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Identification of Molecular Targets for Dietary Energy Restriction Prevention of Skin Carcinogenesis: An Idea Cultivated by Edward Bresnick

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Abstract Dietary energy restriction (DER) has long been known to strikingly inhibit carcinogenesis in many animal models. The animal data has been corroborated by recent and ongoing epidemiological studies demonstrating the importance of energy balance, physical exercise and obesity in human cancer. Dr. Edward Bresnick provided key insights into this important area of research and pivotal direction for the author's research while he served as Director of the Eppley Institute for Research in Cancer, Omaha, NE. These insights moved this research toward demonstrating that DER reduced the expression of key protein kinase C isoforms in mouse skin. More recent studies have uncovered downstream events that are inbibited by DER including blockage of tumor promoter activation of Raf-1, ERK 1,2 and AP-1 expression. Parallel studies have demonstrated the DER inhibition of these key cellular signaling events in mouse skin carcinogenesis are dependent upon an intact adrenal gland because adrenalectomized mice fed DER diet did not have reduced tumor burden or inhibited signaling and blocked AP-1 activation as was observed in DER mice with intact adrenal glands. In addition, the DER inhibition of tumorigenesis and AP-1 signaling was restored in adrenalectomized mice that were given corticosterone in the drinking water. This showed that in mice in the chemical carcinogenesis protocol glucocorticoid hormone plays a major role in mediating DER prevention of cancer. Studies are ongoing to further assess the mechanism of DER modulation of skin cancer by assessing impacts on transcriptional regulation and expression of genes that are critical in skin carcinogenesis. J. Cell. Biochem. 91: 258–264, 2004.

Key words: dietary energy restriction; cancer prevention; glucocorticoid hormone; carcinogenesis; activator protein-1

Long standing research in the Birt laboratory demonstrated that dietary energy restriction (DER) was a potent inhibitor of 7,12-dimethylbenzanthracene (DMBA) induced and 12-0-tetradecanoyl-13-phorbol ester (TPA) promoted mouse skin carcinogenesis [Birt et al., 1991]. Our first venture into assessing the molecular mechanism for this prevention involved assessing the impact of DER on protein kinase C (PKC) activity and isoform expression. Dr. Edward Bresnick, then Director of the Eppley Institute was instrumental in facilitating collaborations that linked the Birt laboratory investigations with the pharmacological studies of Dr. Thomas Donnelly who was interested in PKC and with the molecular carcinogenesis work of Dr. Jill Pelling who continues to consult and collaborate in this endeavor. Ed was truly in his element in serving as a marriage broker between scientists with diverse backgrounds. He particularly encouraged the Birt laboratory to move in the direction that provided the foundation for our work on establishing targets for DER prevention of cancer. Then, as the research became more successful, Ed would seek appropriate appointments to further expand the horizons of his faculty.

I remember in particular a long discussion in Ed's office following a seminar given by Tom Donnelly on PKC, at that time a recently identified calcium and lipid-regulated kinase. Ed kept rapidly stating, "yes, yes, yes" as I was laying out tentative hypothesis. I finally asked him, do you know that there is data in support of this hypothesis or? And he replied meekly that his "yes" was just to show his interest

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and enthusiasm. Talking science with Ed was always fun.

ENERGY INTAKE AND CANCER

Excessive dietary energy intake appears to be an important factor in the high rates of cancer in affluent Western society. Furthermore, the recent trends of increasing energy consumption and obesity in parts of the developing world may be a prelude to increasing cancer rates in these societies. The International Agency for Research in Cancer (IARC) evaluated the role of weight control and physical activity in cancer prevention and concluded "there was sufficient evidence that excess body weight increases the risk of cancers of the colon, breast in postmenopausal women, endometrium, kidney, and adenocarcinomas of the oesophagus." Regarding physical activity, the IARC judged that "there was sufficient evidence for a cancer-preventive effect of physical activity against cancers of the colon and breast" [International Agency for Research on Cancer Handbooks of Cancer Prevention, 2002].

