Effects of Dietary Calorie Restriction or Exercise on the PI3K and Ras Signaling Pathways in the Skin of Mice*

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Weight control by exercise and dietary calorie restriction (DCR) has been associated with reduced cancer risk, but the underlying mechanisms are not well understood. This study was designed to compare the effects of weight loss by increasing physical activity or decreasing caloric intake on tumor promoter-induced Ras-MAPK and PI3K-Akt pathways. SENCAR mice were randomly assigned to one of the following five groups: ad libitum-fed sedentary control, ad libitum-fed exercise (AL+Exe), exercise but pair-fed at the amount as controls (PF+Exe), 20% DCR, and 20% DCR plus exercise (DCR+Exe). After 10 weeks, body weight and body fat significantly decreased in the groups of DCR, DCR+Exe, and PF+Exe when compared with the controls. AL+Exe did not induce weight loss due to, at least in part, increased food intake. Plasma IGF-1 levels reduced significantly in DCR and DCR+Exe but not PF+Exe. The protein H-Ras and activated Ras-GTP significantly decreased in TPA-induced skin tissues of DCR-fed mice but not exercised mice. PI3K protein, phosphoserine Akt, and p42/p44-MAPK were reduced, however, in both DCR and PF+Exe groups. Immunohistochemistry demonstrated that the significantly reduced H-Ras occurred in subcutaneous fat cells, while the reduced PI3K and PCNA took place only in the epidermis. Plasma leptin decreased in PF+Exe, DCR, and DCR+Exe, while the caspase-3 activity increased in DCR+Exe only. Genomic microarray analysis further indicated that the expression of 34 genes relevant to PI3K and 31 genes to the MAPK pathway were microarray analysis further indicated that the expression of 34 genes relevant to PI3K and 31 genes to the MAPK pathway were significantly regulated by either DCR or PF+Exe treatments. The reduced PI3K in PF+Exe mice was partially reversed by IGF-1 treatment. The overall results of this study demonstrated that DCR abrogated both Ras and PI3K signaling, which might inhibit TPA-induced proliferation and anti-apoptosis. Selective inhibition of PI3K by PF+Exe but not AL+Exe seems more attributable to the magnitude of the caloric deficit and/or body fat loss than diet versus exercise comparison.

The National Health and Nutrition Examination Survey indicates growing rates of obesity in American adults and overweight children over the past 20 years (1). Numerous prospective and case-control studies associated with weight control and physical activity estimate that excess body weight and sedentary lifestyle account for about 39% endometrial, 25% kidney, 11% colon, 9% postmenopausal breast cancer, and 5% total cancer incidence (2–3). It has been suggested that those 25% over normal weight have a 33% greater cancer risk than those who maintain ideal body weight (4). Therefore, for many individuals, it would be advisable to maintain weight within the normal range to reduce their risk of cancer.

Overweight/obesity is recognized as a reflection of a positive energy state that results from either over-consumption of energy or low energy expenditure. There is ample evidence that weight control via decreasing caloric intake and/or increasing physical activity reduces cancer risk in animal models. For almost a century, dietary calorie restriction (DCR) has been shown to inhibit the development of spontaneous, transplanted, chemically, and virally induced cancer in animal models (5–6). Many types of cancers, including mammary, liver, colon, skin, pancreas, bladder, and leukemia, have been shown to be prevented by 20–40% DCR (7–8). Alternatively, exercise, an effective method for energy expenditure, has been also shown to reduce both the incidence of DMBA-induced colon tumors and the growth of transplanted tumors in rats (4, 9–10). Voluntary wheel running has been reported to inhibit UVB-induced skin carcinogenesis in hairless SKH-1 mice (9). The inhibition of tumor development by exercise is, however, modest if not combined with dietary restriction (12).

There are several hypotheses proposed to describe mechanisms by which weight control may reduce tumor development, including decreased oncogene expression, assisted DNA repair, scavenged reactive oxygen species, and altered levels of cancer-related hormones. Hormone alteration seems to be a critical factor for cancer prevention by weight control due to the significant role of hormones in regulating cellular growth. Previous researchers have found that the levels of insulin (13), IGF-1 (14–16), and leptin (14–17) decreased significantly in DCR-fed mice.

As a mitogen, IGF-1 is known to stimulate cell proliferation, inhibit apoptosis, and enhance angiogenesis (18). Chronically

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‡ The abbreviations used are: DCR, dietary calorie restriction; AL, ad libitum; DXA, dual-energy X-ray absorptiometry; PF, pair-fed; ERK, extracellular signal-regulated protein kinase; PBS, phosphate-buffered saline; PKC, protein kinase C; TPA, 12-,13-tetradecanoylphorbol-13-acetate; RIA, radioimmunoassay; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase.
high levels of IGF-1 have been linked with an increased risk of developing breast (19–20), colon (21–22), and prostate (23) cancers in several case-control and prospective cohort studies. It has been suggested that the role of IGF-1 in mitogenesis is triggered through cellular signaling cascades leading to the activation of both mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways (24).

