EFFECT OF WEIGHT CONTROL VIA DIETARY CALORIE RESTRICTION AND TREADMILL EXERCISE ON LIPID PROFILE AND OVERALL GENE AND PROTEIN EXPRESSION IN MOUSE SKIN TISSUES

by

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

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Abstract

Weight control via dietary caloric restriction and/or exercise has been demonstrated for cancer prevention. However, the underlying mechanisms are not clear. Previous studies in our lab showed that IGF-1 and IGF-1-dependent signaling were reduced by weight control. To confirm the requirement of IGF-1 reduction for cancer prevention, we restored IGF-1 in the exercised mice, which partially reversed the reduction of TPA-induced PI3K expression and PI3K-related 38:4 PI substrate. To explore the overall mechanistic impact, we further studied the effect of weight control on the profiles of lipid, gene and protein expression in TPA treated skin tissues. The mice were randomly assigned into 4 groups: ad libitum-fed sedentary control (control), ad libitum-fed exercise (AL+Exe), exercise but pair-fed at the amount of control (PF+Exe), and 20% of dietary calorie restriction (DCR). At the end of 10 weeks, the mice were treated with TPA topically for two hours. The body weights were significantly reduced in DCR and PF+Exe but not AL+Exe mice when compared with the control. Plasma and skin tissue triacylglycerides were significantly decreased in PF+Exe and DCR groups but not AL+Exe. Similar impact was found for the diacylglyceride profile in both plasma and skin tissue accordingly. Using Affymetrix microarray, 784, 223, and 152 probe sets were respectively found significantly changed by DCR, PF+Exe, and AL+Exe. PF+Exe and DCR showed similar impact on signaling pathways-related gene expression as analyzed by GenMAPP. Of the total 86 proteins identified by 2D-DIGE proteomics, 20 proteins were significantly changed by DCR. Overall, our results showed weight control via DCR or pair-fed exercise rather than exercise with ad libitum feeding significantly reduced body weight and body fat, resulting in reduction of IGF-

1 and IGF-1-induced signaling such as PI3K and PI-related pathway. The overall impact upon lipid profiling and gene and protein expression by weight loss suggests many other mechanistic targets. Although we could not ambitiously clarify all the changes were related to anticancer mechanisms in the scope of this study, understanding of the relationship between weight control and TPA-induced skin cancer risk as well as IGF-1-dependent signaling pathways may reveal intrinsic mechanisms and provide novel approaches to prevent cancer in the future studies.

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CHAPTER 1 - Review: Mechanism of Weight Control on Skin Cancer Prevention and "-OMICS" Study

Mechanism of Weight Control on Skin Cancer Prevention

The obesity rate in the U.S has been growing rapidly during the past 20 years (CDC, 2006). It has become a serious world wide problem which is associated with increased risks for several chronic diseases, including cancer, diabetes, and cardiovascular disease. Studies show evidence for a positive association between overweight/adiposity and cancer risk in esophagus, pancreas, colon, rectum, endometrium, kidney, and postmenopausal breast cancer (WCRF/AICR, 2007). Weight control, therefore, has become an important strategy against cancer and/or other chronic diseases. Body weight control is carried out by the balance of negative energy, which is tightly associated with dietary calorie intake and/or physical activity (energy expenditure). A positive energy balance, via increased dietary intake and/or decreased energy expenditure, results in increased weight and fat mass, or adiposity. Negative energy balance via decreased calorie intake or increased expenditure in adult may help maintain body weight and thus benefit health status.

Calorie restriction is referred to decrease of energy intake without malnutrition. In calorie restriction regimens, proteins and all the essential micronutrients such as vitamins and minerals are kept same. Only the total amount of energy is reduced usually at 20-40% of the ad libtum-fed controls. The cancer preventive effect of caloric restriction has been known for almost one hundred years. The first animal study was done as early as 1909 by Moreschi, who observed that tumors transplanted into underfed mice grew slower than those in ad libitum fed mice (Moreschi et al., 1909). In the 1940s, Tannenbaum found that reduced food intake decreased tumor incidence in experimental animals (Tannenbaum, 1947). Later on, the preventive effect of calorie restriction on cancer was confirmed in various animal models such as primate and rodent or various organs including mammary gland, prostate, colon, and skin. Calorie restriction has been

shown to be effective in both spontaneously occurring and chemically induced cancers. Calorie restriction is also able to lessen cancer in genetically engineered models, such as p53 knock out mice and APC ^{min} mice (Hursting SD, 1997; Mai V et al., 2003). To data, calorie restriction is found to be the most potent and effective dietary intervention strategy for cancer prevention in animal models (Hursting SD. et al., 2003).