Dietary energy intake has been identified as a potent factor in cancer in population-based [Berg, 1975] and case control [Miller et al., 1983] studies. There were positive correlations between cancer of the breast [Berg, 1975] and colon [Miller et al., 1983], and energy intake, in conjunction with an association between cancer and high dietary fat intake. It has not been possible to clearly separate a high intake of energy from a high-fat diet in human studies. In addition, obesity and, in some cases, sedentary lifestyles (low energy expenditure) have been identified as risk factors for several forms of human cancer including; endometrial [Olson et al., 1995], breast [Zhang et al., 1995], and colon [Giovannucci et al., 1995] cancers.

Experimental animal studies have demonstrated a relationship between reduced dietary energy intake and tumor rates [Kritchevsky, 1992]. Early energy restriction studies often used the well established mouse skin carcinogenesis model [Tannenbaum, 1942]. Inhibition of breast, liver, colon, and pancreas cancer was also been reported in energy-restricted animals [Kritchevsky, 1992]. We have focused our efforts on elucidating the targets for DER prevention of the promotion phase of skin carcinogenesis because prior studies observed an inhibition of TPA induced skin tumor promotion in mice fed diets restricted during promotion [Birt et al., 1991]. However, restriction of 40% energy at the time of DMBA application did not influence skin carcinogenesis [Birt et al., 1991].

DER AND GLUCOCORTICOID HORMONES AS MEDIATORS OF ENERGY RESTRICTION INHIBITION OF SKIN CANCER

Early investigations of two-stage mouse skin carcinogenesis using croton oil as the promoter first demonstrated the ability of oral or topically applied glucocorticoid hormones (GCH) to inhibit mouse skin tumor promotion [Boutwell, 1964]. Further investigations demonstrated that synthetic GCH such as dexamethasone and fluocinolone acetonide were potent inhibitors of TPA induced promotion of skin carcinogenesis [Slaga et al., 1978]. GCH inhibit inflammatory processes and limit 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 [Schwartz et al., 1977].

A role for DER in modulating circulating GCH was suggested by elevated circulating corticosterone in DER mice as shown by us [Yaktine et al., 1998], and as was previously observed in other rodent studies [Pashko and Schwartz, 1992]. Furthermore, in self-imposed underfed humans (anorexia nervosa patients) an elevated circulating cortisol has been observed but the dietary components responsible have not been identified [Kennedy et al., 1991].

The importance of an intact adrenal gland for DER inhibition of DMBA-initiated, TPA promoted skin tumorigenesis was demonstrated by our recent investigation. DER was not effective in the inhibition of skin tumorigenesis in adrenalectomized (ADX) mice while a striking inhibition of papilloma development was observed in the sham-operated mice (Fig. 1). These observations demonstrate that the adrenal gland was required for DER prevention of skin papillomas and carcinomas. Additional ad libitum (AL)/ADX and DER/ADX groups received GCH in drinking water (corticosterone, the primary murine GCH) to mimic the elevated GCH in intact DER mice. The development of the multiplicity of combined carcinoma in situ and invasive carcinoma were modulated in parallel with papilloma development.

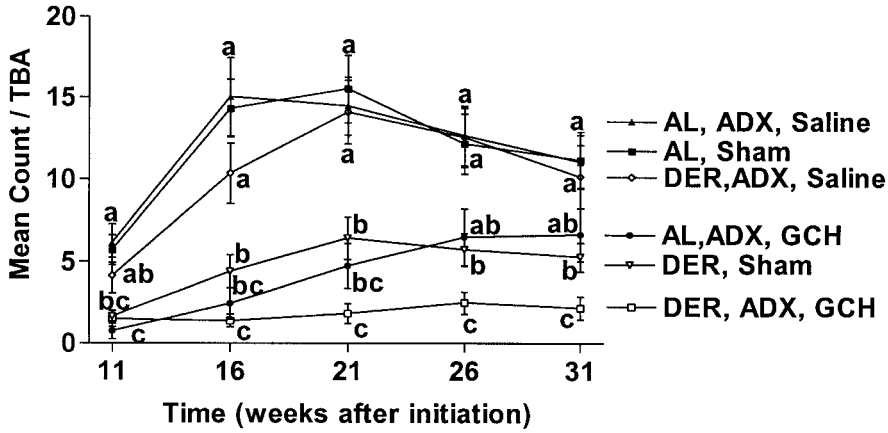

Fig. 1. Papilloma numbers (mean ± SD) per tumor bearing mouse; mice were initiated by DMBA and promoted 2×/week for 10 weeks with TPA. GCH was administered as corticosterone in the drinking water (60 ng/μl). Mean ± SEM. a < b < c at each time point ($P < 0.01$). N = 8–41.

