MECHANISTIC TARGETS OF WEIGHT LOSS-INDUCED CANCER PREVENTION BY DIETARY CALORIE RESTRICTION AND PHYSICAL ACTIVITY

by

JOSEPH TABB STANDARD

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Approved by:

Major Professor
Weiqun Wang

Abstract

Weight control through either dietary calorie restriction (DCR) or exercise is associated with cancer prevention in animal models. However, the underlying mechanisms are not fully defined. Bioinformatics approaches using genomics, proteomics, and lipidomics were employed to elucidate the profiling changes of genes, proteins, and phospholipids in response to weight loss by DCR or exercise in a mouse skin cancer model. SENCAR mice were randomly assigned into 4 groups for 10 weeks: ad lib-fed sedentary control, ad lib-fed exercise (AE), exercise but pair-fed isocaloric amount of control (PE), and 20% DCR. Two hours after topical TPA treatment, skin epidermis was analyzed by Affymetrix for gene expression, DIGE for proteomics, and lipidomics for phospholipids. Body weights were significantly reduced in both DCR and PE but not AE mice versus the control. Among 39,000 transcripts, 411, 67, and 110 genes were significantly changed in DCR, PE, and AE, respectively. The expression of genes relevant to PI3K-Akt and Ras-MAPK signaling was effectively reduced by DCR and PE as measured through GenMAPP software. Proteomics analysis identified ~120 proteins, with 22 proteins significantly changed by DCR, including upregulated apolipoprotein A-1, a key antioxidant protein that decreases Ras-MAPK activity. Of the total 338 phospholipids analyzed by lipidomics, 57 decreased by PE including 5 phophatidylinositol species that serve as PI3K substrates. Although there were many impacts that we still need to characterize, it appears that both Ras-MAPK and PI3K-Akt signaling pathways are the key cancer preventive targets that have been consistently demonstrated by three bioinformatics approaches.

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Dedication

I dedicate this thesis to my parents, John and Grace Standard. They have sacrificed so much to help me pursue my goals, and I thank them for their steadfast love and support. I appreciate all the opportunities they have provided me and for instilling a desire to learn more about this world and its Creator. They have given me a desire to serve others, work hard, and make the world a better place through their example. I thank them for their strong faith and love!

Chapter 1 - Review of Literature

Obesity Epidemic

Rising rates of obesity both in the developed and developing world due to increased caloric intake and/or sedentary lifestyles has placed a major burden on healthcare systems. With obesity rates of greater than one-third for adults and 17% for children in the US in 2009-2010, strategies need to be further developed to stabilize and lower these rates.[1] Obesity is a contributing factor for many chronic diseases such as cardiovascular diseases, diabetes, and many types of cancer,[2,3] and it has been associated with increased risk for colorectal, breast, endometrial, kidney, esophageal, pancreatic, prostate, and liver cancer.[4] Obesity has also been shown to greatly increase the risk for mortality due to cancer.[3] Tannenbaum demonstrated that calorie restriction reduced spontaneous and chemically induced tumors in several mouse or rodent models, demonstrating a direct cancer preventive effect of calorie restriction.[5] Body weight control, through both dietary calorie restriction and exercise, has been identified as a possible preventive means to reduce cancer due to obesity.

Many mechanisms have been studies to better understand the role of obesity in cancer etiology. Growth hormones such as IGF-1 and insulin have been shown to increase in obese mice with possible cancer promotion. Excess body fat from obesity can further lead to production of adipokines such as leptin that have been correlated with increased cancer rates. Inflammatory cytokines also seem to be increased with obesity. Increased oxidation and inflammatory response are other possible changes due to obesity that may promote cancer.[6] Many cellular signaling pathways have been investigated to better understand the etiology of obesity and cancer. As the etiology of weight control to prevent cancer still remains unclear, further investigation is warranted.

Dietary Calorie Restriction for Cancer Prevention

History of Calorie Restriction in Mice

Dietary calorie restriction (DCR) consists of restricting total caloric intake while adequate protein and micronutrient levels. DCR is oftentimes called “undernutrition without malnutrition.” Caloric restriction typically reduces total calories at 20-40% restriction levels.[7]

Moreschi et al. first performed calorie restriction studies in mice in 1909, noting that caloric restriction reduced tumor growth rate.[8] Tannenbaum in 1940 noted that DCR also decreased the number of spontaneous and chemically-induced tumors in mice.[5] Subsequent rodent studies have confirmed that reduction in caloric intake decreases tumor rates.[9] DCR has been shown to decrease cancers such as mammary, skin, colon, pancreas, and leukemia,[10] and it has been identified as the most potent and effective dietary treatment for cancer prevention.[7] While a strong relationship has been established for DCR and cancer prevention, the molecular mechanisms remain unclear.[10]

Molecular Targets of Dietary Calorie Restriction and Cancer Prevention

This clear relationship between calorie restriction and decreased cancer rates has led researchers to search for specific molecular pathways by which DCR causes cancer prevention. Some possible mechanisms by which DCR prevents cancer could include DNA repair, antioxidant activity, apoptosis promotion, and inhibition of cellular proliferation. Nuclear factor (erythroid-derived 2) 45kDa (NF-E2) pathway induces antioxidant activity, and its levels have been increased by calorie restriction.[11] Anti-inflammation is another possible cancer prevention, and peroxisome proliferator-activated receptor-gamma (PPAR-γ) is a possible DNA repair protein seen in calorie restriction for anti-inflammation.[12] Additionally, cancer is induced by cellular pathways that promote cellular proliferation and anti-apoptosis activity. One possible key target is the Phosphatidylinositide 3-kinase/Protein Kinase B/mammalian target of rapamycin (PI3K-Akt-mTOR) towards cell growth and inhibition of apoptosis.[13] Some possible downstream activity related to cancer could include Forkhead box protein O (FOXO), mTOR-S6 kinase, AMP-activated protein kinase (AMPK), Glycogen synthase kinase 3 beta (GSK-3β), silent mating type information regulation 2 homolog 1 (SIRT-1) and Signal transducer and activator of transcription 3 (STAT 3).[13-15] FOXO pathway is believed to promote apoptosis through acting as transcription factor for ricin to exit the G1-S phase of cell cycle. Meanwhile, mTOR-S6 in a downstream pathway of PI3K-Akt that is believed to promote anti-apoptosis activity.[16] AMPK has a possible cancer preventive effect through reducing anti-apoptosis signaling form PI3K-Akt.[17] GSK-3β is a downstream enzyme that promotes anti-apoptosis.[14] STAT 3 is DNA binding protein that is highly phosphorylated in cells with cancer, and it also is affected by PI3K-Akt activity.[15,18] A transgenic mouse with knock-in SIRT1 genes showed

similar phenotypes to DCR mice such as reduced body weight, increased insulin sensitivity, decreased adipokine levels, and increased metabolism through gluconeogenesis.[19] The SIRT1-FOXO3 pathway also has been shown to increase renal cell autophagy in an induced-hypoxia model.[20] Further important mechanisms for SIRT1-FOXO3 and cancer prevention include increased DNA repair, cell cycle arrest, and reduction of oxidative stress.[21] The Ras/MAPK/ERK is another pathway that leads to cancer promotion. Higher levels of IGF-1 and insulin have been seen in obese mice with a corresponding increase in PI3K-Akt activity. Many of the pathways are complicated and are interconnected through crosstalk, and a more global approach using bioinformatics could help better elucidate the key biochemical pathways of weight control for cancer prevention.

