Reduced Susceptibility to Two-Stage Skin Carcinogenesis in Mice with Low Circulating Insulin-Like Growth Factor I Levels

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Abstract

Calorie restriction has been shown to inhibit epithelial carcinogenesis and this method of dietary restriction reduces many circulating proteins, including insulin-like growth factor I (IGF-I). Previously, we identified a relationship between elevated tissue IGF-I levels and enhanced susceptibility to chemically induced skin tumorigenesis. In this study, liver IGF-I–deficient (LID) mice, which have a 75% reduction in serum IGF-I, were subjected to the standard two-stage skin carcinogenesis protocol using 7,12-dimethylbenz(*a*)anthracene as the initiator and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as the promoter. We observed a significant reduction in epidermal thickness and labeling index in LID mice treated with either vehicle or TPA. A significant decrease in both tumor incidence and tumor multiplicity was observed in LID mice undergoing two-stage skin carcinogenesis relative to wild-type littermates. Western blot analyses of epidermal extracts revealed reduced activation of both the epidermal growth factor and IGF-I receptors in response to TPA treatment in LID mice. In addition, reduced activation of both Akt and the mammalian target of rapamycin (mTOR) was observed in LID mice following TPA treatment relative to wild-type controls. Signaling downstream of mTOR was also reduced. These data suggest a possible mechanism whereby reduced circulating IGF-I leads to attenuated activation of the Akt and mTOR signaling pathways, and thus, diminished epidermal response to tumor promotion, and ultimately, two-stage skin carcinogenesis. The current data also suggest that reduced circulating IGF-I levels which occur as a result of calorie restriction may lead to the inhibition of skin tumorigenesis, at least in part, by a similar mechanism. [Cancer Res 2008;68(10):3680–8]

Introduction

Dietary energy balance refers to the balance between caloric intake and energy expenditure (1). Findings from both epidemiologic and experimental studies suggest chronic positive energy balance, which can lead to obesity, heightens the risk of developing multiple cancers, whereas a state of negative energy balance, as induced by calorie restriction (CR), decreases these risks (1). Although the obesity-cancer link remains poorly understood, the anticancer effects of CR have been more extensively examined. CR has been shown to inhibit the formation of spontaneous neoplasia arising in several knockout and transgenic mouse models, suppress tumor growth in tumor transplant models, and inhibit radiation and chemically induced carcinogenesis in rodents (1–4). A number of studies have examined the effect of negative energy balance on epithelial tumorigenesis using the two-stage skin carcinogenesis model. This model allows for the examination of the effect of CR on both the initiation and promotion stages of tumorigenesis. Consistently, CR during the promotion phase was shown to significantly reduce tumor incidence, multiplicity, and papilloma size (2, 5, 6). Despite the well-established anticancer effects of CR, no mechanism of inhibition has been clearly identified. Several studies indicate that changes in globally active circulatory factors, particularly IGF-I or glucocorticoids, may underlie the inhibitory effects of CR (7). We and others have shown that CR reduces circulating IGF-I levels, whereas restoration of circulating IGF-I levels in CR animals abolishes many of the inhibitory effects observed with CR alone (8, 9). These experiments suggest a critical role for circulatory IGF-I levels in the regulation of epithelial tumor development and growth.

Previous research conducted in our lab has suggested a role for IGF-I in skin carcinogenesis. In this regard, overexpression of *IGF-I* under control of the *bovine keratin 5* (*BK5*) promoter dramatically enhanced two-stage skin tumorigenesis (10). In addition, elevated expression of *IGF-I* and activation of the IGF-I receptor (IGF-IR) in keratinocytes led to spontaneous tumor promotion in previously initiated mice (10, 11). Furthermore, we reported up-regulation of epidermal phosphoinositide-3 kinase (PI3K) and Akt activities as well as up-regulation of cell cycle regulatory proteins in these *BK5.IGF-I* transgenic mice (10, 11). Additional studies revealed that the PI3K inhibitor, LY294002, directly inhibited these constitutive biochemical changes observed in the epidermis of *BK5.IGF-I* transgenic mice. Finally, topical application of LY294002 inhibited IGF-I–mediated skin tumor promotion in these mice in a dose-dependent manner. These data support the hypothesis that the PI3K/Akt signaling pathway is involved in regulating skin tumor promotion by IGF-I. Data published by Segrelles et al. (12) reported sustained activation of epidermal Akt throughout two-stage carcinogenesis in mouse skin, supporting a role for activation of this pathway in the development of skin tumors in this model system. Additional data recently published by this same group and others further confirms the involvement of Akt-mediated cellular proliferation in mouse skin tumorigenesis (13–15). We have recently found that diverse tumor promoters lead to the activation of epidermal Akt through a mechanism involving the epidermal growth factor receptor (EGFR; ref. 16), providing additional support

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that Akt signaling plays a critical role in multistage skin carcinogenesis.

To further explore the role of PI3K/Akt signaling in epithelial carcinogenesis, we generated transgenic mice that overexpress either wild-type mouse Akt or a constitutively active, myristoylated form of mouse Akt in epidermal basal cells under control of the BK5 promoter. These mice showed enhanced susceptibility to two-stage skin tumorigenesis (17). Western blot analyses performed on protein lysates prepared from the epidermis of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)–treated Akt transgenic mice showed enhanced signaling through the PI3K/Akt/mammalian target of rapamycin (mTOR) pathway, with heightened activation of signaling molecules found downstream of activated Akt and mTOR. In addition, we observed elevated levels of critical $G_1 \rightarrow S$ phase cell cycle regulatory proteins in the epidermis of these Akt transgenic mice. Collectively, data generated from IGF-I and Akt overexpressing transgenic mouse lines, as well as other studies, indicate that enhanced signaling through the PI3K/Akt/mTOR pathway plays an important role in two-stage skin carcinogenesis, especially during the tumor promotion stage.

In the present study, we have used the liver IGF-I–deficient (LID) mice (18) as a model to replicate the reduction in circulating IGF-I seen during CR. LID mice, through conditional deletion of the IGF-I gene in hepatocytes, have an ~75% reduction in the level of circulating IGF-I (18). Therefore, LID mice were used to examine the effect of reduced circulating *Igf-I* on susceptibility to two-stage skin carcinogenesis and to skin tumor promotion by TPA. Biochemical studies then examined the effect of reduced circulating IGF-I on signaling downstream of both the EGFR and IGF-IR. The results indicate that reduced circulating IGF-I levels dramatically influence susceptibility to two-stage skin carcinogenesis. In addition, reduced circulating IGF-I modulates signaling through the Akt/mTOR pathway. This mechanism may explain, at least in part, the inhibitory effects of CR on skin carcinogenesis.

