Glucocorticoid Mediation of Dietary Energy Restriction Inhibition of Mouse Skin Carcinogenesis\textsuperscript{1,2}

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ABSTRACT Dietary energy restriction (DER) inhibits carcinogenesis in numerous animal models. DER is a potent and reproducible inhibitor of two-stage mouse skin carcinogenesis when administered during the promotion phase. Previous research demonstrated that adrenalectomy abolished cancer prevention by food restriction. Several lines of evidence suggest that glucocorticoid elevation in the DER mouse mediates the prevention of skin cancer. Our research tested the hypothesis that elevated glucocorticoid hormone activates the glucocorticoid receptor (GR) and that this activated receptor interferes with the activator protein-1 (AP-1) transcription factor. Induction of AP-1 by the phorbol ester tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) is essential to tumor promotion. We have been unable to demonstrate elevated activated GR in the epidermis of the DER mouse, perhaps because only indirect strategies have been possible with the use of epidermis from DER mice. However, DER blocked the induction of AP-1 and c-jun, a constituent protein of AP-1, in the epidermis of mice. Current studies are focused on the induction of AP-1 and we propose this as an essential component of the mechanism of DER prevention of mouse skin carcinogenesis. J. Nutr. 129: 571S–574S, 1999.

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Dietary energy restriction (DER)\textsuperscript{4} is a potent and reproducible inhibitor of carcinogenesis at a wide variety of sites, including the mammary gland, colon, liver and skin (Birt et al. 1998). Research in the Birt laboratory has focused on the inhibition of skin carcinogenesis initiated by 7,12-dimethylbenz(a)anthracene (DMBA) and promoted by 12-O-tetradecanoylphorbol-13-acetate (TPA) in DER mice. Our initial studies compared food restriction (feeding less of the control diet) with energy restriction, i.e., feeding a reformulated diet in which reductions in fat and calorie intakes are instituted by dietary energy restriction, i.e., feeding a reformulated diet in which reductions in fat and calorie intakes are instituted with constant intake of all other nutrients. Our results demonstrated a striking inhibition of carcinogenesis with both approaches; however, energy restriction was more effective than food restriction, particularly in reducing the growth of tumors (Birt et al. 1991). Further studies demonstrated that although energy restriction from either fat or carbohydrate was effective in inhibiting skin carcinogenesis, reduction of fat calories was somewhat more effective in the inhibition of papillomas (Birt et al. 1993). Finally, we demonstrated a dose response inhibition in skin carcinogenesis when an intermediate (20% restricted vs. control) and more restrictive (40% restricted vs. control) intake was imposed with control of fat intake. However, the intermediate dietary restriction was less effective in preventing skin carcinogenesis when dietary fat intakes were high (Birt et al. 1996). Numerous mechanisms...
have been suggested for the prevention of cancer by energy restriction; these include reduced oxidative stress, reduced proliferation, increased apoptosis, induction of DNA repair and enhancement of other processes involved in protection (Birt et al. 1998). It has been hypothesized that at least some of the protective properties of dietary energy restriction are due to modulation of the hormonal milieu (Birt et al. 1998). Food restriction and DER have been shown to reduce the secretion of some hormones and increase that of others. This paper will focus on the role of elevated glucocorticoid hormone in DER rodents in the prevention of skin carcinogenesis.