The 12-0-tetradecanoylphorbol-13-acetate (TPA) is a cancer promoter that is topically applied to experimental mice in the skin cancer model (25–26). TPA can induce ras gene expression via the Ras guanyl nucleotide-releasing protein (RasGRP). RasGRP is a family of guanyl nucleotide exchange factors and has been reported to be expressed in mouse epidermal keratinocytes in response to phorbol ester treatment (27–28). The activation of RasGRP by TPA is usually accompanied by subsequent Ras-mediated downstream regulation, especially of the MAPK pathway (28).

Ras, a G-protein effector of IGF-1, is an important upstream regulator of the MAPK and PI3K pathways (29–30). Three ras genes have been identified in the mammalian genome: H-ras, K-ras, and N-ras. They have similar structure and sequences and each codifies a monomeric G protein of 21 kDa (29–30). H-ras is highly expressed in the skin and skeletal muscles, K-ras is mostly expressed in the colon and thymus, and N-ras is expressed in male germinal tissue and thymus (30). H-Ras and K-Ras are reported to be more responsive to the Raf-1/MAPK pathway. Ras becomes activated as a result of extracellular signals by the effect of its interaction with guanine exchange factors such as SOS. The combination of SOS and Ras stimulates GDP release. GTP then shifts from SOS to Ras (31). Activated GTP-bound Ras further initiates the MAPK cascade. Previous studies demonstrated that DCR treatment inhibited TPA-promoted skin carcinogenesis by reducing certain isoforms of PKC, down to the ERK pathway (32), and then repressing transcriptional regulation of AP-1 (34).

PI3K contains the p85 regulatory subunit (which has two SH2 domains) and a 110-kDa catalytic subunit (35). The PI3K constitute a family of eight distinct PI3K isoforms divided into three classes according to their sequence homology and substrate preference. Class Ia, including p110\(_{\alpha}\), p110\(_{\beta}\), and p110\(_{\gamma}\), is associated with the catalytic subunit (37). Class Ib has only one member, p110\(_{\gamma}\), which is triggered by activated \(\alpha\), \(\beta\), and \(\gamma\) heterotrimeric G-protein (37–38). PI3K down to Akt may modulate the function of numerous substrates involved in the regulation of cell survival, cell cycle progression, and cellular apoptosis (39).

This study was designed to compare the effects of weight loss by DCR-fed or exercise-trained mice on Ras-MAPK and PI3K-Akt cascades. We provide novel evidence that these signaling pathways are potential targets by DCR and/or exercise for the inhibition of phorbol ester-induced skin tumor promotion. This is of particular interest because the key role of DCR- or exercise-induced weight control for these signal pathways in cancer promotion by which IGF-1 and/or other hormones such as insulin and leptin may be involved is largely unknown. Better understanding the relationship between body weight status, endocrine hormone changes, and hormone-dependent signal pathway activation may lead to a novel approach for cancer prevention.

**EXPERIMENTAL PROCEDURES**

**Animals and Treatments**—Female SENCAR mice were purchased from National Institutes of Health (Frederick, MD) at 8 weeks of age (30 ± 2 g). The SENCAR mouse was developed by a special breeding of CD-1 with skin tumor-sensitive mice for enhanced susceptibility to TPA-promoted skin carcinogenesis as originally described by Routwell (40). The averaged body weight was reduced (27.6 ± 2.8 g) after 1-week acclimation to new facility and diet change from rodent chow to AIN-93 purified diet. Mice were then randomly divided into one of the five groups: ad libitum sedentary (Control), ad libitum exercise (AL+Exe), Pair-fed plus exercise (PF+Exe), 20% DCR (DCR), and 20% DCR plus exercise (DCR+Exe). The PF+Exe group was paired-fed at the same amount as the control group. The mice were fed either a basal diet (AIN-93) or a 20% DCR diet for 12 weeks. The 20% DCR diet was formulated and provided by Harlan Teklad (Madison, WI). Briefly, the DCR diet consisted of 20% less total calories from carbohydrates and fat in comparison with the basal AIN-93 diet, while the levels of protein and essential micronutrients were kept as same as the basal diet. The amount of food that each mouse consumed was recorded daily and averaged to determine the amount for the following week of the DCR and pair-fed consumption. A zero-grade adjustable-speed rodent treadmill (Boston Gears, Boston, MA) was used to exercise the mice. After a 2-week training period, mice performed treadmill exercise at 13.4 m/min for 60 min/day, 5 day/wk for 10 weeks. Mice were housed individually in an environmentally controlled room maintained at 75 ± 1 °C and 80% relative humidity with a 12-h light/12-h dark cycle. To take into account the biological clocks of nocturnal rodents, the light cycle was adjusted for mice to run nighttime exercise. Body weights were recorded weekly. In the last 2 weeks, some mice in PF+Exe group were received intraperitoneal injection of either saline (sham) or IGF-1 (Novozymes GroPep, Australia) at 10 μg/g B.W. twice per week for IGF-1 treatment. The mice were fed until the last day, but exercise was stopped 24 h after the last bout. In the end of experiment, the dorsal skin of the mice was shaved and topically treated once with TPA at 3.2 nmol. Mice were sacrificed 2 h after TPA treatment. The dorsal skin samples were snap-frozen in liquid nitrogen and kept at −70 °C until further analyses.