Dietary Calorie Restriction and Clinical Trials

Many epidemiological studies have shown a relationship between decreased caloric intake and prevention of chronic disease. Older natives Okinawa islands of Japan who traditionally eat a lower calorie diet compared to the general Japanese population are expected to live approximately 5% longer and demonstrate decreased cardiovascular-related deaths.[22] Following a famine in the Norway during World War II, records have shown decreased deaths from cancer and other chronic diseases due to caloric restriction.[23,24] Western diet, containing more energy dense foods, is associated with higher rates of colon cancer; migrants from Japan to the United States have demonstrated this relationship of Western diet and increased colon cancer risk.[25] Thus, it appears that epidemiological studies point to calorie restriction as an effective treatment for the prevention of chronic diseases such as cancer.

Dietary Calorie Restriction (DCR) has a strong history in reducing cancer rates and improving longevity in a variety of animal and cell culture models. In the late 1980s, DCR studies in rhesus monkeys to have a DCR animal model in a species closer to humans. Recent studies have shown that DCR in monkeys does reduce cancer rates and improves HDL cholesterol. Within the past ten years studies have been conducted to analyze the effectiveness of DCR in human clinical trials. In 2008, Fontana et al. reported that 25% DCR for one year did not reduce IGF-1 levels unless it was accompanied with protein restriction. Thus, it appears that protein restriction could have a major role in providing DCR benefits.[26] Further studies are currently being undertaken to measure the effectiveness of DCR in humans.

Dietary Calorie Restriction Mimetics

With the variety of pathways identified for DCR and cancer, many pharmalogical interventions have been conducted to mimic the effect of DCR on cancer pathways. Higher levels of insulin have been identified with increased risk for cancer, and insulin sensitizers could improve cancer profile. Metformin is a drug currently accepted for treatment of type II diabetes through improving insulin sensitivity, and its use could also protect against cancer through means similar to DCR's insulin sensitivity effect. SIRT-1 and PPAR-γ may also be used to improve the lipid profile through reducing lipid accumulation in adipocytes. Reduction of fat could reduce adipokines and hormones related to obesity that increase cancer. Increased adiponectin has been shown to reduce body weight and increase insulin sensitivity, and adiponectin therapy is another possible DCR mimetic that could prevent cancer. Finally, there are a number of foods that could prevent IGF-1 including: retinoids, soy isoflavones, and flavonoids.[27]

Exercise for Cancer Prevention

Relationship of Exercise to Dietary Calorie Restriction for Cancer Prevention

Exercise is another form of weight control that has been studied for its possible cancer prevention similar to DCR. In humans, long-term exercise has been associated with a decreased risk for cancers such as endometrial and colon cancer.[28,29] In a review on physical activity and cancer, Friedenriech et al. noted that cancer prevention was: convincing and/or probable for colon, breast and endometrial cancers; possibly associated with lung, prostate, and ovarian cancers; and insufficiently supported for other cancers such as leukemia, gastric, kidney, and cervical cancers.[30]

While exercise does have a positive health benefit to many chronic diseases, its effectiveness for cancer prevention still remains unclear. Voluntary wheel running exercise has been shown to decrease tumor size in mouse skin cancer models.[31,32] Moore et al. noted that exercise alone does not decrease intestinal polyps in APC^{Min} mice,[33] similar to our findings that exercise needs to be in conjunction with isocaloric intake for cancer prevention.[34] The impact of exercise on physical activity seems positive, but it is not as consistent as DCR.[10] It appears that negative energy balance plays a key role in exercise for cancer prevention.

Biological Targets of Exercise for Cancer Prevention

Examining potential targets for exercise on cancer prevention is helpful in understanding its possible relationship with DCR in cancer prevention. Friedenreich et al. noted that probable biological mechanism for physical activity and cancer prevention included: decreased body fat, decreased insulin resistance, improved pulmonary function, and decreased sexual hormone activity; meanwhile, evidence was more limited for physical activity and biological mechanisms such as: increased vitamin D levels, decreased IGF-1 levels, decreased adipokines such as leptin and Interleukin 6 (IL-6), decreased inflammation, improved immune function, and increased antioxidant activity.[30] Further study examining both DCR and physical activity is necessary to better understand the complex mechanisms of weight control for cancer prevention.

Physical Activity and Clinical Trials

According to the American Cancer Society, regular exercise may reduce the risk for colon, breast, endometrial cancers, and possibly late-stage prostate and pancreatic cancers. Possible mechanisms for physical activity and cancer prevention include: reduction of oxidation, enhanced DNA repair, suppressed proliferation, increased apoptosis, decreased inflammation, and induction of differentiation.[35] For colon cancer, physical 30-60 minutes of moderate to vigorous exercise per day is associated with a 30-40% reduction in colon cancer risk. It appears that modulation of insulin and IGF-1 pathways, improved antioxidant activity, and decreased transit time are the major biological mechanisms for cancer prevention through physical activity.[36] Physical activity has also been shown to lower breast cancer and increase breast cancer survivor rates. Physical activity of 2-3 hours per week was shown to reduce insulin and IGF-1 levels to prevent breast cancer.[37] Physical activity is thought to have a protective effect of prostate cancer through decreasing IGF-1 levels, improved immunity, and antioxidant activity.[38] Many studies are continuing to examine the possible effect of physical activity on other cancers in humans. Additionally, many studies have shown an improved survival rate for cancer patients who incorporate an exercise routine in recovery following surgery or chemotherapy.[39] Further studies need to be done to determine the effectiveness and chemical pathways through which physical activity reduces cancer.

Hormones Related to Weight Control and Cancer Prevention

IGF-1 and Insulin

Insulin-like growth factor-1 (IGF-1) is an endocrine hormone secreted by the liver that has similarities with insulin, and it is stimulated by Growth Hormone (GH). IGF-1 can bind to six homologues of IGF-1 binding proteins (IGF-BP's) on the IGF-1 receptor, in addition to its ability to bind to insulin receptor.[40] Binding of IGF-1 to its receptor modulates downstream activity of PI3K-Akt and Ras-MAPK, both key cancer pathways for anti-apoptosis and cell proliferation respectively.[41]

IGF-1 serum levels in humans have increased risk for certain cancers such as breast, prostate, colon, and lung cancers.[42] Transgenic mice (HK1.IGF-1) with increased IGF-1 production experienced more rapidly growing tumors and increased number of tumors in a chemically-induced cancer model.[43] DCR has been shown to decrease IGF-1 levels in animals.[23,44] Injection of IGF-1 into DCR mice has been shown to reverse the cancer protective effect as seen in our lab and others.[45,46] Further, our lab has shown that exercise with isocaloric intake, but not ad libitum exercise, also decreases plasma levels of IGF-1.[34] Thus, it appears that negative energy balance modulates decreased IGF-1 levels for possible cancer prevention.