It has long been known that glucocorticoid hormones are potent inhibitors of skin carcinogenesis. In particular, Belman and Troll (1972) reported that topical application of steroidal anti-inflammatory glucocorticoid analogues inhibited skin carcinogenesis in direct proportion to their anti-inflammatory activity. Furthermore, dietary administration of glucocorticoid hormone was also effective in skin cancer prevention (Trainin 1963). More recent studies demonstrated that food restriction with reduction of all components of the diet (Pashko and Schwartz 1992) and energy restriction, with reduction in fat and carbohydrate (Yaktine et al. 1998) elevated glucocorticoid hormone in mice. The DER study demonstrated a 10-fold higher level of plasma corticosterone in the restricted mice than in controls at 0700 h. Research by Pashko and Schwartz (1992) demonstrated the dependence of food restriction prevention of skin carcinogenesis on an intact adrenal gland. Food-restricted sham-operated mice had few papillomas after 12 wk of TPA in DMBA-initiated mice, whereas high rates of papilloma developed in food-restricted adrenalectomized mice as well as in the sham-operated and adrenalectomized fully fed controls. This experiment did not demonstrate that glucocorticoid hormone was the critical hormone. However, there is currently no evidence that other adrenal hormones such as aldosterone or epinephrine contribute to skin carcinogenesis; thus the most direct and rational hypothesis is that elevated glucocorticoid hormone was responsible for the observation. More recent studies indicated that adrenalectomy similarly blocked the inhibition of lung carcinogenesis in diet-restricted animals (Pashko and Schwartz 1996).

Glucocorticoid hormone (GCH) acts by binding to an intercellular receptor, the glucocorticoid receptor, a member of the steroid hormone receptor superfamily (Cato and Wade 1996). Hormone binding alters association with heat shock proteins and translocates the receptor to the nucleus where the activated receptor acts as a transcription factor. A body of research has demonstrated interactions between the steroid hormone receptor superfamily members and members of the activator protein-1 (AP-1) transcription factor family (Pfahl 1993). Such interactions are of particular interest because AP-1 induction by TPA is required for tumor promotion, and the interaction between the activated GR and AP-1 interfered with transcription from both GR- and AP-1-regulated genes (Pfahl 1993). These observations provide the foundation for our hypothesis that dietary energy restriction inhibits mouse skin carcinogenesis by elevating GCH and GR, and the activated GR interferes with AP-1 transcriptional regulation.

Our research on this hypothesis developed along two tracts, one assessing the activation of the GR in the DER mouse and the other assessing the effect of DER on the activation of the AP-1 transcription.

**GLUCOCORTICOID ACTIVATION IN DER MOUSE EPIDERMIS**

Studies assessed the activation of the GR in epidermis from control and DER mice (40% reduction in calories by reducing fat and carbohydrate) by the following three approaches: 1) measurement of total GR protein by Western blot analysis; 2) measurement of activated GR by gel mobility shift by using a labeled GR consensus sequence to identify binding activity; and 3) localization of GR protein in the epidermal cells to determine if distribution in cytoplasm and nucleus differed between the diet groups. For each of these experimental approaches, mice were prefed the control or DER diet for periods of 8–15 wk. For the studies on GR protein, Western blot was performed with the anti-GR antibody, BuGR-2, as described (Yaktine et al. 1998). Both the GRα and GRβ isoforms have been observed in skin; the antibody used for this research detected both of these forms without discrimination by subtype. Mice were killed at 0700, 1600 and 2300 h, and the results of this experiment indicated no consistent change in the GR protein with diet. Binding of protein to the GR consensus sequence to assess activated receptor was conducted on nuclear extracts of mice prefed control and DER diets. These results clearly indicated that nuclear extracts of DER mice had less binding activity than extracts from control mice. This was the opposite of what would be expected if DER resulted in activation of the GR. Finally, localization of GR protein in the epidermal cell was assessed using immunogold and electron microscopy. This experiment indicated no significant difference in the ratio of cytoplasmic:nuclear receptor in epidermis from the control and DER mice (Yaktine et al. 1998). An increase in nuclear localization of GR would have been expected if GR activation was elevated in the epidermis of DER mice.

Thus, none of these three lines of evidence suggested that DER resulted in activated GR. However, it is important to note that these are all indirect measurements of GR activation. Because the aim of these studies was to compare the effect of control and DER protocols, it was necessary to assess GR activation in mouse skin. To measure steroid hormone binding activity of the epidermis, it would be necessary to isolate mouse epidermal cells. However, such isolation would require heat or cold treatment of the epidermis and this would alter the heat shock proteins that are critical in the binding of the receptor with the hormone and activation of the receptor. This alteration would make dietary differences difficult or impossible to detect.