The health benefit of physical activity (exercise), on the other hand, has been known for many decades. Accumulated evidence both in human studies and animal models has shown that physical activity is helpful in decreasing cancer risk. The epidemiologic studies on the relationship between physical activity and cancer prevention as reviewed by Friedenreich and Orensterin (2002) suggested that the evidence for decrease of cancer risk by physical activity be convincing for colon and breast cancer, probable for prostate cancer and possible for endometrium and lung cancer, although some types of cancers seemed less sufficient and conclusive (Friedenreich and Orenstein, 2002). Colon cancer has been studied the most with respect to physical activity in animal models. It was found that physical activity, both by forced treadmill and voluntary wheel, was effective in reducing azoxymethane-induced colon carcinomas in rat (Reddy BS et al., 1988; Thorling EB et al., 1994). However, the results were not conclusive in APC ^{min} mice (Basterfield et al., 2005). The effect of physical activity on breast cancer prevention in animal models was reviewed by Thompson et al. (2004), indicating physical activity might inhibit mammary carcinogenesis, but the effect was less reproducible compared to calorie restriction. Overall, the impact of physical activity on cancer prevention is positive, but not consistent or potent as calorie restriction approach.

Despite the many studies that have been conducted, no mechanism of weight control on cancer prevention has been definitively established. Enhancement of DNA repair and diminution

of oxidative damage to DNA, as well as reduction of oncogene expression have been postulated for calorie restriction. Weight loss, via calorie restriction and/or exercise, has been found to reduce certain circulating growth factors and hormones, such as IGF-1, adipocytokins, glucocorticosteroids, which are critical in maintenance of cellular growth, proliferation, cell cycle, and apoptosis function. Changes of these growth factors and inhibition of these factors-dependent biological processes by weight control may contribute to the overall anti-carcinogenesis.

IGF-1 a key modulator for cell growth and anti-apoptosis

IGF-1 system: key IGF-1, IGF-1 binding proteins, IGF-1 receptor and IGF signaling

Insulin-like growth factors (IGF-1 and IGF-2) are 70-amino-acid polypeptides that have high sequence similarity to insulin. Both IGF-1 and IGF-2 have metabolic functions, and play important roles in cellular proliferation and differentiation. The major function of IGF-2 seems related to embryonic growth and early development (DeChiara et al., 1990), but IGF-1 is more important in post-natal growth. IGF-1 is majority produced in the liver and also all the other cells. In the liver, the synthesis of IGF-1 is mainly regulated by growth hormone.

The circulating levels of IGF-1 and their bioavailability are modulated by a family of IGF binding proteins (IGF-BPs), which have six homologies. IGF-BP3 is the most abundant in humans. It is found that about 90% of IGF-1 in the serum is binds to IGFBP3, a very large complex that can not be transported out of bloodstream. Free IGF-1 or IGF-1 that bind to IGF-BP1 and IGF-BP2 are able to across the capillary endothelium and reach target tissues (Voskuil et al., 2005). IGF-BPs are degraded by proteases both in the tissues and in the circulation, through which IGF-1 is freed and interacts with IGF-1 receptors. Furthermore, IGF-BPs can also

modulate the process of IGF-1 binding to receptors and the IGF signaling (Holly et al., 1993; Clemmons, 1993).

IGF-1 receptor (IGF-1R) is a member of receptor tyrosine kinase super family. IGF-1R binds IGF-1 with the highest affinity, while it can also bind IGF-2 and insulin (Ullrich et al., 1986). In addition to IGF-1R, insulin receptor (IR) and IGF-2 receptor (IGF-2R) are able to bind IGF-1, but with less affinity. Studies showed that insulin receptor and IGF-1 receptor could form heterohybrid (Soos et al., 1993; Pandini et al., 2002).