Insulin is also an important growth factor that could be related to weight control for cancer prevention. Higher insulin levels and insulin resistance are characteristic of type 2 diabetes and obesity. Insulin and IGF-1 are both raised in obese individuals, and they lead to increased signaling through the PI3K-Akt pathway towards anti-apoptosis.[33,47] Negative energy balance through both DCR and exercise is a strong possible protection against cancer promotion from growth factors like IGF-1 and insulin.

Adipokines: Leptin and Adiponectin

Leptin and adiponectin are adipokines that are secreted by adipose tissue for endocrine function. Leptin regulates appetite and body weight through feedback with the hypothalamus.[48] High plasma levels of leptin have been associated with increased risk for cancer. Leptin is correlated with amount of body fat, and leptin is primarily secreted by adipocytes. Leptin receptor activation leads to pro-cancer pathways including: Ras-MAPK, PI3K-Akt, PKC-p38 kinase, and AP-1 transcription factors.[10,49-50] (Garofalo 2006, Fruhbeck 2006). Studies have been conducted demonstrating a similar cancer promoting effect of leptin and IGF-1, indicating that

they may work together in cancer promotion. Studies from our lab have shown that weight control modulates a decrease in leptin. Adiponectin has an inverse relationship with cancer risk. Adiponectin has been shown to increase insulin sensitivity and decrease body weight. Lower levels of adiponectin have been associated with increased risk for breast, prostate, and colon cancers.[10]

Glucocorticoids

Glucocorticoid levels have been shown to increase in DCR animals, and increased glucocorticoid levels is thought to lead to cancer prevention. A diet with increased corticosterone led to decreased in mammary tumors in a rat model. Further, adrenalectomy reversed the cancer preventive effect of corticosteroid levels in DCR mice in SENCAR mice.[10] Glucocorticoid levels may be responsible for possible cancer prevention.

Conclusion

Weight control through both DCR and exercise show strong cancer preventive effects in both animal studies and human clinical trials. Obesity has been identified as a risk factor for cancer, but its mechanism of cancer promotion is unclear. Animal models, especially normal weight models, demonstrate weight-loss induced cancer prevention. Findings from these studies may provide potential mechanisms for future understanding of obesity and cancer. There are many different cancer pathways related to obesity and cancer promotion, so further analysis is needed to better understand the best pathways to identify to focus for cancer preventive measures. Traditional techniques have isolated specific targets to better understand cancer pathways. Bioinformatics tools have been developed within the past decade to better understand a global approach to pathways related to diseases such as cancer. Our study is looking to identify major cancer pathways through analyzing global studies of genes, proteins, and lipids using bioinformatics techniques of microarray analysis, proteomics, and lipidomics to better understand the etiology of weight control for cancer prevention.

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Chapter 2 - Both PI3K-AKT and RAS-MAPK Are the Key Signaling Targets for Weight Loss-Induced Cancer Preventive Mechanisms by Dietary Calorie Restriction or Physical Activity

Introduction

Obesity has been identified as risk factor for many chronic diseases including cancer. Since 1960, obesity rates have doubled in US men and nearly tripled in US women, with both groups at rates over 30%.[1,2] Obesity and overweightness contribute to cancer deaths in up to 14% for men and 20% for women over age 50, with higher rates of mortality for overweight cancer patients.[3] With rising rates of obesity and chronic diseases, many studies have focused on various interventions to prevent obesity-related chronic diseases. In the early 20th century, dietary calorie restriction (DCR) was shown to prevent tumor growth in animal models. Since then, it has been the most robust nutritional intervention for cancer prevention. As sedentary behavior has become endemic worldwide, exercise has been also identified as another lifestyle behavior that could reduce body weight and chronic disease risk. Weight control through DCR and/or exercise may be responsible, at least in animal models, for cancer prevention.

DCR is a weight control measure that decreases caloric intake of fats and carbohydrates while maintaining protein, fiber, and micronutrients. Typical studies have withheld calories between 20 and 40%.[4] After a pilot study in 1909 by Moreschi, DCR has been shown to prevent cancer in a variety of animal models and to be effective in reducing both chemically induced and spontaneous tumors.[5] On the other side, increased energy expenditure through exercise is another form of weight control that may contribute to cancer prevention. Exercise has been strongly associated with cardiovascular health benefits, but the cancer preventive effect of exercise is less consistent. Exercise has demonstrated convincing evidence for cancer prevention in colorectal[6] and breast cancer.[7] Additionally, exercise is probable for prostate cancer prevention, and it is possible for endometrial and lung cancer prevention.[8] Voluntary wheel running exercise provided cancer prevention by decreasing tumor size in a mouse skin cancer model.[9,10] Some studies, however, have shown that exercise may not protect against cancer.[11-13] Furthermore, previous studies in our lab had mixed results of exercise for cancer prevention, with protection only seen with iso-caloric intake.[14] Moore et al. demonstrated that a negative energy balance as

opposed to exercise alone was responsible for inhibiting intestinal polyps in APC^{Min} mice.[15] Thus, our study compared exercised mice with or without iso-caloric intake.

A variety of studies have been conducted to discover the biological mechanisms of weight control for cancer prevention. Many studies have focused on endocrine hormones and adipokines that are modified by increased adiposity. IGF-1 is a growth factor that has been widely studied for cancer promotion. Increased IGF-1 levels from obesity have been associated with increased cancer risks. IGF-1 receptor has been shown to activate downstream pathways such as: MAPK towards increased proliferation, PI3K-Akt towards anti-apoptosis and increased protein synthesis, and JAK/STAT for gene transcription of IGF-1 receptor.[16-17] In addition, adipocyte-secreted adipokines such as leptin have been shown to promote pro-cancer cellular signaling.[18-19]

While traditional studies have been limited to examining a few select genes, proteins, or fat signaling phospholipids of interest to gauge the cancer preventive effect of weight control, recently developed -omics tools have enabled us to employ a more global approach to examine the etiology of cancer development. Genomics, proteomics, and lipidomics are -omics tools that examine the interplay of profiling changes of genes, proteins, and phospholipids on the development of cancer. We hypothesized that application of genomics, proteomics, and lipidomics in this study may provide new information of a mechanistic understanding of cancer prevention by weight control.

Methods and Materials

Animals and Treatment

Six-week-old female SENCAR mice were purchased from NIH (Frederick, MD). Mice underwent a two-week training period to adjust to the new environment and treadmill exercise. Mice were housed individually at 24 ± 1 ˚C with a 12:12 light-dark cycle and given water ad libitum. Mice were divided into four treatment groups consisting of sedentary ad libitum-fed controls (control), ad-libitum-fed exercise (AE), pair-fed exercise (PE), and 20% DCR. Ad libitum controls and ad libitum exercise mice were allowed to freely access food, while the pair-fed exercise group was match-fed to the control's consumption. The basal AIN-93 and 20% DCR diets were made by Harland Teklad (Madison, WI). The 20% DCR diet that withheld calories from fat and carbohydrate is shown in Table 2.1 (see next page). A speed adjustable

rodent treadmill (Boston Gears, Boston, MA) was used for mice in exercise treatment groups. After two weeks training, the exercise groups ran on the treadmill at 13.4 m/min, 60 min per day and 5 days a week for 10 weeks. This exercise level has been rated as moderate intensity.[20] Weekly body weights and food consumption measurements were taken.