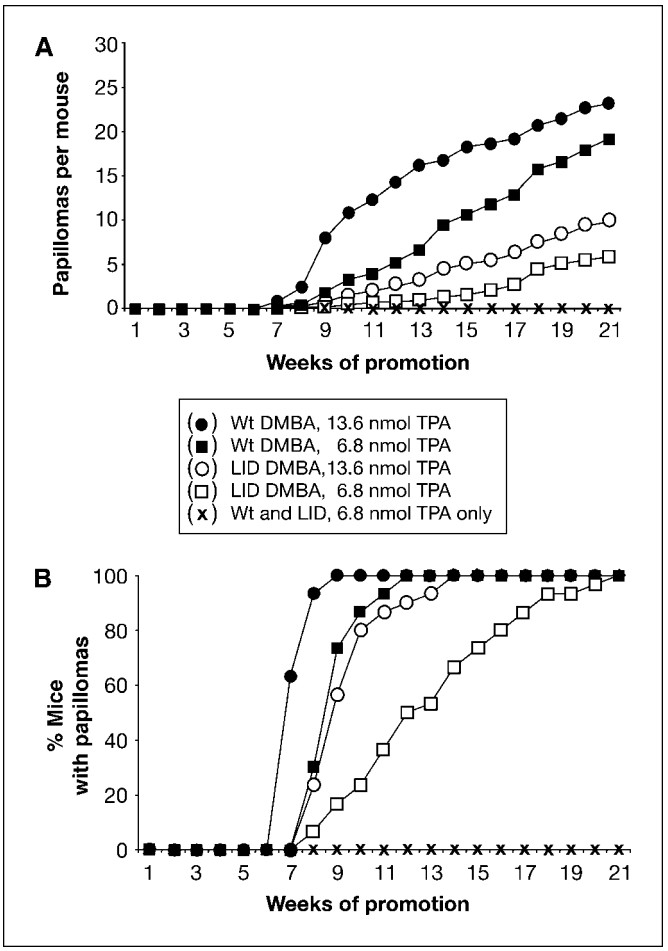

Figure 1. Effect of reduced circulating IGF-I levels on two-stage skin carcinogenesis. Groups of female LID mice and wild-type (Wt) littermates (28–30 mice per group) were initiated with either acetone or 100 nmol of DMBA. Two weeks following initiation, mice were promoted with either 6.8 nmol of TPA or 13.6 nmol of TPA twice weekly for 21 weeks. *A,* tumor multiplicity (papillomas per mouse). *B,* tumor incidence: (●) wild-type DMBA, 13.6 nmol TPA; (■) wild-type DMBA, 6.8 nmol TPA; (○) LID DMBA, 13.6 nmol TPA; (□) LID DMBA, 6.8 nmol TPA; (×) wild-type and LID, 6.8 nmol TPA only (i.e., no initiation). Note that neither wild-type nor LID mice treated with TPA only developed any tumors and were thus represented by the same symbol in the graphs. Differences in the average number of papillomas per mouse at 21 weeks between LID groups and corresponding wild-type littermate groups for each dose of TPA were statistically significant ($P < 0.05$, Mann-Whitney *U* test).

Materials and Methods

Chemicals and biologicals. TPA was purchased from Alexis Biochemicals. Bromodeoxyuridine (BrdUrd) was purchased from Sigma Chemicals Co., and 7,12-dimethylbenz(*a*)anthracene (DMBA) was obtained from Eastman Kodak Co. The antibody against phosphorylated EGFR (Y1086) was purchased from AbCam, whereas antibodies against phosphorylated IGF-IR/insulin receptor (IR; Y1135/1136), phosphorylated Akt (S473), Akt total, phosphorylated mTOR (S2448), mTOR total, phosphorylated p70S6K (T389), phosphorylated 4E-BP1 (T37/46), and phosphorylated S6 ribosomal proteins (S235/236) were all purchased from Cell Signaling Technology, Inc. Anti–β-actin, as well as anti-rabbit and anti-mouse secondary antibodies, were obtained from Sigma Chemicals. Antibodies against phosphorylated IRS-1 (Y989), phosphorylated IRS-1 (Y632), phosphorylated IRS-1 (Y465), and phosphorylated IRS-1 (Y941) were purchased from Santa Cruz Biotechnology and were combined to generate an antiphosphorylated IRS-1 antibody cocktail.

Animals. Age-matched LID mice (FVB/N genetic background) and wild-type littermates were obtained from Taconic Transgenics, where a contract production colony was maintained. Mice were generated and genotyped as previously described (18, 19). Mice were weighed prior to randomization into experimental groups, and then again at the conclusion of the tumor induction experiment. Mice were fed *ad libitum* for the duration of the experiments.

Histologic analysis. Dorsal skin samples were fixed in formalin and embedded in paraffin prior to sectioning. Four-micrometer-thick sections were cut and stained with H&E. Mice received an i.p. injection of BrdUrd (100 μg/g body weight) in 0.9% NaCl 30 min prior to sacrifice. For analysis of epidermal labeling index, paraffin sections were stained with anti-BrdUrd antibody as previously described (20). To examine the effects of TPA, the dorsal skin of LID and wild-type littermates (nine per genotype; 6–8 weeks of age) was shaved, and then topically treated with either 0.2 mL of acetone (vehicle), 3.4 nmol of TPA, or 6.8 nmol of TPA 2 days later. Mice were treated twice weekly for 2 weeks and sacrificed 48 h after the last treatment. Thirty minutes prior to sacrifice, mice were injected with BrdUrd, as described above. The dorsal skin was removed, fixed in formalin, and processed as described above. Epidermal thickness and labeling index were then determined as previously described (21).