**Body Fat Analysis**—Total body composition and bone mineral density were measured with a dual-energy x-ray absorptiometer scan (DXA) using small animal software (v5.6, Prodigy, Lunar-General Electric, Milwaukee, WI). Mice at the final week were anesthetized with sodium pentobarbital prior to scanning. Body fat and bone mineral density were recorded from the DXA and presented as a percentage of the controls.
Assessment of Plasma IGF-1 and Leptin Levels—Blood samples were obtained directly by decapitation and collected in heparin-coated tubes. Plasma was isolated by centrifugation at 1,000 \times g for 10 min at 4 °C. Total IGF-1 in plasma was extracted by Acid-ETOH method and was then measured by a RIA kit (Nichols Institute Diagnostic, San Clemente, CA). Plasma leptin levels were determined using a commercial murine leptin RIA kit (Linco Diagnostics, St. Charles, MO).

Assessment of Protein Levels by Western Blotting—Western analysis was performed as described previously (41). Briefly, mouse skin tissues were homogenized in Triton lysis buffer. Protein concentration was measured in the supernatant and 50 μg of whole cell protein was electrophoresed on 12% SDS-polyacrylamide gel. The protein bands were transferred to a nitrocellulose membrane, and then the transferred bands of H-Ras (21 kDa), K-Ras (21 kDa), P13K (110 kDa), and β-actin (internal control, 43 kDa) were, respectively, bound to their monoclonal antibodies purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The monoclonal antibodies against IGF-1R (98 kDa) was provided by Upstate Inc. (Waltham, MA). Antibodies for p42/p44-MAPK (42/44 kDa), Akt (62 kDa), and p473-Akt (62 kDa) were provided by Cell Signaling Technology, Inc. (Danvers, MA). The bound proteins were treated with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc.) and visualized by the FluorChem™ 8800 Advanced Imaging System (Alpha Innotech, San Leandro, CA). The expression levels of the proteins were qualified by a same imaging system. The relative density of the target band was normalized to the loading control β-actin and then expressed as a percentage of the controls.

Assessment of Activated Ras-GTP Protein Levels by Pull-down Assay—Total activated Ras was determined by a pull-down assay kit from Upstate Inc. (Waltham, MA). Briefly, the whole skin cell lysate was prepared in Triton lysis buffer. The activated Ras in each 100 μg of cell lysate was then pelleted with 10 μl of Raf-1 RBD-agarose beads (Upstate Inc.) at 12,000 \times g for 15 min. The pellet was washed in washing buffer three times. Pelleted samples were eluted in 2× loading buffer and boiled for 5 min. Samples were then loaded onto an 12% SDS-PAGE and transferred to a nitrocellulose membrane. The level of target protein was determined by anti-Ras IgG2α, specifically recognizing p21 H-, K- and N-Ras, followed by a goat anti-mouse-horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology). The level of the activated GTP-bound Ras was visualized by the FluorChem™ 8800 Advanced Imaging System as described before.

Assessment of Local Protein Expression in Skin Tissue Cells by Immunohistochemistry—The flash-frozen dorsal skin tissues of mice were fixed in absolute ethanol overnight at −70 °C, followed by 70% ethanol at 4 °C, 50% ethanol at room temperature, and then rinsed with PBS before adding 10% formaldehyde. Then the skin tissues were set on edge in paraffin blocks to best demonstrate full skin thickness. Five micrometer sections of each skin sample were cut and mounted on positive prechachred slides to ensure optimal adhesion. The sections were deparaffinized and rehydrated through three xylene incubations, twice with absolute ethanol, twice with 70% ethanol wash, and one PBS rinse. After endogenous peroxidase quenching (0.3% H2O2 in PBS for 20 min), antigens were retrieved by steaming at 95 °C for 30 min with a commercial antigen retrieval solution (Dakocytomation, Carpinteria, CA). Following PBS washes, sections were incubated with 1:200 dilution of either one of the primary monoclonal antibodies that recognized mouse H-Ras (Santa Cruz Biotechnology), P13K (Santa Cruz Biotechnology), PCNA (Invitrogen, Carlsbad, CA), caspase-3 (Sigma), and leptin (Affinity Bioreagents, Golden, CO), respectively. The incubation was set for 2 h at room temperature in Tris-HCl buffer with 1% goat serum. Negative control slides were incubated with goat IgG only and without primary antibodies. Immunohistochemistry was performed using a biotin-streptavidin peroxidase technique (BioGenex, San Ramon, CA). Staining was developed with diaminobenzidine chromogen (BioGenex). Staining density in epidermis, dermis, follicles, and subcutaneous fat of each section was blindly graded by the internal medicine residents using computer standards under guidance of a pathologist without knowledge of tissue treatment. The standards of staining intensity were respectively established at 400× by grading up to 40 cells in 5-unit increments from 3–5 mice per group. Data were statistically calculated and group difference scores with p ≤ 0.05 were considered significant.

Caspase-3 Activity Assay—The activity of caspase-3 was measured using the fluorogenic substrate method (Kamiya Biomedical Company, Seattle, WA). Briefly, mouse skin tissue was homogenized in HEPES lysis buffer. The cell lysate at 500 μg of total protein content was incubated with the freshly prepared colorimetric substrate (N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin) at 5 μM, 37 °C for 30 min. The release of 7-amino-4-trifluoromethyl coumarin was then measured by a fluorescence spectrophotometer at 400 nm excitation and 505 nm emission.