At the end of the experiment, mice were sacrificed and the dorsal skin samples were snap-frozen in liquid nitrogen and stored at -70 ˚C until further analyses.

Table 2.1 Experimental Diet Composition

Diet Components[a]	Control Diet	20% DCR[b]
Corn Oil	5.0	3.7[c]
Casein	20.0	20.0
DL Methionine	0.3	0.3
Dextrose	15.0	12.3[d]
Dextrin	49.9	37.1[d]
Fiber	5.0	5.0
AIN-93 Mineral Mix	3.5	3.5
AIN-93 Vitamin Mix	1.0	1.0
Choline bitartrate	0.25	0.25
Total amount of food	100.0	82.0

[a]AIN-93 custom made diet by Harlan Teklad (Madison, WI)
[b]DCR mice were fed 0.82 g of diet for every gram consumed by control mice
[c]Dietary calorie restriction from fat source
[d]Dietary calorie restriction from carbohydrate source

Microarray Analysis

Microarray analysis was performed as described in our previous studies.[14,21] Briefly, labeled cRNA was applied to an Affymetrix GeneChip Mouse Genome 430 2.0 Array containing 39,000 transcripts and 45,101 probe sets (Santa Clara, CA). The images were quantified by using GeneChip operating software 1.0 (GCOS 1.0; Affymetrix, Santa Clara, CA). The raw image readings were analyzed using Simpleaffy package from BioConductor at http://www.bioconductor.org. The data were normalized using either MAS or RMA algorithms. The genes that were differentially expressed between treatment groups were identified by using

pair wise comparison. The data were filtered by using 1.5-fold difference and a p-value less than 0.05 as a cutoff.

Cytoscape v2.6.0 coupled with plug-in BiNGO v2.0 was used to map the predominant gene ontology categories of the differentially expressed genes.[22] The GO annotations p-values were obtained by hypergeometric statistical test for cluster verse whole annotation. The test was adjusted by Benjamin and Hochberg false discovery rate, which is included in the BiNGO package. The dataset consisting of the significantly altered genes was loaded into GenMAPP2.0 (Gene Map Annotator and Pathway Profiler, www.genmapp.org) to analyze the effect of target gene expression on specific pathways. As reported previously,[14,22] RT-PCR on select genes was tested to confirm the microarray results (data not shown).

Proteomics Analysis

Mouse skin tissues were homogenized, and the protein concentration was determined by utilizing Protein RC DC assay (Bio-Rad, Hercules, CA). The protein lysis was purified by ReadyPrep 2-D cleanup kit (Bio-Rad, Hercules, CA). The spots of interest were excised and subjected to in-gel digestion using proteomics grade trypsin (Sigma, St Louis, MO). The digested peptides were analyzed on a MALDI TOF/TOF instrument (Bruker, MA) using α-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO) as matrix. Peak annotation was carried out automatically using software Proteinscape supplied by the instrument manufacturer (Bruker,MA). The m/z-lists were submitted to MASCOT to search the NCBI protein sequence database.

Sample labeling with cyanine minimal dyes was carried out according to the manufacturer's instructions (GE healthcare, Piscataway, NJ). Protein (25 μg) was used for CyDye labeling and the ratio of protein to CyDye is 1 μg protein: 5 pmol CyDye. The internal standard was always labeled with cy2, and the samples were labeled with Cy3 and Cy5 alternatively. The isoelectric focusing was carried on a PROTEAN IEF Cell following manufacture's instruction (Bio-Rad, Hercules, CA). SDS PAGE was conducted using a precast 8-20% gradient gel (Bio-Rad, Hercules, CA). After running, the gels with Cydye labeled proteins were scanned using a Typhoon 9410 scanner (GE Healthcare, NJ) with a resolution of 50 μm. Spot detection was performed on the gel images using the DeCyder 6.5 software. Before the matching process, up to 20 landmarks were defined all over the gel. After a match, the cycle of

reviewing and confirming the matches and re-matching was repeated manually until no new level 1 mismatches were found. The differences between the two groups were analyzed by t-test, which is provided by Decyder 6.5. The gels containing non-labeled protein were stained with Commassie blue for protein identification. The proteomics data were also filtered by using 1.5-fold difference and a p-value less than 0.05 as a cutoff. Western blot analysis for some select proteins was tested to confirm the proteomics results (data not shown).

Lipidomics Analysis

Phospholipid analysis was performed as described in our previous publications.[22,31] In short, each frozen dorsal skin tissue was ground with liquid nitrogen. Then, 1 g of tissue was mixed with 2 ml solvent [chloroform/methanol (1:2) + 0.01% butylated hydroxytoluene], an additional 1 ml of chloroform, and 1 ml of water. The mixture was centrifuged for 15 min at 1,000 rpm, and the lower layer was extracted. Another 1 ml of chloroform was added and the mixture was centrifuged as previously described, collecting the new lower layer. The two lower layer extracts were combined for phospholipid analysis using an automated ESI/MS-MS. Phospholipid analysis was able to determine phospholipid classes/subclasses such as Phosphatidic acid, PI, PC, lysoPC, alk(en)yl/acyl phosphocholine (ePC), PE, lysoPE, alk(en)yl/acyl phosphoethanolamine (ePE), phosphatidylserine (PS), alk(en)yl/acyl phosphoserine, sphingomyelin (SM), and ceramide PE. Phospholipid identification was based upon total mass/charge and fragment mass/charge consistent with the head group.

Statistical Analysis

Body weights were compared between treatment groups using one-way analysis of variance (ANOVA). Microarray and proteomics spots were considered statistically significant at 1.5-fold change using the student t-test with significance at $p<0.05$. Cytoscape v2.6.0 coupled with plug-in BiNGO v2.0 was used for mapping the predominant gene ontology categories and the significantly altered genes were loaded into GenMAPP (Gene Map Annotator and Pathway Profiler, www.genmapp.org) to analyze the specific pathways. We also provide information on protein descriptions and biological pathways using Affymetrix NetAffx web site, GeneSpring, and GenMapp relevant software platforms and databases. Lipidomics analysis was performed

using one-way ANOVA and F test for significance, with pairwise comparison by the least significant difference method.

Results

Body Weight Change

Body weight changes for the 13 weeks of the study are shown in Figure 2.1 (see below). The control group and AE groups demonstrated weight gains, at about 27 g final body weights at the end of the study. Both PE and DCR mice groups had significantly lower body weights of approximately 22 and 19 g, respectively, before sacrificing, demonstrating weight control. Thus, DCR had the most pronounced weight loss, followed by PE.