Two-stage carcinogenesis experiment. The backs of female LID and wild-type littermates (88 per genotype; 6–8 weeks of age) were shaved 2 days prior to initiation. Twenty-eight mice of each genotype received a topical application of 0.2 mL of acetone to the shaved dorsal skin, whereas the remaining mice were initiated with a topical application of 100 nmol of DMBA in 0.2 mL of acetone. Two weeks following initiation, TPA promotion was begun by topically applying either 6.8 nmol of TPA or 13.6 nmol of TPA in 0.2 mL of acetone twice weekly, until papilloma multiplicity reached a

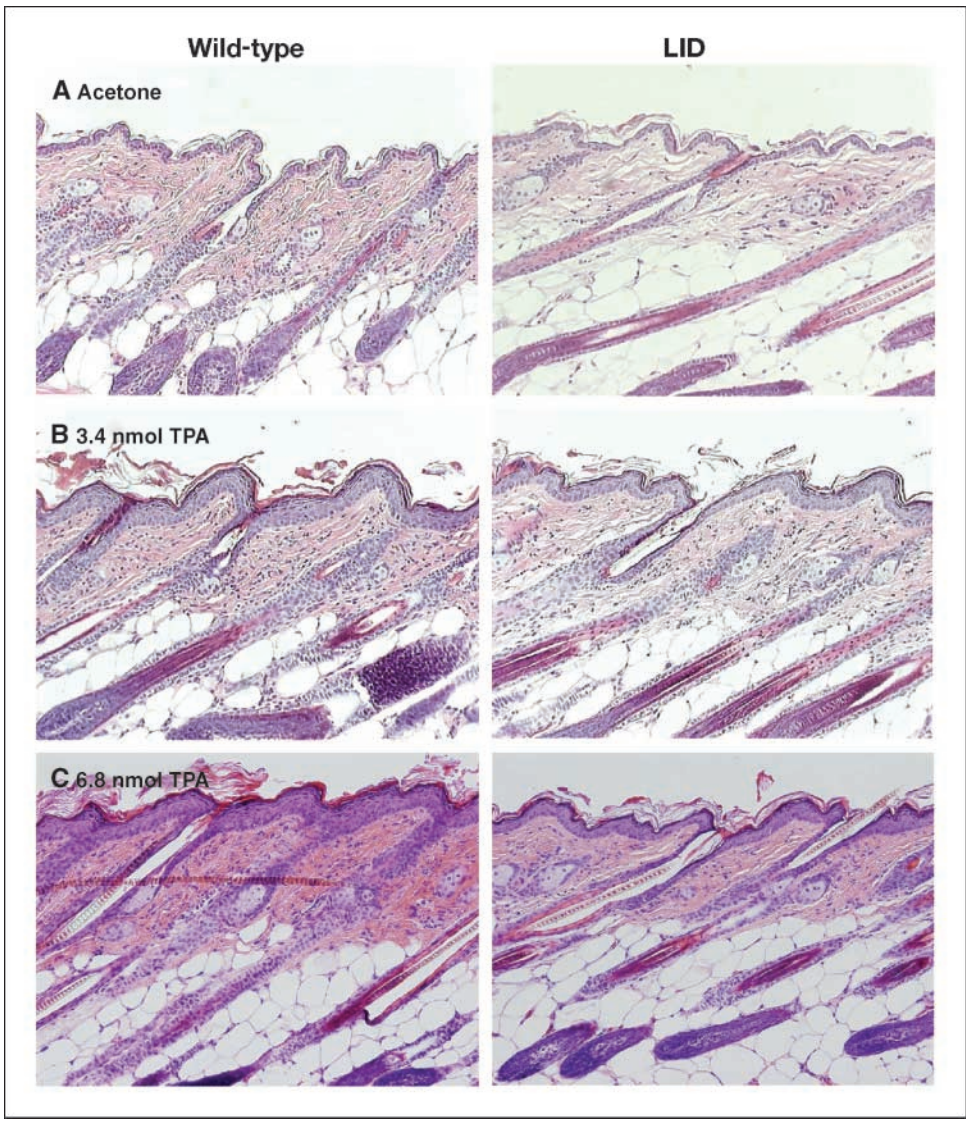

Figure 2. Representative H&E-stained sections of dorsal skin collected from 8-week-old LID mice and wild-type littermates after multiple treatments with either acetone, 3.4 nmol of TPA, or 6.8 nmol of TPA. *A,* wild-type and LID mice after treatment with acetone. *B,* wild-type and LID mice after treatment with 3.4 nmol of TPA (twice weekly for 2 wk). *C,* wild-type and LID mice after treatment with 6.8 nmol of TPA (twice weekly for 2 wk).

plateau. LID and wild-type mice received treatment regimens as follows: acetone (6.8 nmol TPA), DMBA (6.8 nmol TPA), and DMBA (13.6 nmol TPA). Tumor incidence (percentage of tumor-bearing mice) and multiplicity (papillomas per mouse) were recorded weekly throughout the experiment.

Preparation of epidermal lysates. An additional group of female LID and wild-type littermates (35 per genotype; 6–8 weeks of age) were dorsally shaved and treated with a single application of acetone vehicle (0.2 mL), 3.4 nmol of TPA, or 6.8 nmol of TPA 2 days later. Mice were sacrificed by cervical dislocation at 4, 6, and 8 h following treatment. A depilatory agent was applied to the dorsal skin (30 s) and then removed. The skin was then excised and the epidermal layer removed by razor blade into prepared lysis buffer [0.5% Triton X-100, 1% NP40, 10% glycerol, 50 mmol/L HEPES (pH 7.5), 150 nmol/L NaCl, 1 mmol/L EGTA, 1.5 mmol/L $MgCl_2$, 10% Sigma inhibitor cocktail, 10% phosphatase inhibitor cocktail I, and 10% phosphatase inhibitor cocktail II] and homogenized using an 18-gauge needle. Epidermal scrapings from five mice were pooled to generate lysates. The lysates were then centrifuged at 14,000 rpm for 15 min and the supernatant aliquoted for Western blot analysis.