Two recent studies measured circulating GCH in sham operated and ADX mice, with and without corticosterone supplements, which were fed AL and DER diet. Blood was collected at 10–15 weeks of treatment and in sham operated mice, DER elevated circulating corticosterone while corticosterone was not elevated in DER/ADX mice [Przybyszewski et al., 2001]. Furthermore, addition of GCH to ADX mice elevated circulating GCH and this is more apparent in measurements later in the day [Liu et al., 2001]. The tumor study demonstrated that supplementation with GCH restored the inhibition in the DER/ADX mice and caused an inhibition of carcinogenesis in the AL/ADX diet mice, the greatest reduction in carcinomas and papillomas throughout the study was in the DER/ADX/GCH group (Fig. 1). Our tumor study determined that DER inhibition of skin tumor promotion was in large measure mediated through increasing GCH.

DER AND GCH AND PKC

Our early studies on the potential role of PKC modulation by diet in the prevention of skin carcinogenesis looked at dietary fat and energy intake on PKC activity. High fat diets increased [Donnelly et al., 1987; Birt et al., 1989] while DER diets dramatically reduced [Birt et al., 1989] PKC activity in mouse epidermis. We later assessed fat and energy effects on PKC isoform expression by Western blot [Birt et al., 1994]. We observed a reduction in PKCα and ζ in epidermis of mice fed 40% DER diets while PKCε and δ levels were not influenced by DER [Birt et al., 1994]. Further studies demonstrated a reduction in PKCα and PKCζ in the epidermis of ADX mice with corticosterone replacement in comparison with ADX mice without hormone replacement [Birt et al., 2001].

DER, AP1, AND SKIN CANCER

Compelling data have demonstrated that AP1 transcription factor induction is essential for mouse skin tumor promotion. Since AP-1 is down-stream of PKC it was reasonable to think that DER might alter AP-1 signaling. An investigation in the Colburn laboratory found that transgenic mice carrying a transactivation defective mutant c-Jun (TAM67) driven by the keratin-14 promoter developed few papillomas when treated in a phorbol ester tumor promotion protocol in comparison with the background mice ($B6D2/F_1$) or negative siblings where mice experienced an average of 8–9 papillomas/mouse at 18–20 weeks. This showed that transactivation by c-Jun was required for tumor promotion [Young et al., 1999]. In addition, studies with mice expressing a c-Jun mutant that lacked the phosphorylation sites (Ser 63 and Ser 73) had reduced sensitivity in mouse skin tumorigenesis models, further suggesting that the activation of c-Jun was critical for tumor promotion [Behrens et al., 2000]. These studies demonstrate that ERK1,2, c-Jun, and AP1 induction are essential for mouse skin tumor promotion.

Our studies established that TPA inducible AP1 transcription factor binding activity was reduced in the epidermis of the DER

mouse. Furthermore, adrenalectomy restored AP1:DNA binding activity in the DER mouse epidermis and corticosteroid supplementation in the drinking water inhibited AP1:DNA binding in both AL and DER mice [Przybyszewski et al., 2001]. This investigation demonstrated the importance of changes in glucocorticoid hormone in the DER mouse for the inhibition of AP1:DNA binding and investigations revealed that DER blocked c-jun protein and mRNA induction by TPA but that ADX did not restore the AL pattern of c-jun induction [Przybyszewski et al., 2001]. This result indicates that DER and ADX may differentially influence the constituent proteins of AP1.