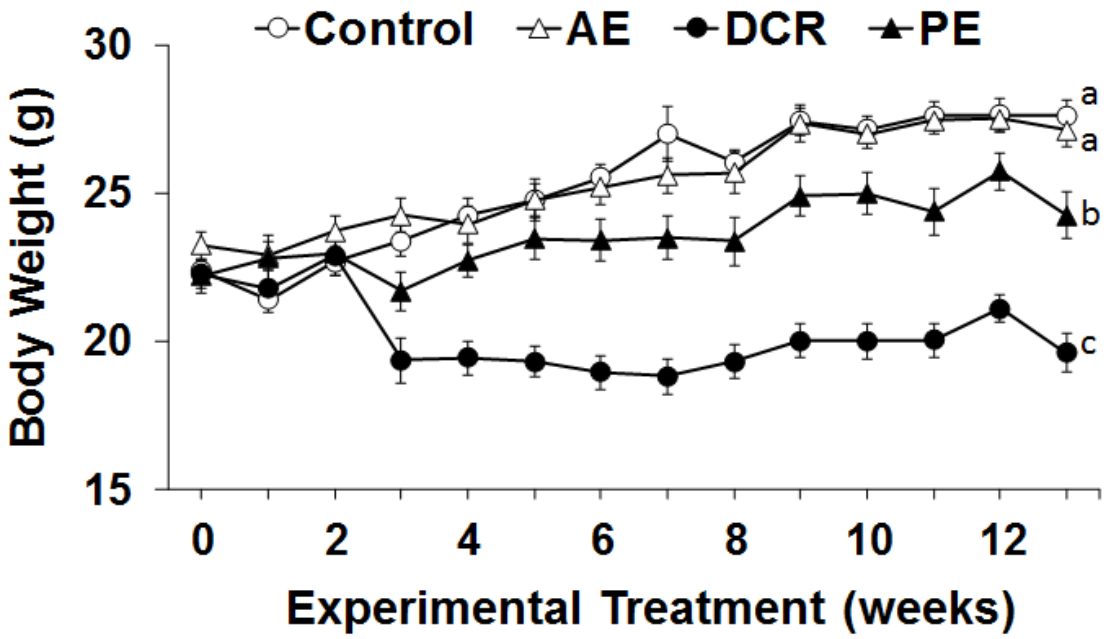

Figure 2.1 Body Weight changes. Data are shown for mice in the four groups: sedentary control (Control), ad-libitum exercise (AE), pair-fed exercise (PE), and dietary calorie restriction (DCR). Values are represented as mean ± SE, n=13-17. Means without a common letter differ, P<0.05.

Effect of Weight Loss on Gene Expression Profile

Among 39,000 genes run, 411 transcripts by DCR, 67 transcripts by PE, and 110 transcripts by AE were significantly changed versus the control. The gene sets identified by microarray analysis that were significantly changed by weight control were further categorized using GO

annotations. The over represented GO categories were identified using BiNGO. Figure 2.2 (see below) shows the visualization of significantly changed gene network by Cytoscape. The shading of the node indicates the degree of statistical significance at black node > grey node > white node. There is an overall impact of gene change by DCR > PE > AE. In comparing the two weight control groups, DCR>PE in cancer genomic events related to cell homeostasis, cell growth, biological regulation and metabolic events such as primary and lipid metabolism. For the two exercise groups, PE>AE in cancer-related functions of cell death, cell differentiation, biological regulation, plasma membrane; however, cell metabolism genes changed little between PE and AE. DCR had distinctly more genetic changes in nearly all the GO categories as opposed to AE. Furthermore, Figure 2.3 (see next page) shows examples of the pathway analysis by GenMapp, indicating Raf MAP-Kinase pathway was significantly down-regulated by 0.64-fold and 0.66-fold change in PE and DCR groups, respectively, but upregulated by 1.55-fold in AE.

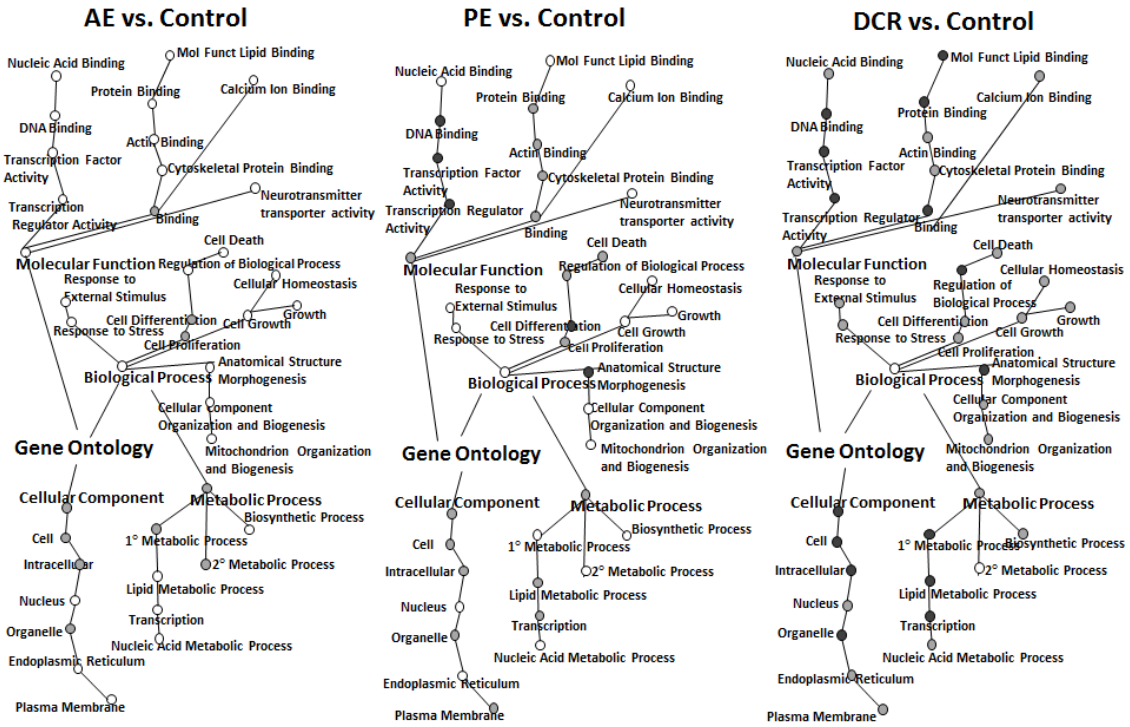

Figure 2.2 BiNGO software representation showing which Gene Ontology (GO) sets of genes that are higher expressed in (A) AL+Exe Vs Control, (B) PF+Exe Vs Control, (C) DCR Vs Control. The coloring of the node indicates the statistical difference in gene expression of treatment group versus control (black node>grey node>white node)