Microarray Analysis—Microarray analysis was performed by the Microarray Core of the Mental Retardation Research Center at the University of Kansas Medical Center. Four samples from four groups including the control, AL + Exe, PF + Exe, and DCR groups were analyzed as follows. 10 μg of total RNA was annealed with 100 pmol of T7(dT)24 at 70 °C for 10 min. Then the annealed mRNA was reverse-transcribed into cDNA using the Superscript Choice System kit (Invitrogen). Biotinylated antisense cRNA was prepared using the Enzo BioArray High Yield RNA Labeling kit (Enzo Diagnostics, Farmingdale, NY). After purification of labeled cRNA using Rneasy RNA Purification Mini kit (Qiagen), 20 μg of biotin-labeled cRNA was incubated in fragmentation buffer (40 mM Tris-acetate, pH 8.1, 100 mM potassium acetate, 30 mM magnesium acetate) at 94 °C for 35 min. The labeled cDNA then was applied to an Affymetrix oligonucleotide array, a GeneChip® Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA) containing 39,000 transcripts with 45,101 probe sets. The genechip was hybridized, washed, and scanned using Affymetrix equipment and protocols (Affymetrix). Image data were quantified using genechip operating software 1.0 (GCOS 1.0). The detection calls of probe sets were determined using default settings (x1, 0.04; α2, 0.06; δ, 0.015; scale factor, 1.0; norm factor, 1.0). If a particular probe set was marked as absent, the probe set was removed from further analysis. To detect the differentially expressed genes between treatment and control, a nonparametric bootstrap analysis was performed using the Affymetrix oligonucleotide array, a GeneChip® Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA) containing 39,000 transcripts with 45,101 probe sets. The genechip was hybridized, washed, and scanned using Affymetrix equipment and protocols (Affymetrix). Image data were quantified using genechip operating software 1.0 (GCOS 1.0). The detection calls of probe sets were determined using default settings (x1, 0.04; α2, 0.06; δ, 0.015; scale factor, 1.0; norm factor, 1.0). If a particular probe set was marked as absent, the probe set was removed from further analysis. To detect the differentially expressed genes between treatment and control, a nonparametric bootstrap
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FIGURE 1. Effect of DCR and exercise on body weight and percent body fat. Female SENCAR mice at 9 weeks of age (27.6 ± 2.8 g) were divided into five groups: ad libitum sedentary (Control), ad libitum exercise (AL + Exe), Pair-fed plus exercise (PF + Exe), 20% DCR (DCR), and 20% DCR plus exercise (DCR + Exe). The mice were fed either a basal diet (AIN-93) or a 20% DCR diet for 12 weeks. The PF + Exe group was paired-fed at the same amount as the ad libitum sedentary group. Mice performed treadmill exercise at 13.4 m/min for 60 min/day, 5 days/week for 10 weeks. A, body weights of the mice at the final week were significantly reduced in DCR, DCR + Exe, and PF + Exe groups. Results are means of n = 8–12 per group. Means with different letters differ significantly (p ≤ 0.05). B, body composition of the mice at the final week was measured by DXA scan. Percent body fat significantly decreased in DCR, DCR + Exe, and PF + Exe groups. Results are mean ± S.E. n = 8–12; *, p ≤ 0.05 versus the controls.

method (42) was used to compute the raw p value for each gene. The eight chips from both the treatment groups and the control groups were re-sampled for 9,999 times with nonparametric bootstrapping, and t statistics of each gene was calculated for each re-sampling. The raw p value for each gene was obtained by ranking the original t statistics value in the 9,999 re-sampling t statistics values. The differentially expressed genes were then determined by controlling the false-discovery rate (FDR) at the 1% level (43). From the total data pool, 48 genes relevant to IGF-1 signaling were found to be significantly impacted and reported in this study.

Gene Expression Confirmation by RT-PCR—RT-PCR was carried out by using purified total RNA obtained as described above. The cDNA was synthesized by RT-PCR using one-step RT-PCR kit (Qiagen). The primers are derived from published gene sequences as follows: MAPK1: sense primer 5’-TCT CCC GCA CAA AAA TAA GG-3’, antisense primer 5’-TCG TCC AAC TCC ATG TCA AA-3’; H-ras: sense primer 5’-TGT TAC CAA CTG GGA CGA CA-3’, antisense primer 5’-TCT CAG CTG TGG TGG TGA AG-3’; PI3Kca: sense primer 5’-TGC TTG CAA AGA AGC TGT GG-3’, antisense primer 5’-TAT GAC CCA GAG GGA TTT CG-3’; IGFBP3: sense primer 5’-AAG TTC CAT CCA CTG CAT GC-3’, antisense primer 5’-AGC TCT CCT TGC GTG CGT-3’; beta-actin: sense primer 5’-ACG CCC AGA CAT TTT TCC TT-3’, antisense primer 5’-TCT TGG AGG ATT CTG ATG TC-3’; beta-actin: sense primer 5’-TGT TAC CAA CAT GGA CGA CA-3’, antisense primer 5’-TCT CAG CGI TGG TGG TGA AG-3’. 50 μl of PCR reaction were run with a final concentration of 200 μM dNTP mix, 1× PCR buffer, 1 μM of each primer, and 1.0 units of Taq polymerase. Thermal cycling conditions, following an initial denaturation at 94 ºC for 4 min, were as follows: 30 s at 95 ºC, annealing at 55 ºC for 30 s, and extension at 72 ºC for 1 min. Then samples were incubated at 72 °C for 7 min. Amplified products at 8 μl were loaded and separated on a 1.5% agarose gel. The RT-PCR products were visualized under UV light by the FluorChem™ 8800 Advanced Imaging System (Alpha Innotech, San Leandro, CA). The relative density of the target band was normalized to the loading control β-actin and then expressed as a percentage of the controls.