Western blot analysis. For analysis of receptor tyrosine kinase activation and activation of Akt/mTOR signaling molecules, 100 μg of epidermal lysate was electrophoresed in 4% to 15% SDS polyacrylamide gels according to the method of Laemmli (22). The separated proteins were then electrophoretically transferred onto nitrocellulose membranes and blocked with 5% bovine serum albumin in TBS with 1% Tween 20 (TTBS). Blots were then incubated overnight with the antibodies described above in 5% bovine serum albumin in TTBS. Blots were washed thrice with TTBS for 15 min each, and then incubated in anti-rabbit and anti-mouse secondary antibody in 5% bovine serum albumin in TTBS for 2 h. Blots were again washed thrice with TTBS for 15 min each, and protein bands were visualized by enhanced chemiluminescence (Pierce). Protein quantification was then determined using an alpha imager system. The relative density of each protein band was normalized to the density of the corresponding β-actin band. In the case of phosphorylated Akt and phosphorylated mTOR, the phosphorylated protein band density was additionally normalized to the total protein band density, thus normalizing phosphorylated protein expression to total protein expression. All normalized values were then expressed as relative phosphorylation to the acetone LID control.

Serum analysis. LID and wild-type mice (eight per genotype) were sacrificed at 6 to 8 weeks of age using CO_2 asphyxiation. Blood was collected following sacrifice using cardiac puncture, allowed to sit at room temperature for 2 h, and then spun at 7,500 rpm for 7 min. Supernatant was collected and spun again under the same conditions. The final supernatant was collected, flash-frozen in liquid nitrogen, and then stored at −80°C until use. Total mouse serum IGF-I concentration was then measured using 25 μL samples with a RIA kit (Diagnostic Systems Laboratories, Inc.).

Statistical analysis. To compare the effects of serum IGF-I levels on tumor multiplicity, differences in the average number of papillomas per mouse were analyzed using the Mann-Whitney *U* test. This statistical test was also used to analyze mean differences in epidermal thickness and labeling index. For comparison of papilloma incidence (tumor incidence), the χ^2 test was used. Differences in mean serum IGF-I levels were analyzed using the Student's *t* test. SPSS 11.0 statistical software was used for all analyses. In all cases, significance was set at $P \leq 0.05$.

Results

Serum IGF-I levels and body mass of LID mice. As previously described, conditional knockout of *igf-1* in the liver led to a significant reduction in circulating IGF-I levels, with no effect on postnatal growth and development (18). In the current study, we analyzed both variables at the beginning of the two-stage carcinogenesis experiment. No significant differences in body weight distribution between LID and wild-type mice were observed (18.1 ± 0.15 g average in LID mice versus 18.8 ± 0.15 g average in wild-type mice). Serum analyses were also performed to confirm the effects of targeted deletion in the liver on serum IGF-I levels. Circulating levels of total IGF-I were markedly lower in LID mice versus wild-type mice (115 ± 22.95 ng/mL versus 642 ± 41.16 ng/mL; $P < 0.05$, Student's *t* test).

Effect of reduced circulating IGF-I levels on two-stage skin carcinogenesis. To evaluate the effect of circulating IGF-I levels on epithelial carcinogenesis, a two-stage skin carcinogenesis experiment was conducted using both LID and wild-type mice. Groups of LID and wild-type littermates were initiated with either acetone (vehicle) or 100 nmol of DMBA, and then 2 weeks later, promoted topically with either 6.8 or 13.6 nmol of TPA given twice weekly. Based on previous published (17, 23) and unpublished studies from our laboratory, FVB mice do not develop skin tumors when initiated with DMBA alone at doses up to 400 nmol per mouse, thus, these control groups were not included. Tumor promotion in all groups was continued for 21 weeks. The incidence of papillomas (tumor incidence) and the average number of papillomas per mouse (tumor multiplicity) were determined weekly for each group. As shown in Fig. 1, LID mice initiated with DMBA and promoted with either dose of TPA, as compared with similarly treated wild-type mice, had a significantly reduced tumor response