DER AND AP1 CELL SIGNALING

Phorbol esters, UV radiation, tumor necrosis factor-alpha, and serum growth factors stimulate different signal transduction pathways, all of which ultimately converge onto the transcription factor AP1, causing its activation [Jonat et al., 1990]. AP1 activation by external stimuli such as the induction by TPA is mediated by several kinase pathways (Fig. 2). Furthermore, DER did not modify the basal JNK or p38 kinase activity. JNK is responsible for phosphorylation of c-Jun protein on serine 63 and serine 73 and phosphorylation of these residues induces c-Jun incorporation into AP1 and this AP1 then drives the further transcription of c-Jun [Angel and Karin, 1991]. The p38 HOG MAP-kinase pathway was found to be necessary for transcriptional activation of p53 by toxic stress genes such as those induced by chemotherapeutic agents [Sanchez-Prieto et al., 2000]. Furthermore, selective inhibitors of p38 kinase blocked apoptosis in colonic cancer cells suggesting a role for p38 in apoptosis in this cell line [Miki et al., 1999]. These studies suggest that the p38 kinase pathway may be more important in the control of cell proliferation through apoptosis than it is in the support of cell proliferation.

Raf-1/MAP-kinase pathway is also instrumental in the induction of the AP1 transcription factor. The importance of this kinase pathway in transformation is apparent from the constitutive activity of MAP-kinase kinase (MEK) and ERK1,2 in v-raf transformed cells [Kyriakis et al., 1992]. Tumor promoter TPA activation of AP1 may involve PKC phosphorylation of Raf-1, which leads to activation of the MAP-kinase pathway of MEK, ERK1, ERK2, and ERK3 that activate c-fos transcription and phosphorylation. Then, c-fos may act by forming AP1 to induce c-Jun transcription. Conventional PKC (α, β, and γ) but not novel PKC (δ, ε, or η) activated c-Raf in a TPA dependent manner in baculovirus systems using overexpression of the PKC isoenzymes and cRaf [Sözeri et al., 1992]. Glucocorticoid mediation of PKC effects on the Raf-1/MAP-kinase pathway was suggested by studies in which a low concentration of dexamethasone inhibited a step before Raf in the activation of MAP-kinase in mast cells [Rider et al., 1996].

Investigations in SENCAR mice demonstrated that DER blocked activation of ERK1,2 by a single treatment of TPA but DER did not influence the activity of JNK or p38 kinase in TPA treated mice, suggesting that ERK is the

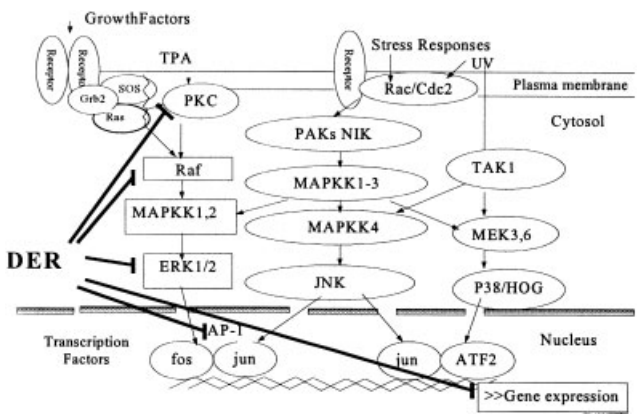

Fig. 2. Critical pathways in AP-1 activation that are inhibited by DER. TPA, 12-tetradeconylphorbol-13-acetate; UV, ultraviolet light; PKC, protein kinase C; MAPKK, mitogen-activated protein kinase kinase; ERK, extra cellular response kinase; JNK, jun-N-terminal kinase; AP-1, activator protein-1; PAKs, p21-activated kinases; NIK, NF-κB-inducing kinase; TAK, TGF-activated kinase; ATF2, activating transcription factor-2.

pathway blocked by DER that results in inhibition of the induction of AP1 by TPA [Liu et al., 2001] (Fig. 2). A similar blockage by DER of ERK1,2 activation by TPA was observed in Sencar mice treated with DMBA and multiple TPA applications [Liu et al., 2002] and in transgenic mice with an activated Ha-ras gene (TG.AC mice) (Liu et al., submitted). In the TG.AC model with genetically activated ERK1,2, the basal ERK1,2 activity was also reduced by DER and Raf-1 kinase was similarly inhibited by DER in the epidermis of the TG.AC DER mouse (Liu et al., submitted) suggesting that DER inhibits signaling at both RAF-1 and ERK1,2.