Statistic Analysis—The overall effects of treatments on body weight, body fat, and IGF-1 levels were analyzed by one-way analysis of variance (ANOVA), and then the least significant difference method (Fisher’s LSD) was used to compare each of the treatment groups and the control group. Each set of comparisons for a given variable will be conducted at the overall 0.05 level of significance by applying Dunnett’s adjustment. In the experiments of RT-PCR, activated Ras-GTP, Western blotting, and immunohistochemistry, a complete randomized block design was used with each running gel as a block effect and analyzed by ANOVA and contrast analysis. All the statistical processes were carried out with SAS software.

RESULTS

Impact of DCR and Exercise on Body Weight and Body Fat—Fig. 1A shows the effects of 20% DCR and exercise with or
without diet intake limitation on body weight. Adult SENCAR mice in the control group gradually gained weight throughout the experimental period ($p < 0.01$, body weight at week 12 versus week 1); however, mice at 20% DCR or 20% DCR plus exercise consistently lost weight during the experimental period ($p < 0.01$, body weight at week 12 versus week 1). The PF + Exe mice had significantly lowered body weight at the end of the experiment when compared with the controls or AL + Exe group. By the end of the experiment, the mean weights of PF + Exe, DCR and DCR + Exe mice were significantly lower than the controls. The weight of AL + Exe group was not significantly lower when compared with the sedentary counterparts, which might be, at least in part, due to their increased food intake (4.0 ± 0.2 g/day for control mice versus 4.3 ± 0.4 g/day for AL + Exe mice). Consequently, percent body fat, as shown in Fig. 1B, significantly decreased in PF + Exe, DCR, and DCR + Exe groups, but not AL + Exe group, when compared with the controls. No significant change of bone mineral density was found among groups (data not shown).

Impact on Plasma IGF-1 and Skin Tissue IGF-1R Levels—As shown in Fig. 2A, plasma IGF-1 levels were significantly reduced in DCR and DCR + Exe groups when compared with control counterparts. Statistical analysis of the exercise factor with three exercise groups (AL + Exe, PF + Exe, and DCR + Exe) combined together demonstrated a significant decrease of IGF-1 levels in exercise-treated mice, but individual group alone did not show any significant change between exercise and control (data not shown). The protein levels of IGF-1R in skin tissues were not significantly different among groups (Fig. 2B).

Impact on Ras-MAPK Pathway—The protein levels of H-Ras, but not K-Ras, were significantly reduced in DCR and DCR + Exe mice. However, exercise did not show a significant effect (Fig. 3A). The activated Ras-GTP, as measured by a pull-down assay, was also significantly reduced in DCR and DCR + Exe groups when compared with the controls (Fig. 3B). RT-PCR analysis indicated no significant alteration of H-ras gene expression among groups (Fig. 3C). The p42/p44-MAPK levels were reduced significantly in DCR, DCR + Exe, and PF + Exe groups in comparison with the controls (Fig. 3D).

Impact on PI3K-Akt Pathway—The protein levels of PI3K, but not Akt, significantly decreased in PF + Exe, DCR, and DCR + Exe groups. Nevertheless, p-Akt(Ser473), but not Akt, significantly decreased in DCR and DCR + Exe groups when compared with the controls. The expression levels of PI3K, but not Akt, were significantly reduced in DCR and DCR + Exe groups, but not AL + Exe group, when compared with the controls. The expression levels of IGF-1R in skin tissues were not significantly different among groups (Fig. 2A).

Impact on Tissue IGF-1R Levels—As shown in Fig. 2B, plasma IGF-1R levels were significantly reduced in DCR and DCR + Exe groups when compared with control counterparts. Statistical analysis of the exercise factor with three exercise groups (AL + Exe, PF + Exe, and DCR + Exe) combined together demonstrated a significant decrease of IGF-1R levels in exercise-treated mice, but individual group alone did not show any significant change between exercise and control (data not shown). The protein levels of PI3K were significantly reduced in DCR and DCR + Exe groups when compared with the controls (Fig. 2B). RT-PCR analysis indicated no significant alteration of H-ras gene expression among groups (Fig. 3C). The p42/p44-MAPK levels were reduced significantly in DCR, DCR + Exe, and PF + Exe groups in comparison with the controls (Fig. 3D).

Impact on PI3K-Akt Pathway—The protein levels of PI3K, but not Akt, significantly decreased in DCR, DCR + Exe, and PF + Exe mice when compared with the controls (Fig. 4A). While the levels of Akt pro-
tein did not change, the levels of phosphoserine Akt were significantly lower in DCR, DCR/H11001Exe, and PF/H11001Exe groups (Fig. 4B).