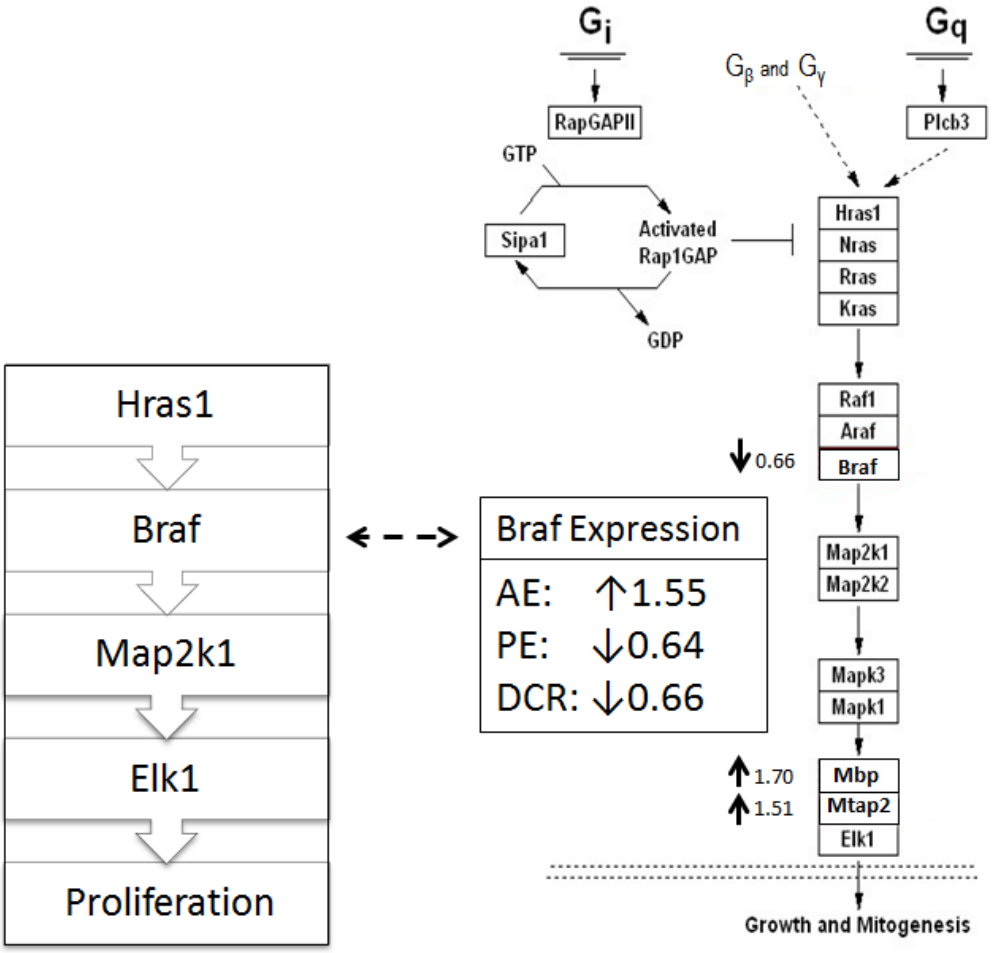

Figure 2.3 The Hras-Braf MAP-Kinase pathway gene expression was visualized using GenMAPP software. Gene expression is indicated as a ratio versus the control, and either up or down regulated (arrows). P<0.05.

Effect of Weight Loss on Protein Expression Profile

We were able to identify ~120 proteins using proteomics and 22 proteins that were significantly changed by CyDye labeling. Table 2.2 (see next page) lists the 10 proteins that were up-regulated and 12 proteins that were down-regulated by DCR. Some of the proteins had multiple spots including albumin (2), carbonic hydrase 3 (3), enolase 3:beta muscle (3), and ATP Synthase (H+ transporting, mitochondrial F1 complex) (2).

Table 2.2 Proteins that were up-regulated or down-regulated by Dietary Calorie Restriction (DCR) versus the Control utilizing a 2D-DlGE gel.[a]

Proteins Up-Regulated by DCR[b]	Proteins Down-Regulated by DCR[c]
6-phosphogluconolactonase	PDZ and LIM domain protein
trisephosphate isomerase	myosin light chain (phosphorytable)
kininogen 1 precursor	myosin A2 catalytic light chain
albumin	enolase 3: beta muscle
ornithine aminotransferase	gelsolin-like capping protein (capG)
carbonic anhydrase 3	carbonic anhydrase 3
apolipoprotein A-1	ATP Synthase (H+ transporting, F1)
heat shock protein (cystallin related)	aldose reductase
Flavin reductase (NADPH-dependent)	UGP2 protein
peroxiredoxin 6	phosphoglycerate kinase
	aconitase 2
	adenylate kinase isoenzyme 1

[a]Proteins were scanned using a Typhoon 9410 scanner with resolution of 50 μm

[b]Up-regulated proteins had fold-change of ≥1.50, $p<0.05$

[c]Down-regulated proteins had fold-change of ≥1.50, $p<0.05$

Effect of Weight Loss on Phospholipid Expression Profile

Among the 338 phospholipid species analyzed, 57 species were significantly changed by exercise. Compared to sedentary controls, most phosphatidylinositol (PI), ether phosphatidylcholine (ePC), and some lysophosphatidylcholine (lysoPC) molecular species decreased significantly in exercise with pair feeding mice. It should be noted that five PI groups decreased in isocaloric exercise PE as opposed to ad libitum feeding (AE). Figure 2.4 (see next page) illustrates five PI species that were significantly decreased in PE.