An intact adrenal gland was required for the DER inhibition of ERK1,2 in the skin of Sencar mice that were treated with DMBA and multiple (2×/week) TPA applications as in a tumor study. In addition, corticosterone supplementation of ADX mice inhibited TPA induction of ERK1,2 [Liu et al., 2002].

DER, GCH, AP-1, AND GENE EXPRESSION

Steroid hormones have long been studied as potent regulators of gene expression and steroid hormone responsive sequences have been reported for numerous hormone-regulated genes [Beato, 1989]. The glucocorticoid receptor has been shown in cell culture studies to be capable of interfering with transactivation by the AP1 transcription factor, whose induction is essential for mouse skin tumor promotion. The list of TPA-inducible genes has grown to include over 30, including *c-myc*, *c-fos*, *c-jun*, cyclin D1, interleukin-2 (IL-2), and plasminogen activator [Lerman and Colburn, 1988; Henderson et al., 2002]. Many of the matrix metalloproteases (MMP) are AP-I regulated including collagenase (MMP-13), stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), gelatinase A (MMP-2), and gelatinase B (MMP-9). Recently, MMP-9 knockout mice were used to show that MMP-9 was required for hyperproliferation, and progression to invasive and end stage malignant cancers [Coussens et al., 2000]. TPA-inducible genes have in their regulatory regions a binding site(s) for the transcription factor AP1. The DNA consensus site for AP1 binding is called a TPA-response element, or TRE [Angel et al., 1987a,b], and has the DNA sequence motif TGAGTCAG.

In order to begin to assess if the reduction in AP1:DNA binding caused a reduced transcription of AP1 responsive genes we used a AP-1 reporter mouse. In addition to determining if DER inhibited TPA induced AP-1 transcription, we assessed the ability of DER to block AP-1 transactivation by UVB-light, another known inducer of AP1. Mice were pre-fed AL and DER diet prior to activation of AP1 signaling by TPA or UVB light treatment. The transgene is known to be induced by UVB-light and by TPA in the AP1/luciferase mice. Results in Figure 3 show that both TPA and UVB light increased the expression of the AP1 reporter luciferase mRNA in the epidermis of AL mice but that