Binding of IGF-1 to the receptors will induce autophosoporylation and activation of downstream signaling network, such as the phosphatidylinositol-3-kinase (PI3K) pathway. The phosphorylation of PI3K results in activation of Akt. Activated Akt will then inhibit the activation of interleukin-1β-converting enzyme (ICE)-like protease, therefore suppresses apoptosis. Binding of IGF-1 to its receptor is also found to activate other pathways, such as MAPK pathway. Activation of MAP kinase will lead to an increase in cell proliferation (Raushan et al., 2006).

IGF-1 and cancer

The IGF-1 system has been found to be involved in human development and the maintenance of a normal function and homeostasis of cell growth in the body. Increased IGF-1 levels lead to break down of normal cell homeostasis and function, which usually found during cancer development. Studies showed that the neoplasia process may due to the elevation of IGF-1 in the circulation and/or the increased sensitivity of IGF-1R to the hormone. Increased IGF-1 stimulates cell proliferation and inhibited apoptosis in various cancer cells. The relationship of IGF-1 and cancer and the potential corresponding mechanism have also been studied extensively in human subjects and animal models.

In the study of colon cancer, it was found that gene expression of IGF-1 was elevated in colon carcinomas (Tricoli et al., 1986). Similar results were found in the human breast and lung tumors (Yee et al., 1989). Later on epidemiological studies showed plasma IGF-1 levels were positively associated with higher risk of cancer, especially prostate and breast cancer. Chan et al. (1998) first demonstrated a link between circulating IGF-1 and prostate cancer risk using a nested case-control study. The study showed that plasma IGF-1 levels were positively associated with prostate cancer risk. Comparing to men in the lowest quartile of plasma IGF-1 levels, men in the highest quartile had a 4.3 folder higher risk of prostate cancer (Chan et al., 1998). In women, IGF-1 was found to be positively associated with pre-menopausal breast cancer rather than post-menopausal breast cancer (Renehan et al., 2004 lancet; Voskuil et al., 2005a; Canzian et al., 2006). The correlation between circulating IGF-1 levels and cancer risk was also found in colon cancer and bladder cancer (Giovannucci et al., 2001; Zhao et al., 2003). In addition to cancer risk, elevated plasma IGF-1 was associated with benign prostatic hyperplasia, proliferation of colorectal mucosa, and colorectal adenomas (Khosravi J et al., 2001; Chokkalingam AP et al., 2002; Cats A et al., 1996; ReneHan AG et al., 2001; Teramukai S et al., 2002). Overall, the reported studies support that relatively high circulating IGF-1 levels may have a causal role in cancer development.

IGF-1 and IGF-1 signaling are also found to play an important role in skin cancer. Rho et al. (1996) found that the mRNA level of IGF-1 and IGF-1 receptor in dermal and epidermal of mouse skin was significantly increased in the skin papillomas and carcinomas. In order to detect the potential role of IGF-1 signaling in the multistage mouse skin carcinogenesis, DiGiovanni lab developed transgenic mice HK1.IGF-1, in which IGF-1 is over expressed in epidermis driven by a human keratin 1 promoter (Bol DK et al., 1997). The authors found that HK1.IGF-1 transgenic

mice were more sensitive to tumor promoters such as TPA, chrysarobin, okadaic acid, and benzyl peroxide after initiated by DMBA than wild type mice. Compared to wild type animals which received the same dose of carcinogen treatment, transgenic mice developed tumors more rapidly and the number of tumors per mouse was dramatically increased (Bol DK et al., 1997; Wilker E et al., 1999). In addition, squamous papillomas and carcinomas were found to developed spontaneously in a similar transgenic mouse model BK5.IGF-1, which over expresses IGF-1 in the basal layer of skin epidermis (DiGiovanni et al., 2000). Activation of IGF-1 receptor, epidermal hyperplasia and increased labeling index were also observed in these mice. Not only in chemically induced skin carcinogenesis, an altered IGF system were also found to contribute to HaCaT keratinocyte UV susceptibility (Thumiger et al., 2005). The above data suggested that constitutive expression of IGF-1 and activation of IGF-1 receptor signaling pathways in basal epithelial cells lead to tumor promotion, in which IGF-1 played an important role in skin cancer development. More recently, it was found that PI3K/Akt pathway is important in IGF-1 mediated skin promotion (Wilker et al., 2005). Inhibition of PI3K activity significantly blocked epidermal proliferation, as well as skin tumor development in DMBA initiated IGF-1 transgenic mice (Wilker et al., 2005).