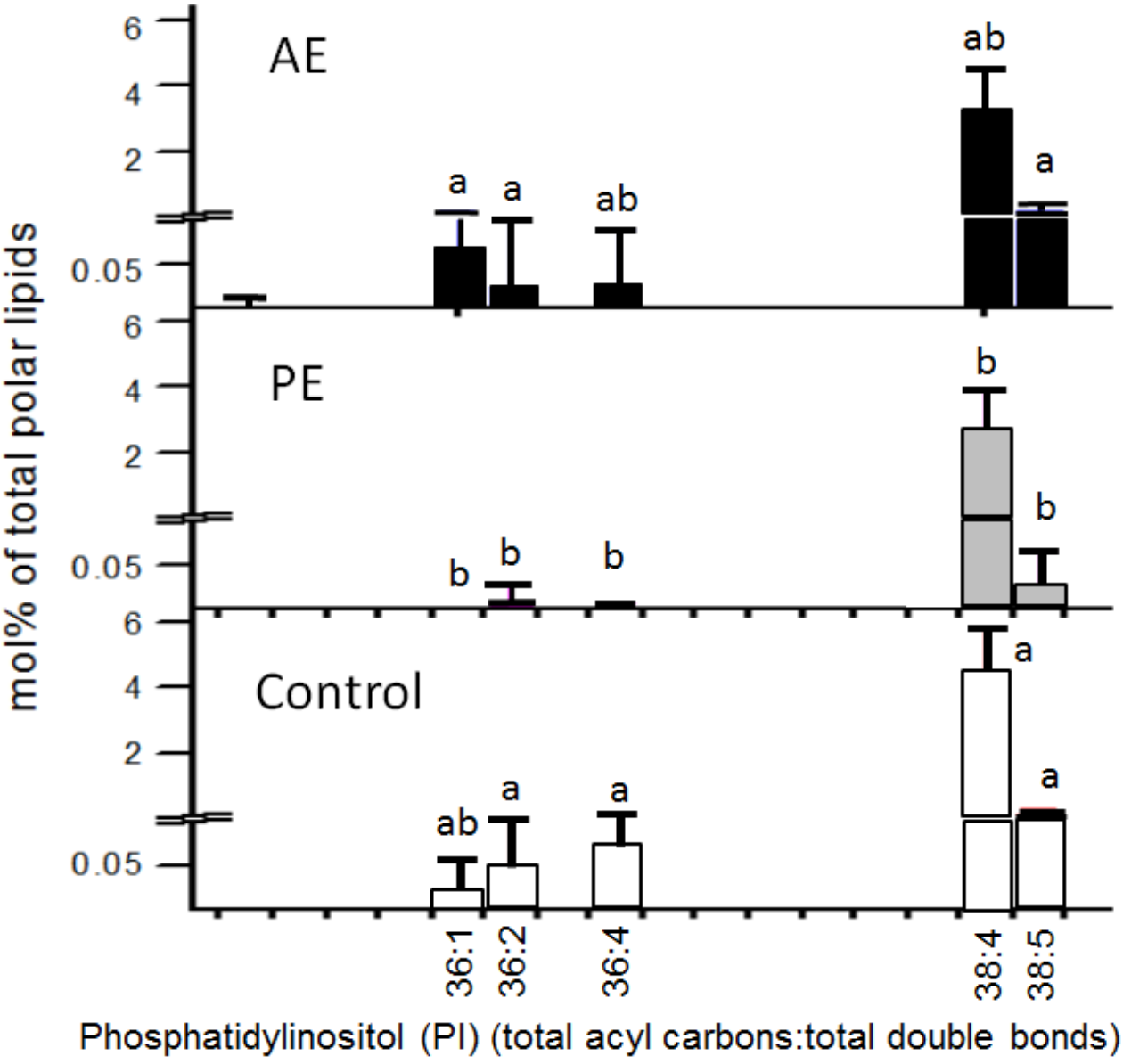

Figure 2.4 Molar percentage of phosphatidylinositol species between DCR, PE, and control as seen through ESI/MS-MS. Values are shown as mean±SE, $p<0.05$, n=8-15. Letters indicate higher mol% of each lipid species amongst the treatment groups (a>b).

Discussion

Identical twin studies have identified cancer risk due to genetics at a rate of approximately 5-10%, thus emphasizing the importance of environmental factors such as those leading to obesity and its pro-cancer effect.[23] This study demonstrated that both DCR and PE were effective in significantly lowering body weight as compared to the control, with DCR having the most impact on body weight. Weight loss in isocaloric exercise (PE) was not as pronounced as 20% DCR, possibly because the exercise was not strenuous enough to reach 20% calories burned in exercise.[14] It appears that excess food intake in the ad-libitum group is responsible for the increased body weight that was comparable to the sedentary control group body weights. Meanwhile, AE was not able to reduce body weight and had similar weight levels to the control. It seems that exercise alone is not able to contribute to weight loss and its cancer preventive effect. Thus, DCR and isocaloric exercise (PE) were effective in reducing body weight for potential cancer prevention.

Out of the 39,000 transcripts measured, 411 genes were changed by DCR, 67 genes by PE, and 110 genes by PE, illustrating the largest total number of genes changed occurred through DCR. To better grasp the specific molecular targets of DCR, BiNGO software was employed to analyze gene functions such as cellular components and regulations of biological processes like apoptosis, cell proliferation, and cell differentiation. DCR had the most change in functions related to cellular components and biological processes, while PE demonstrated moderate change in these functions versus AE. There was a progressive increase in gene change by treatment groups (DCR > PE > AE). While both DCR and PE modulated weight control, the genetic response shows a major distinction between DCR and PE. Cellular proliferation and anti-apoptosis genes were decreased in both DCR and PE, with many genes related to MAPK and PI3K activity. DCR fostered more pronounced protection in genes related to these pro-cancer pathways. For example, insulin-like growth factor binding protein 3 reduces IGF-1 and cancer activity, and it was reduced by 1.89-fold change in PE and 2.50-fold change in DCR. In looking for specific cellular pathways, we utilized GenMAPP software to identify specific pathways that are changed by diet. DCR and PE both reduced the Braf/MAPK pathway towards cell proliferation, whereas this pathway was up-regulated by AE. Braf/MAPK pathway is a cellular pathway towards increased cell proliferation that has been shown to be a mechanism for cancer promotion. It appears that PE has better genomic expression similar to DCR that is not mimicked

by AE.[21] Additionally, DCR shows a unique metabolism genomic response as compared to the two exercise groups. DCR demonstrated the lowest body weight, and the noted change in metabolism-related genes provides an in vivo response that correlates with the phenotype of body weight change. This change of metabolism-related genes may provide not only insight into what negative energy balance affects but also indicator of how negative energy balance acts. While further studies need to be done to elucidate how DCR's gene ontology differs from PE, it appears that both down-regulate the TPA-induced Ras-MAPK pathway.

Using 2D DIGE proteomics, we identified near 120 proteins. Among these proteins identified, we found that 22 proteins were significantly changed in DCR versus the control. The cancer-related functions of the proteins analyzed showed common functions such as energy metabolism/glycolysis and cellular stress responses. In analyzing the data, we identified proteins that may be key targets for cancer prevention and promotion such as Apolipoprotein A-1 (APOA1) that was up-regulated by DCR (Table 2.2). APOA1 has been studied extensively for its cancer protective properties through reducing inflammation.[24] Over-expression of APOA1 mimetic peptides was associated with increased survival rates and inhibition of size and number of tumors in a mouse ovarian cancer model. APOA1 may foster cancer protection via binding lysophosphatidic acid (LPA), a proinflammatory lipoprotein that leads to cellular proliferation through the Ras-Rho GTPase crosstalk towards cancer promotion.[24,25] This anti-activity of APOA1 on Ras-MAPK pathway provides a possible link between cancer prevention observed in microarray and proteomics data. Finally, oxidized phospholipids contribute to pro-inflammation,[26] and it is postulated that APOA1 may reduce this inflammatory response and exert pro-cancer effect through reducing MAPK activity.[24] In contrast, gelsolin-like capping protein (capG), was an oncogenic protein that was down-regulated by DCR for cancer prevention. Bahassi et al. demonstrated that capG is important for tumor cell motility and cell proliferation, and they believe that down-regulation of capG by AP-1 transcription factor complex leads to cancer prevention.[27] Increased cell motility in cancer has been shown to be correlated with increased Ras-MAPK activity,[25] and the downregulation of capG may aid in DCR's cancer prevention. Thus, it appears that DCR decreases pathways towards cancer promotion including Ras-MAPK and PI3K-Akt pathways, reduction of inflammation, and modulation of phospholipids by DCR for cancer prevention.[22]