**AP-1 ACTIVATION IN THE EPIDERMIS OF DER MICE**

Because AP-1 is induced by the tumor promoter used in our mouse skin carcinogenesis studies and because AP-1 induction has been identified as critical to tumor promotion by TPA, we compared AP-1 induction by TPA in control and DER mouse epidermis. Mice were prefed control or DER protocols for 8–15 wk before treatment with 3.2 nmol TPA in 0.2 mL acetone, the same dose used in the carcinogenesis studies described above. AP-1 induction was assessed by measuring the binding of a labeled consensus sequence to proteins in the nuclear extract from control and DER epidermal cells at 1 and 4 h after treatment with TPA (unpublished data). Maximal induction of AP-1 binding was observed at 1 h after TPA treatment in epidermis from both control and DER mice. In control epidermis, TPA induced a 70–80% increase in binding of nuclear proteins to the AP-1 binding consensus se-
quence on DNA in comparison with untreated control epidermis. In nuclear extract from DER mice, the induction by TPA was only 20–30% above untreated epidermis (~33% of the induction indicated above in control mice). At 4 h after TPA treatment, AP-1::DNA binding activity was not significantly induced in either control or DER epidermis.

The studies described above on GR activation in the DER mouse epidermis did not suggest that DER causes activation of GR that would interfere with AP-1. Thus, we assessed a constituent protein of AP-1, c-Jun, over the period of TPA induction in control and DER mice. This study was designed to determine if some of the inhibition in AP-1 induction was due to an inhibition in the induction of the constituent proteins for AP-1. Studies were conducted on c-jun at the message and protein levels by using Northern and Western blot strategies, respectively. Our results demonstrated an inhibition of c-jun message at 1 and 3 h after TPA in the epidermis of DER mice in comparison with control mice and a parallel inhibition in c-jun protein at 1, 6 and 24 h in these mice (unpublished data). These results suggest that the observed inhibition in AP-1 activation in the DER mouse epidermis was due to reduced induction in the constituent proteins of AP-1. Additional studies will be required to assess other constituent proteins of AP-1 and determine if our observations with c-jun are representative of other proteins that partner to form the AP-1 transcription factor.

### CELLULAR SIGNALING THAT INDUCES AP-1 TRANSCRIPTION

Three interacting pathways are under investigation for the induction of AP-1 transcription (Fig. 1). These are generally referred to as the stress-activated protein kinase pathways and more specifically as the c-Jun N-terminal kinase (JNK), the mitogen-activated protein kinase (MAPK) and the p38 HOG pathways. We have conducted preliminary studies on the JNK and MAPK pathways, but we have not yet assessed the p38 HOG pathway.

JNK was of particular interest to our studies because it results directly in the induction of c-jun mRNA and protein. JNK activation by UV light results in phosphorylation of c-jun protein on Ser 63 and 73, and c-jun protein with these sites phosphorylated binds to form a homodimer that activates AP-1 transcriptional activation and drives the production of additional c-jun mRNA and protein. Our preliminary studies assessed JNK in the epidermis of control and DER mice and we observed no change in the activation of this kinase (unpublished data). Thus, it did not appear that DER inhibition of c-jun and AP-1 induction could be caused by inhibition of JNK.

The MAPK pathway was of interest for our investigations because protein kinase C (PKC) plays a role in the activation of the MAPK pathway; our earlier investigations, as described below, demonstrated striking inhibition of specific PKC isoforms in the epidermis of DER mice. MAPK is activated by convergence of ras and PKC on Raf-1, a serine kinase. Raf-1 activates MAPK kinase (MEK) that induces extracellular response kinase (ERK). Activation of ERK results in induction of nuclear ELK that directly induces c-fos and indirectly induces c-jun. Studies are underway to determine whether TPA induction of ERK 1 and 2 is inhibited in the epidermis of DER mice.