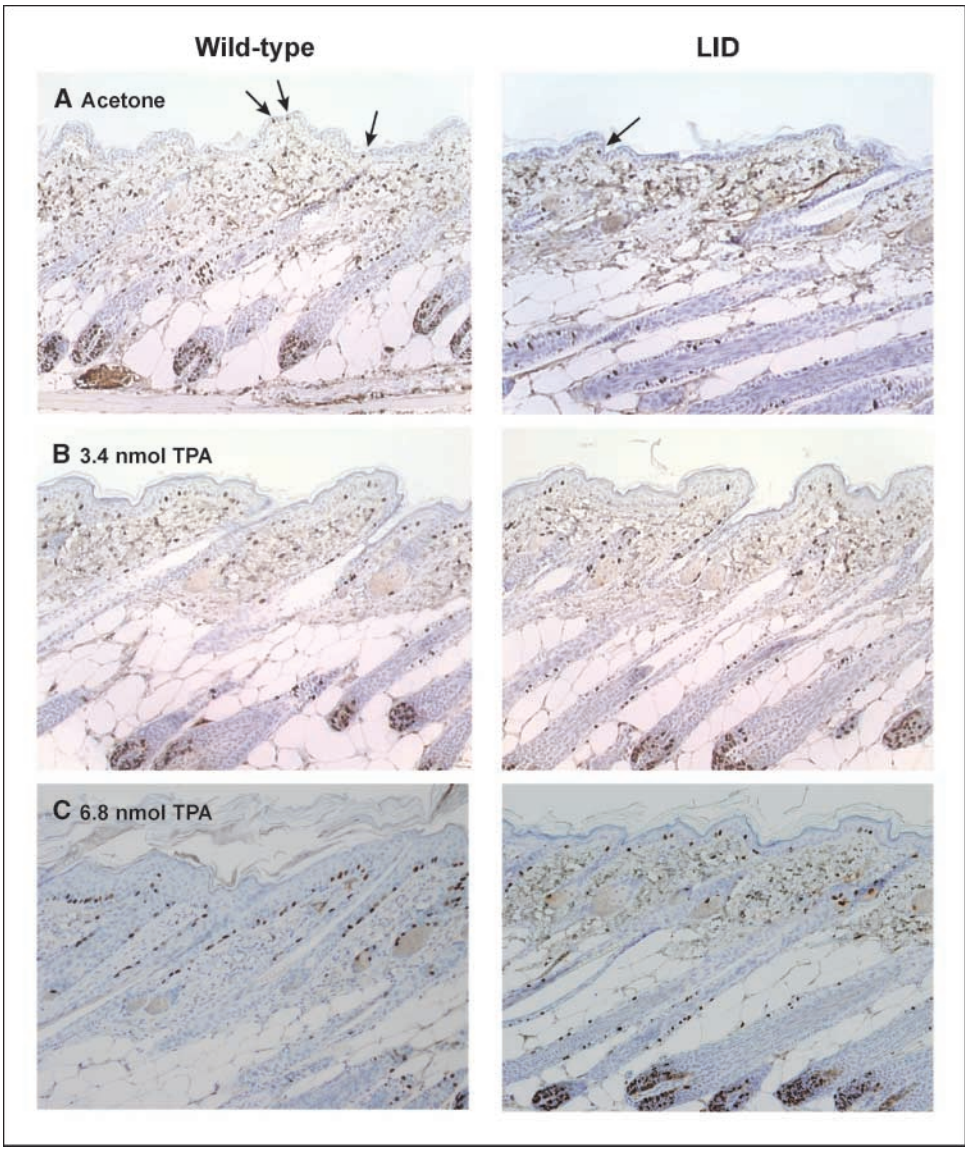

Figure 3. Representative sections of staining for BrdUrd incorporation in dorsal skin collected from 8-week-old LID mice and wild-type littermates after multiple treatments with either acetone, 3.4 nmol of TPA, or 6.8 nmol of TPA. *A,* wild-type and LID mice after treatment with acetone; *arrows,* BrdUrd-labeled epidermal cells. *B,* wild-type and LID mice after treatment with 3.4 nmol of TPA (twice weekly for 2 weeks); *C,* wild-type and LID mice following treatment with 6.8 nmol of TPA.

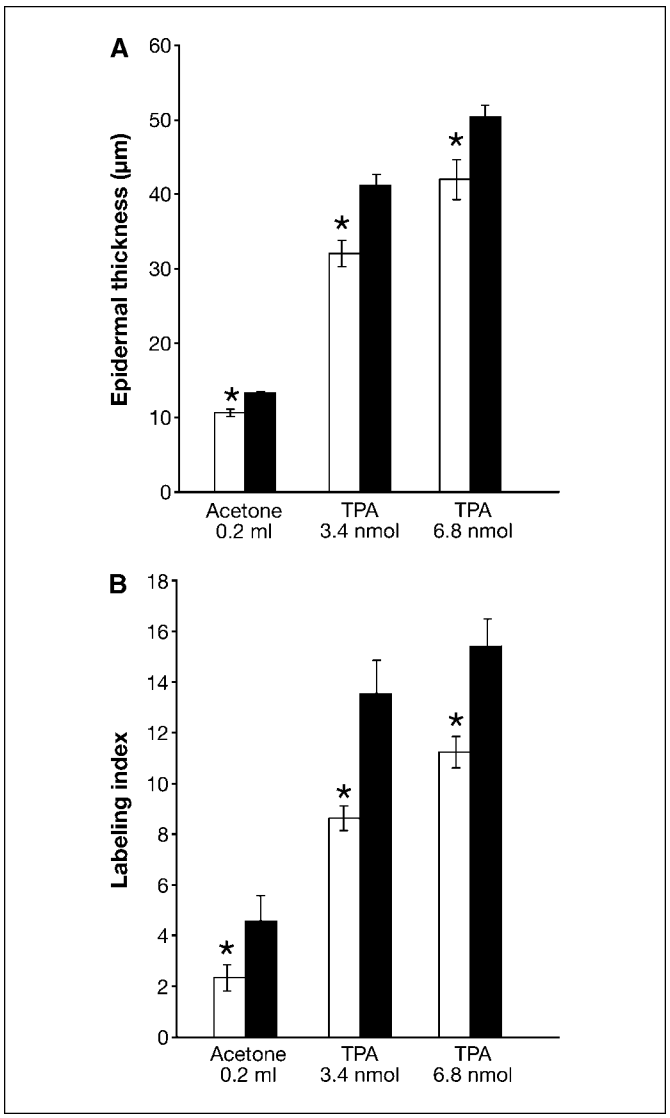

Figure 4. Quantitative evaluation of the effects of TPA on epidermal hyperplasia and labeling index in LID mice and wild-type littermates. *A,* epidermal thickness (μm) measured in LID mice (*white columns*) and wild-type littermates (*black columns*) treated with acetone, 3.4 nmol of TPA, or 6.8 nmol of TPA. *B,* labeling index values (percentage of cells which stained positive for BrdUrd) measured in LID mice (*white columns*) and wild-type littermates (*black columns*) treated with acetone, 3.4 nmol of TPA, or 6.8 nmol of TPA. Groups of three mice of each genotype received four treatments with either acetone, 3.4 nmol of TPA, or 6.8 nmol of TPA. *Columns*, mean; *bars,* SE; *, $P < 0.05$, significantly different than corresponding values from the wild-type littermate group (Mann-Whitney *U* test).

($P < 0.05$, Mann-Whitney *U* test). In this regard, at week 21, a 68% inhibition of papilloma formation was observed in the 6.8 nmol TPA treatment group and a 55% inhibition was observed in the 13.6 nmol TPA treatment group, relative to the corresponding wild-type littermates (Fig. 1*A*). Tumor volumes were measured in each group after 14 and 20 weeks of promotion with TPA to determine whether circulating IGF-I levels affected the growth of papillomas once formed; however, no significant differences were observed at either time point examined (data not shown). No papillomas developed in either group initiated with acetone followed by twice-weekly treatments with TPA (6.8 nmol). Tumor latency was correspondingly affected in both of the LID groups initiated with DMBA and promoted with TPA as compared with similarly treated wild-type mice. The time to 50% tumor incidence in wild-type versus LID mice promoted with 6.8 nmol of TPA was 8.0 versus 13.0 weeks, whereas for mice promoted with 13.6 nmol TPA, these values were 6.5 versus 9.0 weeks, respectively (Fig. 1*B*). Differences in tumor latency were statistically significant ($P < 0.05$, χ^2 test). These data show that circulating IGF-I levels significantly affected susceptibility to chemically induced epithelial carcinogenesis in mouse skin.