IGF-1 as a mediator in cancer prevention by weight control

As discussed above, the high IGF-1 levels seem associated with the risk of cancer development and lowering IGF-1 levels via weight control appears to be related to a decreased cancer incidence. Thus, manipulating plasma IGF-1 levels have been applied in cancer prevention strategies. In order to test this hypothesis, a mouse model that has a genetic deletion of liver IGF-1 gene was generated (Wu Y et al., 2003). In these mice, IGF-1 levels are 25% of that in none transgenic mice. Lowering circulating IGF-1 significantly delayed mammary gland

tumor development by carcinogen DMBA or C3 (1)/SV40 large T- antigen induced carcinogenesis (Wu Y et al., 2003). Fibroblasts lacking IGF-1 receptor were found to be highly resistant to transformation by simian virus 40 T antigen (Pietrzkowski Z et al., 1992; Sell C et al., 1993). Moore et al. (2008) found that the activation of the Akt and mTOR signaling pathways by tumor promoter TPA were significantly reduced in IGF-1 deficient mice, resulting a blockage of epidermal response to tumor promotion. Kari et al. (1999) found that the functional disruption of IGF-1R markedly inhibited breast cancer metastasis in the nude mice by suppressing cellular adhesion, invasion, and metastasis of breast cancer cells to the lung, lymph nodes, and lymph vessels.

Reducing plasma IGF-1 by weight control has been investigated in a number of studies. Ruggeri et al. (1989) first reported that dietary calorie restriction decreased serum IGF-1 significantly at the first and third week after the experiment started in female Sprague-Dawley rats. Hursting et al. (1993) found serum IGF-1 in 40% of calorie restricted rats was only 44% of *ad libitum* fed controls. The author also infused human recombinant IGF-1 back to the dietary restricted rats by using osmotic minipumps. Infusion of IGF-1 restored cell proliferation activity and enhanced mitogen responsiveness in dietary restriction treated rats (Hursting et al., 1993). In a tumor study of p53 deficient mice, 20% of calorie restriction decreased circulating IGF-1 by 26% and restoration of IGF-1 in calorie restricted mice did not change the tumor incidence significantly, but increased cell proliferation and inhibited apoptosis dramatically (Dunn et al., 1997). Study by Thompson also found that 40% of calorie restriction reduced circulating IGF-1 by half in rats and restoration IGF-1 failed to have effect on mammary tumor incidence (Zhu et al., 2005). Studies in our lab also found that IGF-1 was significantly decreased by dietary calorie restriction and restoration of IGF-1 significantly abolished PI3K reduction in treadmill exercised

mice with limited feeding at same amount as sedentary control (Xie et al., 2007). Overall, the above results showed that reduction of IGF-1 levels and thus down-regulation of IGF-1 signaling pathways as a consequence of dietary restriction could contribute to anti-tumorigenesis. Restoration of IGF-1 abrogated, at least in part, the protective effect of calorie restriction on carcinogenesis.

The impact of physical activity on IGF-1 reduction and cancer prevention is complicated. As reviewed by Kaaks et al. (2004), physical activity decreased IGF-1 level in children and adolescents. But for adults, the plasma IGF-1 levels were not decreased either by short bouts of exercise or physical training. Studies showed that weight control by long term exercise could decrease IGF-1. For example, a recent published paper found that plasma concentrations of IGF-1 were significantly lower in endurance runners than sedentary controls (Fontana et al., 2006). In animal models, our lab found that exercise alone with ad libitum feeding was not sufficient to decrease plasma IGF-1 levels. When the exercised mice were fed the same amount as their sedentary counterpart, plasma levels of IGF-1 were modestly but significantly reduced (unpublished data). Nevertheless, the evidence by us and others indicate a negative energy balance appears to be a fundamental requirement for IGF-1 reduction and potential cancer prevention.