Impact on Local Protein Expression in Skin Tissues—Expression of PI3K, H-Ras, PCNA, and caspase-3 proteins in epidermis and H-Ras in subcutaneous fat was respectively assessed by immunohistochemistry. Representative images of PI3K, PCNA, and caspase-3 staining in epidermis and H-Ras staining in subcutaneous fat cells, respectively. TPA significantly enhanced the densities of PI3K, PCNA, and caspase-3 in epidermis as well as H-Ras in subcutaneous fat cells compared with acetone-treated controls. The TPA-induced densities of PI3K, PCNA, and H-Ras but not caspase-3 staining significantly decreased in DCR and PF/H11001Exe groups when compared with the TPA-treated controls. *p*/H11349.05, *n*/H11005.3–5.

Effect on Plasma Leptin Levels—In contrast to an unchanged local leptin production in the skin subcutaneous fat cells, plasma leptin levels in PF+Exe, DCR, and DCR+Exe groups but not AL+Exe group were significantly reduced in comparison with ad libitum-fed controls (Fig. 6).

Effect on Activated Caspase-3 Levels in Skin Tissues—Although the expression of caspase-3 protein in local skin cells as measured by immunohistochemistry was not changed, the caspase-3-like proteolytic activity in the cell lysates of skin tissues was potentially but insignificantly elevated following exercise and/or DCR treatments. A significant increase was found in DCR+Exe mice only when compared with that in the controls (Fig. 7).

Effects of Gene Expression Relevant to Ras and PI3K Signaling Pathways—By using Affymetrix microarray, we detected the effects of treatments on the expression of 39,000 transcripts with 45,101 probe sets in the skin tissues 2 h after TPA application. As shown in Table 1, 34 marker genes relevant to PI3K and 31 genes related to Ras-MAPK pathway were significantly changed. Eight of the marker genes relevant to PI3K and eleven genes to MAPK significantly decreased in DCR-fed mice, while five genes to PI3K and three to MAPK were considerably lowered in pair-fed but exercised mice. DCR treatment also up-regulated expression of 15 genes related to PI3K and 14 genes to MAPK. In exercised mice with pair feeding, 8 genes relevant to PI3K and 7 to MAPK significantly overexpressed when compared with the sedentary controls.

RT-PCR Confirmation—The microarray data were further validated by using RT-PCR for five randomly selected genes in IGF-1-related categories. As shown in Fig. 8 (MAPK1, PI3Kca, IGFBP3, and lepr) and Fig. 3C (H-ras), the gene expression of cutaneous fat, respectively. However, TPA-enhanced densities of PI3K and PCNA in epidermis as well as H-Ras in subcutaneous fat significantly decreased in DCR and PF+Exe groups. There were no significant differences in AL+Exe group when compared with TPA-treated positive control only. There were no statistical differences of leptin (Fig. 6) and caspase-3 staining between treatment groups.
FIGURE 6. **Effect of DCR and exercise on plasma leptin and local leptin production in skin subcutaneous fat.** Plasma leptin levels as measured by a RIA kit in DCR and DCR + Exe groups were significantly reduced in comparison with ad libitum-fed controls. The production of local leptin in subcutaneous fat cells as measured by immunohistochemistry staining was not significantly changed between treatment groups. Results are mean ± S.E. n = 6–9; *, p ≤ 0.05 versus the controls.

FIGURE 7. **Effect of DCR and exercise on proteolytic activity of caspase-3.** The cell lysates of skin tissues taken 2 h after TPA application were incubated with fluorogenic caspase-3 substrate as described under “Experimental Procedures.” A significant increase in caspase-3-like proteolytic activity in DCR + Exe mice was found when compared with the controls. n = 3–4; *, p ≤ 0.05 versus the controls.

MAPK1, PI3Kca, IGFBP3, and lepr was significantly decreased in DCR and DCR+Exe groups in comparison with the control group. PI3Kca expression was also reduced in PF+Exe group. The expression of H-ras was not significantly changed between experimental groups. The RT-PCR confirmation rate to the microarray data set, as estimated using a Bayesian statistical method, is about 93.3%.

**Impact on PI3K by IGF-1 Treatment**—The mice in PF+Exe group were received intraperitoneal injection with either saline (sham) or IGF-1 at 10 µg/g B.W. twice per week in the last 2 weeks. As shown in Fig. 9, the levels of expression in skin samples were significantly reduced in PF+Exe when compared with the controls. However, the reduced PI3K in PF+Exe group was partially reversed by IGF-1 injection.

**DISCUSSION**

The major foci of this study are to elucidate the potential mechanisms of cancer prevention by weight loss and to compare the impact between DCR and physical activity. By using a combined weight control protocol with DCR and exercise components in an epidermal carcinogenesis-sensitive mouse strain, we assessed the effects of DCR and exercise on body weight, body fat, and plasma IGF-1 levels. The mechanistic impact of body weight reduction by DCR or exercise on Ras-MAPK and PI3K-Akt signal pathways was further assessed in cancer promoter phosphor ester-promoted skin tissues.