Reduced serum IGF-I and TPA-induced epidermal hyperproliferation. In light of the dramatic reduction in tumor development seen in LID mice undergoing two-stage skin carcinogenesis (as shown in Fig. 1), we examined their responsiveness to TPA-induced epidermal hyperproliferation. For these experiments, groups of both LID mice and wild-type littermates received four treatments with either vehicle, 3.4 nmol of TPA, or 6.8 nmol of TPA (given twice weekly) over the course of 2 weeks. Forty-eight hours following the last treatment, whole skin sections were processed and then examined for epidermal hyperplasia (measured as epidermal thickness) and epidermal labeling index. Figures 2 and 3 show representative H&E-stained sections and BrdUrd-stained sections, respectively, for acetone, 3.4 nmol TPA, and 6.8 nmol TPA–treated groups. Visual inspection of these sections reveals that LID mice displayed reduced epidermal thickness and labeling index in both control (acetone) and TPA-treated groups compared with wild-type mice. Quantitative analyses of the effect of TPA on both epidermal thickness and epidermal labeling index are summarized in Fig. 4. LID mice exhibited statistically significant reductions in epidermal thickness both in the presence and absence of TPA treatment when compared with wild-type littermates (Fig. 4*A*; $P < 0.05$, Mann-Whitney *U* test). Examination of the labeling index showed similar reductions that were also statistically significant (Fig. 4*B*; $P < 0.05$, Mann-Whitney *U* test). Thus, reduced circulating IGF-I decreased epidermal proliferation in the absence of any treatment as well as following TPA treatment.

Effect of reduced circulating IGF-I levels on EGFR, IGF-IR, and Akt/mTOR signaling in the epidermis. To further explore potential mechanisms whereby a reduction in circulating IGF-I leads to the inhibition of epidermal proliferation and skin tumor promotion, experiments were conducted to evaluate alterations in epidermal signaling molecules in the presence and absence of TPA treatment. For these experiments, groups of LID and wild-type mice were treated with a single dose of either acetone, 3.4 nmol of TPA, or 6.8 nmol of TPA and were sacrificed at 4, 6, and 8 hours following treatment. To evaluate alterations in cellular signaling occurring in both vehicle-treated and TPA-treated mice, epidermal lysates were prepared for Western blot analysis. As shown in Figs. 5 and 6, topical treatment of both wild-type mice and LID mice with either 3.4 or 6.8 nmol of TPA led to rapid activation (i.e., phosphorylation) of the EGFR, IGF-IR/IR, IRS-1, Akt, mTOR, and downstream effectors of mTOR (e.g., p70S6K, p-eIF4B, p-4E-BP1, and p-S6-ribosomal protein). These data are consistent with previously published data from our laboratory (16, 17, 24, 25). Notably, the level of activation as assessed by phosphorylation of all signaling molecules examined was reduced in LID mice following TPA treatment compared with wild-type littermates (Figs. 5*A–C* and 6*A–C*). In contrast, comparison of the groups that received acetone alone revealed only minor differences in the phosphorylation levels of EGFR, IGF-IR, IRS-1, Akt, mTOR, p70S6K, eIF4B, 4E-BP1, and S6 ribosomal protein in the epidermis of LID mice as

compared with the wild-type mice (again see Figs. 5*A–C* and 6*A–C*). Thus, reduced circulating IGF-I levels seemed to attenuate signaling through the EGFR and IGF-IR following treatment with TPA. In particular, a marked reduction in signaling through both Akt and mTOR was observed. Note that nearly identical results were obtained in repeat experiments using both doses of TPA.

Discussion

The current study was designed to examine the effect of reduced circulating IGF-I on epithelial carcinogenesis in a well-established model system. In this regard, LID mice exhibited a significantly reduced tumor response in terms of both incidence and multiplicity following two-stage skin carcinogenesis using DMBA as the initiator and TPA as the promoter. Histologic evaluation of dorsal skin from LID mice showed statistically significant reductions in epidermal thickness and labeling index compared with wild-type littermate controls, both in the absence and presence of treatment with TPA. The reduction in epidermal proliferation observed in LID mice following TPA treatment may explain, in part, the significant inhibition of skin tumorigenesis seen in LID mice. As noted in the Introduction, previous work from our laboratory using transgenic mice in which IGF-I expression was elevated at the tissue level through targeted overexpression in skin keratinocytes showed that these mice have an increased susceptibility to two-stage skin carcinogenesis (10, 25, 26). The current results clearly show that levels of circulating IGF-I can also directly affect the susceptibility to skin tumor promotion and two-stage skin carcinogenesis.

The LID mouse model has been used in previous studies to examine the effect of reduced circulating IGF-I on mammary tumor development (both chemically induced as well as tumors that develop in a transgenic model; ref. 27). Furthermore, LID mice were used to study the effect of reduced circulating IGF-I levels on growth and metastasis of colon 38 adenocarcinoma cells following orthotopic transplantation (28). In both of these reports, reduced circulating IGF-I levels had a significant inhibitory effect on tumor development. In the colon cancer study by Wu et al. (28), there was also a reduction in liver metastases in LID mice relative to wild-type controls and these authors suggested that altered vascular endothelial growth factor levels and differential vascularization might have played a role in some of the observed differences in tumor progression. When serum IGF-I levels were restored in LID mice using rh-IGF-I supplementation, the inhibitory effects on colon cancer were abolished. Although these studies suggested an important role for IGF-I in mediating tumor growth and metastasis, neither study provided significant insight regarding the molecular mechanisms evoked as a consequence of physiologic reduction of IGF-I and its effect on tumor development.

In an effort to further elucidate the mechanisms for the inhibitory effect of reduced circulating IGF-I on epithelial carcinogenesis, Western blot analyses were performed using epidermal lysates from LID mice and wild-type littermates. Previous studies from our laboratory have shown that activation of EGFR and erbB2 is an early event in mouse keratinocytes following exposure to tumor promoters, including TPA, either *in vivo* or *in vitro* (16, 24, 25, 29). Activation of the EGFR and erbB2 seems to be due to increased availability of EGFR ligands through a combination of ectodomain shedding and increased synthesis (16, 30–32). Activation of EGFR and erbB2 in the epidermis following TPA treatment leads to the activation of downstream signaling pathways such as src (29), Stat3 (33), and Akt (16).