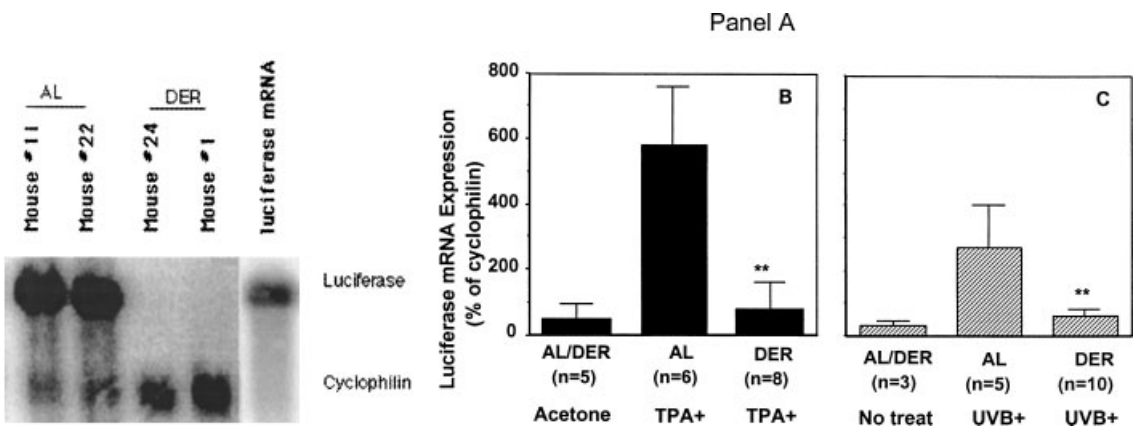

Fig. 3. Inhibition of transactivation of AP1 in DER transgenic luciferase mice. Mice were fed 40% DER for 10 weeks and treated with 17 nmol TPA, or UVB-light (6 kJ/m^2), 50 h later, they were killed and epidermal mRNA was analyzed by Northern blot for both luciferase and cyclophilin (as loading control). Band intensity was quantitated by PhosphorImager analysis. Acetone treated AL and DER values were combined because they did not differ by diet. **Panel A**: representative Northern blot of luciferase mRNA induction by TPA in AP1 and luciferase transgenic mice. **Panel B/C**: blockage of luciferase induction by TPA (panel B) and UVB (panel C) in DER mice. The data shown are mean ± SD. $^{**}P < 0.001$ vs. TPA or UVB-treated, AL-fed mice.

induction of this gene was prevented in the epidermis of the DER mice. Studies of DER effects on TPA induction of luciferase in the transgenic mouse primarily provided information on Raf-1/MAP-kinase signaling because this is the principal pathway for AP1 induction by TPA. The comparative studies with UVB-light induction of luciferase provide evidence that DER may inhibit the JNK induction by UVB-light since this is a primary signaling pathway for the induction of AP1 by UV-light.

In an effort to determine if endogenous AP1 regulated genes in SENCAR mice, the primary mouse model that we have used in our studies, are also inhibited by DER we initiated Northern blot analysis of an endogenous AP1 regulated gene, *MMP3*, and a non-AP1 regulated gene, uterocalin/24p3. Our results demonstrated a clear induction of both MMP3 and uterocalin/24p3 relative to GADPH and cyclophilin in AL mice (5 and 20 fold, respectively relative to acetone) at 24 h after TPA and lower induction of uterocalin by TPA in DER epidermis (3.5 fold, respectively, relative to acetone) ($P < 0.05$). MMP3, the AP1 regulated gene, was not induced by TPA in the DER epidermis in agreement with our hypothesis that AP1 regulated genes will be uniquely sensitive to inhibition by DER. Uterocalin induction by TPA is reflective of the association of uterocalin gene expression with the inflammatory response and our results suggest that DER may have more potent inhibitory potential against AP1 regulated processes (MMP3 induction) than against generalized inflammatory processes (uterocalin induction). The TPA induced changes relative to the β-actin loading control followed the same pattern but were not as reproducible.

SUMMARY

Our early studies on the impact of DER on PKC that were encouraged, facilitated, and supported by Ed Bresnick provided the foundation for assessing the mechanism of DER and GCH inhibition of skin carcinogenesis. In our recent studies, we determined that DER elevation of GCH is necessary for the inhibition of skin carcinogenesis and that this occurs through inhibiting signaling down PKC and the extracellular signal regulated kinase ERK 1,2, reducing AP1:DNA binding, and inhibiting transcriptional regulation through activator protein 1 (AP1). The inhibition of ERK1,2 and the reduction in AP1:DNA binding by DER were eliminated in ADX mice demonstrating the dependence of these DER effects on an intact adrenal gland. Supplementation with GCH in the ADX animals restored the inhibition of TPA-induced ERK1,2 and AP1:DNA binding. Studies of AP1 regulated genes indicate that DER inhibits TPA induced transcriptional activation of an AP1-luciferase reporter gene in a transgenic mouse model. The known importance of TPA induced ERK1,2 signaling and AP1 transcriptional regulation in mouse skin carcinogenesis suggests that DER blockage of these events causally contributes to DER prevention of skin carcinogenesis. DER may serve as a "gold standard" for dietary cancer prevention that will be useful as we develop other dietary strategies of cancer prevention and control. Mechanistic information will help strengthen recommendations for cancer prevention and may result in strategies to block tumor promotion by overeating. Furthermore, mechanistic information may assist in identifying markers of optimal dietary intake for cancer prevention. Ed Bresnick contributed in a profound and significant way to the growth of these studies in dietary prevention of cancer both through his facilitation of collaboration and also through his contributions to the professional development of his faculty.

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