Previous data have shown DCR at 10% does not affect DMBA-induced tumor incidence in rats, but reduces tumor
size (44). At 20% DCR, tumor incidence fell by one-third, and tumor size was reduced similar to that of 10% DCR (44). The 30 and 40% DCR reduced both tumor incidence and size even more (45). However, certain undesired side effects occurred at the level greater than 30% DCR (46). In this study, we chose 20% DCR and introduced treadmill exercise for the first time to compare the impact of weight control between decreasing calorie intake and increasing energy expenditure.

As expected, we found that body weight significantly decreased with 20% DCR, which corresponded to a decrease in body fat and plasma IGF-1 levels. In contrast to DCR treatment, the impact of treadmill exercise demonstrated a modest but intricate result for weight maintenance. Exercise alone with ad libitum feeding was not sufficient to decrease body weight due to, at least in part, the corresponding increase in dietary intake. However, the dietary energy increase in AL + Exe might not necessarily match the treadmill exercise-induced energy expenditure, because the energy expenditure could have been altered due to the changed spontaneous activity in the cage and/or resting energy metabolism, etc. If the food intake of the exercised mice was limited by pair-feeding with sedentary counterpart, then body weight and body fat were significantly reduced. Plasma levels of IGF-1 seemed lowered, but were not significantly reduced by exercise. It should be noted that the caloric deficit was not matched between the exercise and DCR interventions, and thus the results should be interpreted accordingly. The estimated increase in energy expenditure by treadmill exercise at a moderate intensity in this study (13.4 m/min for 60 min/day, 5 days/wk for 10 weeks) seems much lower than the 20% reduction in dietary calorie intake. Particularly, the lack of an exercise effect in AL + Exe mice on the body weight/fat, IGF-1, and various signaling markers might be in part due to the insufficient energy expenditure of exercise. In comparison with DCR, therefore, a partial inhibition of signaling pathways by PF + Exe and no inhibition by AL + Exe may be more attributable to the magnitude of the calorie deficit than the dietary calorie intake versus exercise comparison.

Although negative energy balance is a fundamental cause of weight loss, the body weight in the DCR group combined with exercise treatment did not show any additive decrease when compared with DCR alone. Clearly, the role of physical activity in total energy expenditure and energy homeostasis is complicated. Badman and Flier recently summarized that the total energy expenditure should consist of physical activity, adaptive thermogenesis, and obligatory energy expenditure (46). It should be noted that both calorie restriction and exercise have been found to suppress plasma thyroid hormones (47–48). It is well known that thyroid hormones enhance energy conservation and resist loss of weight. It is possible that the combination of calorie restriction and exercise could suppress thyroid hormone action to a greater extent than calorie restriction alone and therefore explain the similar weight loss for both types of treatments.

The signaling transduction pathways promoted by TPA in skin carcinogenesis are well characterized. Briefly, TPA activates PKC-Raf-MAPK signal transduction pathways and then promotes proliferation. Previous studies have demonstrated that DCR inhibited skin carcinogenesis by suppressing TPA-induced AP-1 transcriptional activation via blocking PKC-Raf-ERK signal (32–34). Our current data provide significant novel information that the reduction of circulating IGF-1 levels may induce an abrogated IGF-1-dependent signaling, and thereby co-inhibit TPA-induced cancer promotion.

While the basal levels of most signaling events tested in acetone-treated TPA-free skin tissues were not significantly impacted by DCR intervention (33–34), we found that 20% DCR for 10 weeks significantly inhibited TPA-promoted Ras

![Figure 8. Confirmation of microarray data by RT-PCR. Five genes were randomly chosen from cancer-related categories and their expression pattern in comparison with the microarray data were validated by RT-PCR as shown in Figs. 8 and 3C, respectively. Identical results were obtained with MAPK1, PI3Kca, IGFBP3, and lepr genes but nor H-ras gene that down-expressed significantly in DCR and DCR + Exe groups. The gene expression of PI3K was also significantly reduced in PF + Exe group.](image-url)
MAPK and PI3K-Akt pathways as well as proliferative marker PCNA. The finding that H-Ras protein levels and activated Ras levels are depressed by DCR is in agreement with the skin tissue specificity of Ras. It is interesting that immunohistochemistry staining indicates that the decrease of H-Ras expression occurred in subcutaneous fat cells, but not in epidermis. To our knowledge, this is the first study to report that DCR reduces TPA-induced H-Ras expression in subcutaneous fat cells. It is not clear how a reduction of H-Ras in subcutaneous fat may impact skin carcinogenesis that originally develops from epidermis. The role of subcutaneous fat on malignant melanoma, however, has been suggested via tumor-stroma interaction required during metastases (49) or early micrometastases (50). Recent data also suggest that adipocytes not only respond to hormonal signals, but also produce hormone-like factors such as adiponectin or pro-inflammatory adipocytokines that may enhance oncological risk (51–52). Therefore, we further measured the levels of caspase-3 as an apoptosis marker and found that the caspase-3-like proteolytic activity did potentially increase but not significant following physical activity or DCR treatments. A significant increase in caspase-3 activity by the combination of DCR and exercise together may further support an involvement of an apoptosome-mediated mechanism.

It is well known that not only IGF-1 but also some other hormones or growth factors such as insulin and leptin could activate both PI3K-Akt and Ras-MAPK signal cascades. It is interesting that IGF-1 injection partially abolished the suppression of PI3K in pair-fed and exercised mice, suggesting a requirement of IGF-1 reduction on down-expression of p110 PI3K protein observed in those mice. We still cannot exclude the involvement of other hormones that have been found to be significantly changed in response to weight control (13, 14–17). Future studies to elucidate the individual or the combined effects of IGF-1 with other changed hormones on PI3K and Ras signaling in weight-controlled mice appear to be warranted.