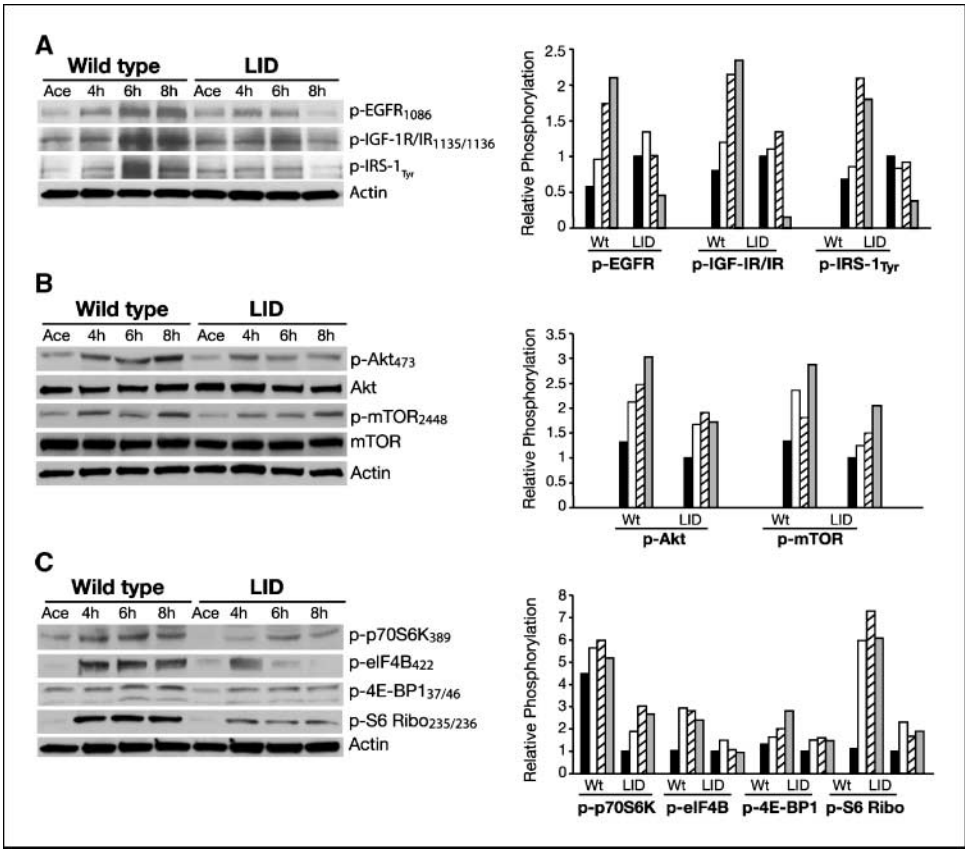

Figure 5. Effect of reduced circulating IGF-I on basal and TPA-induced epidermal signaling pathways in LID mice and wild-type (Wt) littermates. Female LID mice and wild-type littermates received a single treatment of either acetone or 3.4 nmol of TPA. Mice treated with TPA were sacrificed at 4 h (*white columns*), 6 h (*diagonal columns*), or 8 h (*gray columns*) following treatment (five mice per genotype at each time point), whereas mice treated with acetone (*black columns*) were sacrificed 4 h following treatment (five mice per genotype). Pooled epidermal lysates were prepared for Western blot analysis as described in Materials and Methods. *A,* Western blot analysis and quantification of the effect of reduced circulating IGF-I on EGFR and IGF-IR activation. *B,* Western blot analysis and quantification of the effect of reduced circulating IGF-I on Akt/mTOR activation. *C,* Western blot analysis and quantification of the effect of reduced circulating IGF-I on mTOR downstream signaling. Densitometric graphs represent the Western blots shown following normalization to the corresponding actin. Representative from one of two completely independent experiments giving nearly identical results.