Cancer prevention was further demonstrated through reduction of PI phospolipid species via PE treatment. Our lipidomics data indicated that weight control through PE reduced 5 PI species as opposed to the AE and control groups. The predominant form of PI in mouse tissue is PI 38:4.[22,28] PI species can be a substrate of PI3K for cellular signaling pathway towards increased cancer promotion via downstream PI3K-Akt signaling. This decrease in PI phospholipids is consistent with previous research that indicates that PIs are decreased in exercised groups.[14] PE had the most pronounced reduction in PI substrates compared to AE or the control. Our lab has also shown that exercise-induced reduction in PI phospholipids leads to protection from further downstream cancer promoting events through PI3K-related signaling.[22]

A major hormone decreased for downstream reduction in this PI signaling is IGF-1, which is higher in excess fat/obese conditions and lower in weight controlled mice by DCR or exercise.[29,30] In iso-caloric intake exercise-trained mice, our lab showed that IGF-1 restoration reversed the reduction of PI phospholipids and PI-associated PI3K down-expression for cancer prevention.[31] Hence, it appears that IGF-1 is a required growth factor that promotes cancer etiology in overweight conditions, in part through promoting PI phosphorylation by PI3K and downstream Akt activity towards anti-apoptosis.[30,32] Through demonstrating that PIs decreased in the phospholipid membrane, our study helps to better understand the mechanism for IGF-1 reduction and inactivated PI3K-Akt activity. Morimura et al. demonstrated that IGF-1 promotes colocalization of IGF-1 receptor and PIP3, which is phosphorylated PIs by PI3K.[33] This colocalization of IGF-1 receptor and phosphorylated PIs could explain how PIs could amplify the signal for anti-apoptosis from IGF-1. Reduced IGF-1 could lead to reduced localization of PIs and subsequent reduction of downstream signaling towards PI3K-Akt and anti-apoptosis. Figure 2.5 illustrates the possible mechanism through which weight control could lead to reduced IGF-1, PI3K activity, and anti-apoptosis. Thus, our phospholipidomics data further illustrates weight control's important cancer prevention mechanism through the PI3K-Akt pathway.

Although there are many other impacts that have not been figured out yet, it seems that cellular signaling pathways of Ras-MAPK and PI3K-Akt are the key cancer preventive targets that have been consistently demonstrated by three bioinformatics approaches. A possible limitation, for example, is that proteomics analysis did not detect many proteins especially for protein kinases. A more recently developed technique of phosphoproteomics may aid in identifying more kinases related to signaling pathways.

Taken together, this study identified PI3K-Akt and Ras-MAPK as two major pathways related to weight control and cancer prevention seen through all three bioinformatics approaches. Microarray data showed that the Ras-MAPK pathway was down-expressed in DCR and PE, but increased in AE. Our proteomics data showed that APOA1 and capG are proteins that are modified for cancer prevention by Ras-MAPK. APOA1 leads to a decrease in proinflammatory response that may be helpful for cancer prevention through modulating Ras-MAPK. CapG was also a protein reduced by DCR that is indicative of reduced Ras-MAPK and PI3K-Akt activity. Finally, lipidomics data showed reduced levels of PI species with isocaloric exercise (PE), suggesting how weight control can reduce the PI3K-Akt pathway towards anti-apoptosis. The three areas of bioinformatics utilized give us a more global overview for the protective effect of weight control through both isocaloric exercise and calorie restriction on cancer prevention. It seems that weight control helps to prevent against cancer through reduction in hormones such as IGF-1 and cross-talk between IGF-1-dependent and TPA-induced downstream signaling as concluded in Figure 2.5.

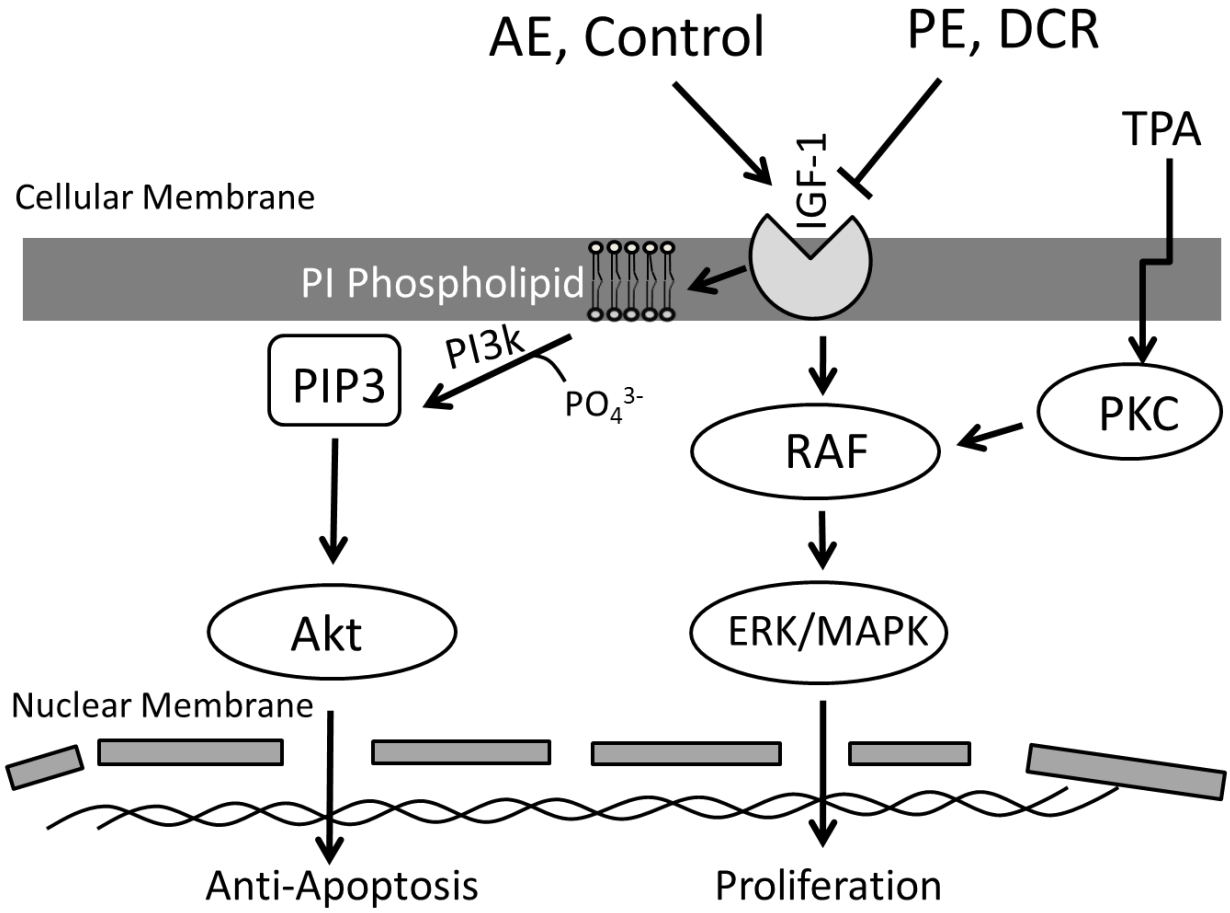

Figure 2.5 Overview of the study illustrating the molecular pathway through which weight control leads to cancer prevention.

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