By using our established strategies to control body weight, we measured the genomic gene expression in TPA-promoted skin tissues and compared the TPA-induced gene expression profiles between exercise and DCR treatments. Of the 39,000 transcripts with 45,101 probe sets measured, we identified 559 genes that showed at least 1.5-fold significant change by DCR and/or exercise treatments in comparison with the controls. Of these 559 genes, 411 genes were altered by DCR, 110 genes were changed by AL+Exe, and 67 genes were changed by PF+Exe. By using gene ontology annotation, we identified the altered genes in 21 major biological processes including cell growth, cell maintenance, cell communication, DNA binding, transcription factor, and transcription activity categories. A list of all the genes that significantly changed by either DCR or exercise treatment with all the gene ontology categories has been published separately (55). The results of 34 genes relevant to PI3K pathway and 31 genes related to MAPK pathway were selected to report here. Much of the altered genes were consistent with the results of protein and active protein measurements, which showed the treatments of DCR and exercise with pair-feeding suppressed the expression of 16 genes relevant to events in AL+Exe group in this study might be related to absence of body fat loss, which is consistent with the results in the PF+Exe group. Follow-up studies in the Wang laboratory are on-going to look for the association of reduced body fat with plasma leptin levels as well as lipoproteins profiling in exercised mice.

In contrast to DCR that suppressed both Ras-MAPK and PI3K-Akt pathways in TPA-treated skin tissues, treadmill exercise with pair-feeding selectively reduced only the PI3K-Akt pathway. The activation of the PI3K-Akt pathway, as marked by p110 PI3K protein levels, activated phosphor Akt, p110 PI3K protein expression in epidermis, and their corresponding gene expression, was significantly lower in DCR, DCR+Exe, and PF+Exe groups in comparison with the controls. The selective inhibition of PI3K-Akt signaling in phorbol ester-promoted skin samples by exercise with pair-feeding may be associated with anti-apoptosis. Depressing this signaling pathway may increase apoptosis and thus protect against cancer development (53–54). We further measured the levels of caspase-3 as an apoptosis marker and found that the caspase-3-like proteolytic activity did potentially increase but not significant following physical activity or DCR treatments. A significant increase in caspase-3 activity by the combination of DCR and exercise together may further support an involvement of an apoptosis-mediated mechanism.

FIGURE 9. Impact on p110-PI3K protein levels by IGF-1 treatment. As mentioned above in Fig. 1, the PF+Exe mice were pair-fed at the same amount as the ad libitum sedentary group and performed treadmill exercise at 13.4 m/min for 60 min/days, 5 days/week for 10 weeks. In the last 2 weeks, they were intraperitoneal injected with saline (sham) or IGF-1 at 10 μg/g B.W. twice per wk. Levels of p110-PI3K protein as measured by Western blotting were significantly reduced in PF+Exe when compared with the controls. However, the reduced PI3K in PF+Exe group was partially reversed by IGF-1 injection. Results are mean ± S.E. (n = 5–7). Bars with different letters significantly differ, p ≤ 0.05.

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p110-PI3K
β-actin

i.p. injection saline IGF-1

![Graph showing p110-PI3K protein levels by IGF-1 treatment.](Image 243x26 to 270x38)
PI3K or MAPK pathways. However, some of the marker genes were unexpectedly overexpressed. The precise reason underlying this discrepancy is unknown. Considering that the microarray data were detected at one time point, a time course of gene expression may help to explain any potential inconsistencies. An alternative explanation for the unexpected gene overexpression observed from microarray data may be associated with potential cross-talk by other undetected pathways. This could also explain the data by which exercise with pair feeding did not affect Ras protein and activity levels, but attenuated downstream MAPK activity and then TPA-induced PCNA staining.

Taken together as illustrated in Fig. 10, the well-known cancer prevention by DCR treatment, therefore, appears to be a consequence of the reduced Ras/MAPK in subcutaneous fat and PI3K/Akt signaling in epidermis, and results in decreased sensitivity to TPA stimuli in skin tissues leading to suppressed proliferation and expressed potential apoptosis. Exercise with limited dietary intake, but not exercise alone, induced a decrease of PI3K/Akt signaling in epidermis. These reductions seem to be related to the decrease of circulating IGF-1 levels, but an involvement of other hormones such as insulin and leptin might not be excluded.

In conclusion, this study demonstrated for the first time that a successful strategy to control body weight via decreasing caloric intake or increasing physical activity led to the reduction of corresponded Ras-MAPK and/or PI3K-Akt pathways. DCR induced substantial weight loss and significantly decreased IGF-1 levels for a comprehensive inhibition of both MAPK and PI3K pathways. DCR alone was as effective as DCR + Exe, indicating that DCR, independent of exercise, is a potent inhibitor for tumor-promoting pathways. Exercise with pair-feeding but not exercise alone, however, only selectively restrained the PI3K pathway, which seemed more attributable to the magnitude of the caloric deficit and/or body fat loss than diet versus exercise comparison.

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