, J. J., Oehler, D. T., Lacson, R. G., and Hofert, J. F. Effects of glucocorticoids on circulating levels and hepatic expression of insulin-like growth factor binding proteins and IGF-I in the adrenalectomized streptozotocin-diabetic rat. Endocrinology, *133:* 2531–2539, 1993.

13. Vrtovsnik, F., Jourdain, M., Cherqui, G., Lefebvre, J., and Friedlander, G. Glucocorticoid inhibition of Na-P_i cotransport in renal epithelial cells is mediated by protein kinase C. J. Biol. Chem., *269:* 8872–8877, 1994.

14. Jun, C-D., Hoon-Ryu, Um, J-Y., Kim, T-Y., Kim, J-M., Kang, S-S., Kim, H-M., and Chung, H-T. Involvement of protein kinase C in the inhibition of nitric oxide production from murine microglial cells by glucocorticoid. Biochem. Biophys. Res. Commun., *199:* 633–638, 1994.

15. Boutwell, R. K. Some biological aspects of skin carcinogenesis. Prog. Exp. Tumor Res., *4:* 207–250, 1964.

16. Schwartz, J. A., Viaje, A., Yuspa, S. H., Hennings, H., and Lichti, U. Fluocinolone acetonide; a potent inhibitor of mouse skin tumor promotion and epidermal DNA synthesis. Chem.-Biol. Interact., *17:* 331–347, 1977.

17. Jonat, C., Rahmsdorf, H. J., Park, K-K., Cato, A. C. B., Gebel, S., Ponta, H., and Herrlich, P. Antitumor promotion and anti-inflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. Cell, *62:* 1189–1204, 1990.

18. Leakey, J. E. A., Chen, S., Manjgaladze, M., Turturro, A., Duffy, P. H., Pipkin, J. L., and Hart, R. W. Role of glucocorticoids and "caloric stress" in modulating the effects of caloric restriction in rodents. Ann. NY Acad. Sci., *719:* 171–194, 1994.

19. Kennedy, S. H., Brown, G. M., McVey, G., and Garfinkel, G. Pineal and adrenal function before and after refeeding in anorexia nervosa. Biol. Psychiatry, *30:* 216–224, 1991.

20. Birt, D. F., Pelling, J. C., White, L. T., Dimitroff, K., and Barnett, T. Influence of diet and calorie restriction on the initiation and promotion of skin carcinogenesis in the SENCAR mouse model. Cancer Res., *51:* 1851–1854, 1991.

21. Birt, D. F., Pinch, H. J., Barnett, T., Phan, A., and Dimitroff, K. Inhibition of skin tumor promotion by restriction of fat and carbohydrate calories in SENCAR mice. Cancer Res., *53:* 27–31, 1993.

22. Koizumi, H., Kohno, Y., Osada, S., Ohno, S., Ohkawara, A., and Kuroki, T. Differentiation-associated localization of nPKCη, a Ca^{++}- independent protein kinase C, in normal human skin and skin diseases. J. Investig. Dermatol., *101:* 858–863, 1993.

23. Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahili, H., Mischak, H., Finkenzeller, G., Marmé, D., and Rapp, U. R. Protein kinase Cα activates RAF-1 by direct phosphorylation. Nature (Lond.), *364:* 249–242, 1993.

24. Dlugosz, A. A., Cheng, C., Williams, E. K., Dharia, A. G., Denning, M. F., and Yuspa, S. H. Alterations in murine keratinocyte differentiation induced by activated *ras*^{Ha} genes are mediated by protein kinase C-α. Cancer Res., *54:* 6413–6420, 1994.

25. Kampfer, S., Hellbert, K., Villunger, A., Doppler, W., Baier, G., Grunicke, H. H., and Überall, F. Transcriptional activation of c-*fos* by oncogenic Ha-Ras in mouse mammary epithelial cells requires the combined activities of PKC-λ, ϵ, and ζ. EMBO J., *17:* 4046–4055, 1998.

26. Ho, P. D., Zechner, D. K., He, H. P., Dillman, W. H., Glembotski, C. C., and McDonough, P. M. The Raf-MEK-ERK cascade represents a common pathway for alteration of intracellular calcium by Ras and protein kinase C in cardiac myocytes. J. Biol. Chem., *273:* 21730–21735, 1998.

27. Marais, R., Light, Y., Mason, C., Paterson, H., Olson, M. F., and Marshall, C. J. Requirement of Ras-GTP-Raf complexes for activation of Raf-1 by protein kinase C. Science (Wash. DC), *280:* 109–112, 1998.

28. Kyriakis, J. M., App, H., Zhang, X-F., Banerjee, P., Brautigan, D. L., Rapp, U. R., and Avruch, J. Raf-1 activates MAP kinase-kinase. Nature (Lond.), *358:* 417–421, 1992.

29. Liu, Y., Duysen, E., Yaktine, A., Au, A., Wang, W., and Birt, D. F. Dietary energy restriction inhibits ERK but not JNK or p38 activity in the epidermis of SENCAR mice. Carcinogenesis (Lond.), *22:* 607–612, 2001.

30. Beato, M. Gene regulation by steroid hormones. Cell, *56:* 335–344, 1989.

31. O'Sullivan, A. J., and Jamieson, J. D. Protein kinase A modulates Ca^{2+}- and protein kinase C-dependent amylase release in permeabilized rat pancreatic acini. Biochem. J., *287:* 403–406, 1992.

32. Yang-Yen, H-F., Chambard, J-C., Sun, Y-L., Smeal, T., Schmidt, T. J., Drouin, J., and Karin, M. Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. Cell, *62:* 1205–1215, 1990.

33. Schüle, R., Rangarajan, P., Sliewer, S., Ransone, L. J., Bolado, J., Yang, N., Verma, I. M., and Evans, R. M. Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. Cell, *62:* 1217–1226, 1990.

34. Miner, J. N., Diamond, M. I., and Yamamoto, K. R. Joints in the regulatory lattice: composite regulation by steroid receptor-AP1 complexes. Cell Growth Differ., *2:* 525–530, 1995.

35. Akana, S. F., Cascio, C. S., Shinsako, J., and Dallman, M. F. Corticosterone: narrow range required for normal body and thymus weight and ACTH. Am. J. Physiol., *249:* R527–R532, 1985.

36. Häusler, A., Persoz, C., Buser, R., Mondadori, C., and Bhatnagar, A. Adrenalectomy, corticosteroid replacement, and their importance for drug-induced memory-enhancement in mice. J. Steroid Biochem. Mol. Biol., *41:* 785–789, 1992.

Downloaded from http://aacrjournals.org/cebp/article-pdf/10/6/679/3257723/ce0601000679p.pdf by guest on 25 June 2024