### INFLUENCE OF DER ON PKC ACTIVITY AND EXPRESSION

Our earlier studies determined the effect of DER on PKC activity and expression of specific isoforms of PKC. Studies on the influence of restriction of energy from fat or carbohydrate on PKC activity in epidermal cell cytosolic and particulate fractions were conducted. Particulate PKC activity was reduced in rats prefed diets restricted in fat or carbohydrate in comparison with the control groups (Kris et al. 1994). The reduction was greatest in cells from mice fed the high carbohydrate/restricted diet. These data show that particulate PKC activity was reduced to the greatest extent in the diet groups that developed the fewest papillomas and carcinomas in the tumor study. To determine whether dietary modulation of PKC activity as described above and reported previously by our laboratory (Choe et al. 1992, Donnelly et al. 1987, Kris et al. 1994) was due to changes in PKC protein, we assessed the presence of PKC isoenzymes in the Senear mouse epidermis and determined the influence of diet on the isoenzymes found (Birt et al. 1994). Mice were fed control or DER (60% of dietary energy) diets for 6–29 wk, their epidermis was collected and Western blot was used to assess PKC protein expression. We detected PKC α, ε, δ and ζ; dietary energy did not modify the presence of PKC ε or δ (Birt et al. 1994). We observed a reduction in PKC α and ζ in the epidermis of DER mice in comparison with control mice in cytosolic and particulate protein. For example, DER reduced the overall amount of PKC ζ in cytosol and particulate by 42 and 59%, respectively.

Recent observations from our laboratory provide evidence that corticosterone has a role in the regulation of PKC protein expression. We conducted preliminary studies on the effect of corticosterone in the drinking water on PKC α, ε, δ and ζ expression in mouse epidermis. Mice were bilaterally adrenalectomized and corticosterone was provided in the drinking water. They were maintained with 0, 3, 30 or 60 μg cortico-
sterone/mL drinking water for a period of 6–12 wk. Circulating corticosterone and epidermal PKC α, ε, δ and ζ were measured by radioimmunoassay and Western blot, respectively. A dose-response relationship was observed in plasma corticosterone, with the highest dose resulting in an average of ~270 ng corticosterone/mL plasma. Adrenalectomized mice that were not given corticosterone in the drinking water experienced no change in PKC ζ expression in comparison with sham-operated mice. Corticosterone in the drinking water resulted in decreased expression, and the difference was significant with treatments of ≥3 μg/mL. PKC α was similarly reduced by increasing doses of corticosterone, but PKC ε and δ were not consistently influenced with increasing doses of corticosterone. These results parallel our earlier observations on DER effects on PKC protein (PKC α and ζ, reduced in DER mice; Birt et al. 1994) and support our hypothesis that expression of some isoforms of PKC (α and ζ) in mouse epidermis are responsive to circulating corticosterone and reduced by the elevated hormone in the DER mouse, whereas other isoforms (e.g., PKC ε and δ) are not responsive.

**SUMMARY AND CONCLUSIONS**

These results suggest that the inhibition of the induction of AP-1:DNA binding activity and c-jun mRNA and protein may be through inhibition of MAPK signaling. Our earlier results suggest that this may be caused by the inhibition of the expression of specific PKC isoforms (α and ζ). Our studies on PKC expression in control and DER mice that are either intact or adrenalectomized suggest that elevated GCH may play some role in the inhibition of PKC isoforms in the DER mouse. Our present working hypothesis is that elevated GCH in the DER mouse reduces the amount and activity of PKC isoforms important in the activation of the Raf-1/MAPK pathway. We further hypothesize that this reduction in kinase activity attenuates the induction of c-jun and AP-1. Because activation of AP-1 transcription by TPA was obligatory for tumor promotion, we suggest that inhibition of this pathway by DER contributes to the inhibition of skin carcinogenesis. We suggest that DER prevention of skin cancer through hormonal mediators of transcription may be extrapolated to other models in which DER inhibits carcinogenesis, but the hormonal players and relevant signaling pathways may differ, depending on those pathways that are active in the tissues of interest.

**LITERATURE CITED**