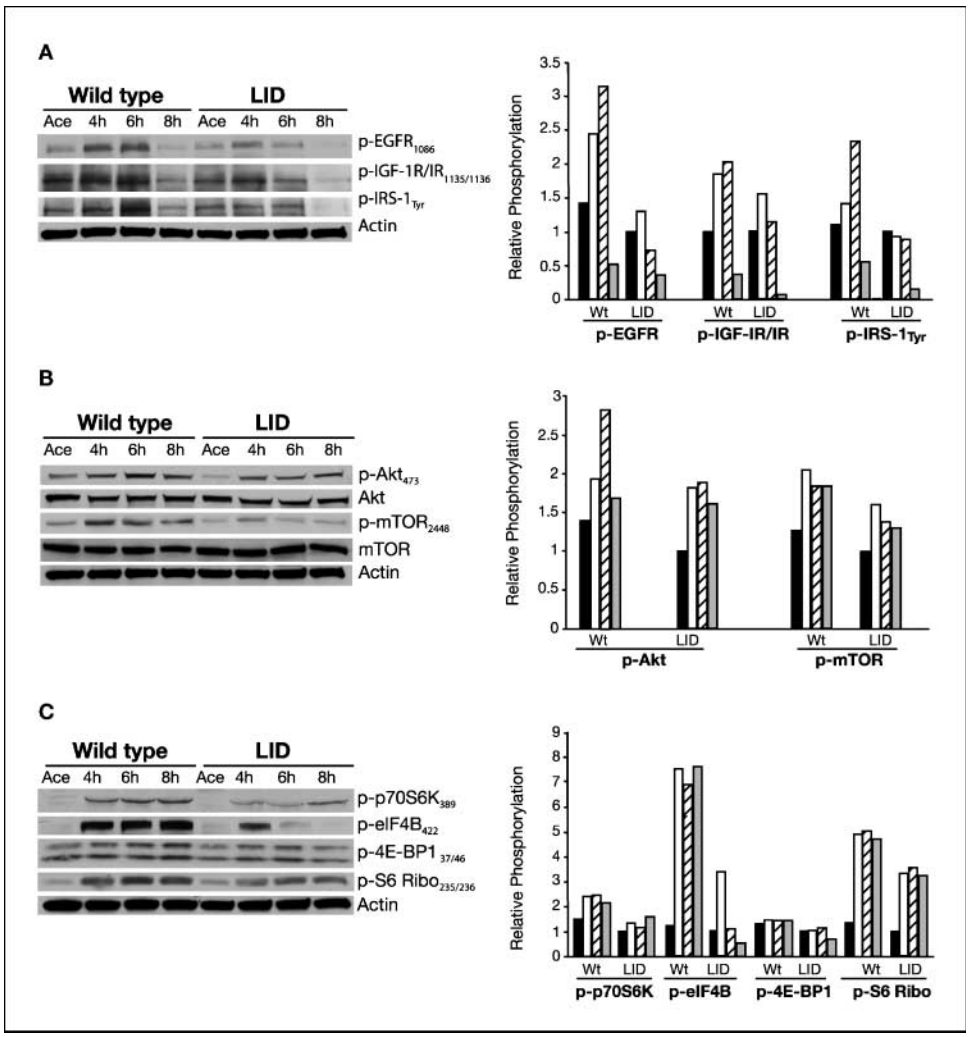

Figure 6. Effect of reduced circulating IGF-I on basal and TPA-induced epidermal signaling pathways in LID mice and wild-type littermates. Female LID mice and wild-type littermates received a single treatment of either acetone or 6.8 nmol of TPA. Mice treated with TPA were sacrificed at 4 h (*white columns*), 6 h (*diagonal columns*), or 8 h (*gray columns*) following treatment (five mice per genotype at each time point), whereas mice treated with acetone (*black columns*) were sacrificed 4 h following treatment (five mice per genotype). Pooled epidermal lysates were prepared for Western blot analysis as described in Materials and Methods. *A,* Western blot analysis and quantification of the effect of reduced circulating IGF-I on EGFR and IGF-IR activation. *B,* Western blot analysis and quantification of the effect of reduced circulating IGF-I on mTOR activation. *C,* Western blot analysis and quantification of the effect of reduced circulating IGF-I on mTOR downstream signaling. Densitometric graphs represent the Western blots shown following normalization to the corresponding actin. Representative from one of two completely independent experiments giving nearly identical results.

Furthermore, following topical treatment with TPA, the IGF-IR becomes activated, although up-regulation of ligands (i.e., IGF-I or IGF-II) does not seem to account for this activation (34). As shown in Figs. 5*A* and 6*A*, the EGFR, IGF-IR, and IRS-1 were activated in the epidermis early after topical treatment of LID and wild-type mice with TPA. However, the level of activation of these receptors was reduced in LID mice compared with wild-type littermates (~50% overall reduction in phosphorylation). These data suggest that reduced circulating IGF-I levels influenced signaling through both the EGFR and IGF-IR following TPA treatment. In support of this idea, we previously reported the development of *HK1.IGF-I* transgenic mice in which expression of *IGF-I* was targeted to the epidermis using the *human keratin 1* (*HK1*) promoter (25, 26). Following treatment with TPA, there was a significant increase in EGFR activation in the epidermis of *HK1.IGF-I* transgenic mice compared with wild-type mice. These data suggested that tissue levels of IGF-I, and presumably, the activation state of the IGF-IR could influence the activation of EGFR. A number of mechanisms have recently been proposed whereby cross-talk between the EGFR and IGF-IR may occur (35–39). Current experiments are exploring possible mechanisms whereby reduced circulating IGF-I levels may disrupt cross-talk between the IGF-IR and EGFR.

Further analysis of signaling downstream of the EGFR and IGF-IR showed reduced activation of Akt and mTOR and signaling downstream of mTOR in LID mice relative to wild-type littermates following TPA treatment. As noted in the Introduction, TPA induces the activation of Akt in mouse epidermis very early after topical treatment (16). Furthermore, Akt becomes constitutively activated in papillomas and remains constitutively activated throughout multistage carcinogenesis in mouse skin (12, 14–16). This activation of Akt has been shown to critically regulate proliferation throughout epithelial carcinogenesis (12, 17). In the present study, decreased signaling through the Akt and mTOR pathways in LID mice may account, in part, for the reduced susceptibility of LID mice to tumor promotion and two-stage skin carcinogenesis. This conclusion is supported by the following lines of evidence: (*a*) the PI3K inhibitor LY294002 blocks IGF-I–mediated skin tumor promotion in *BK5.IGF-I* transgenic mice (11); (*b*) Akt knockout mice display reduced sensitivity to two-stage skin carcinogenesis using the DMBA-TPA protocol (40); (*c*) overexpression of Akt sensitizes the epidermis to TPA-induced epidermal hyperplasia and skin tumor promotion (17); and (*d*) topical application of rapamycin effectively blocks TPA-induced activation of mTOR and epidermal hyperproliferation in wild-type mice.[4]

[4] B. Hammann, T. Moore, and J. DiGiovanni, unpublished data.

Finally, LID mice were used in the current study to mimic the reduction of circulating IGF-I induced by CR (7, 18). Although the effects of CR extend beyond reductions in serum IGF-I levels and include alterations in many serum hormones, adipokines, and cytokines (7), the exact role of IGF-I levels in the anticarcinogenic effects of CR remain to be determined. The current data, taken together with previously published studies (27, 28), suggest that reduced circulating levels of IGF-I can dramatically influence the susceptibility to tumorigenesis in multiple epithelial tissues. Our study provides one plausible mechanism for this effect, i.e., reduced signaling through the EGFR and IGF-IR and subsequent attenuation of downstream signaling through Akt and mTOR. Very recently, Xie et al. (41) reported reduced PI3K and ras signaling in response to TPA in the skin of SENCAR mice on CR diets. In this study, phosphorylation of Akt in the epidermis following TPA treatment was reduced by CR and, to a greater extent, by CR in addition to exercise. In preliminary experiments, we have found reduced signaling through the EGFR, IGF-IR, Akt, and mTOR in the epidermis of mice on CR diets following TPA treatment compared with mice on control diet.[5] Collectively, these data support the hypothesis that reduced circulating IGF-I during CR affects cell signaling at the tissue level during tumor promotion, thereby reducing susceptibility to two-stage skin carcinogenesis.

In conclusion, we have shown that reduced circulating IGF-I (induced through genetic manipulation) in the absence of dietary energy restriction dramatically reduces susceptibility to two-stage skin carcinogenesis. The mechanism for this effect seems to involve, at least in part, reduced epidermal proliferation in response to the tumor promoter TPA. This also seems to be due to reduced signaling through the EGFR and IGF-IR and is manifested, at least in part, by reduced signaling through Akt and mTOR. The observation that a decrease in the circulating level of IGF-I can affect signaling through cell surface receptors in tissue such as skin is novel and provides further support that such changes contribute to the overall susceptibility to carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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[5] T. Moore, S. Hursting, and J. DiGiovanni, unpublished studies.

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