Adipocytokines: a linkage of adipose and cancer risk

Adipocytokines are secretary products of adipose tissue and have metabolic and endocrine functions. They include leptin, adiponectin, resistin, and visfatin, etc., which have been identified and studied recently for a potential relationship between obesity and cancer risk (Korner et al., 2005).

Leptin

The leptin gene, which is also called the obese (ob) gene, encodes a 16 kDa protein (Zhang et al., 1994). As an adipocytokine, leptin is secreted mainly by adipose tissue. Other tissues, such as placenta, ovaries, skeletal muscle, pituitary gland, stomach, and liver, are also able to produce leptin. The major factor that affects circulating leptin levels is adipose tissue mass (Maffei et al., 1995). Increased body weight has been shown to be positively associated with high level of plasma leptin (Frederich RC et al., 1995). Leptin was found to regulate appetite and control body weight by affecting the hypothalamus, suppressing food intake and stimulating energy expenditure (Muoio et al., 2002). In addition to the central cirtuits, leptin also has effects in the periphery tissues, such as lung, intestine, skin, stomach, heart and other organs, though binding to leptin receptors (Margetic et al., 2002; Cornish et al., 2002).

Leptin receptors contain extracellular, transmembrane and intracellular domains. The extracellular domain is responsible for leptin binding and intracellular domain recruits and activates downstream substrates. Activation of leptin receptors was found to stimulate signaling pathways, such as JAK2/STAT3, Ras/ERK1/2 and PI3K/Akt/GSK3. Other signaling proteins induced by leptin were also found, including protein kinase C, p38 kinase, and AP-1 component c-fos, c-jun, and junB, etc. (reviewed by Garofalo et al., 2006).

Leptin is important in the regulation of energy balance. Obese (ob/ob) mice, which have a leptin gene mutation, are found to be morbidly obese, infertile, hyperphagic, hypothermic, and diabetic (Huang et al., 2000). Infusion of recombinant leptin into these mice reduced food intake and decreased body weight (Campfield et al., 1995; Halaas et al., 1995). In diet induced obese mice, the circulating leptin was significantly elevated with the increase of body weight. Studies also showed these mice are resistant to peripherally administrated leptin (Van Heek et al., 1996).

Compared to normal weight people, obese people usually developed hyperleptinemia and leptin resistance, which might be due to a defect in transporting of leptin across the blood barrier (Banks et al., 1996; Caro et al., 1996).

Epidemiologic studies showed that moderately elevated serum leptin was associated with prostate cancer development (Stattin et al., 2001). People that have high leptin levels tend to have a large tumor (Chang et al., 2001, Saglam et al., 2003). However, some studies found there was no relationship between circulating leptin and prostate cancer risk (Lagiou et al., 1998; Stattin et al., 2003). In vitro, leptin is found to be a promoter in cancer cells. Studies showed that leptin induced cell proliferation in breast cancer ZR75-1 and HTB-26 cells via the activation MAPK and PI3K (Frankenberry et al., 2006). Leptin also simulated estrogen synthesis by increasing aromatase gene transcription and protein activity, which implied that leptin might be responsible for the resistance to anti-estrogens during hormonal treatment of breast cancer (Sulkowska et al., 2006). In colon cancer cells, leptin induced cell growth and blocked apoptosis of human cancer HT29 cells via stimulation of ERK1/2 and NFκB pathway (Hardwick et al., 2001; Liu et al., 2001). In addition, the mitogentic activity of leptin has also been demonstrated in prostate, pancreatic, ovarian and lung cancer cells. Taken together, leptin seems to be important in tumor progression. Manipulation of plasma leptin might be effective in cancer prevention and treatment.

As discussed above, leptin was positively associated with body weight and body mess index. Weight control seems to be effective in lowering circulating leptin. Fontana et al (2006) showed that plasma concentrations of leptin were significantly lower in endurance runners than sedentary controls. In animal models, it was found that 40% of calorie restriction significantly decreased the serum leptin levels in APC^{min} mice compared to the control (Mai et al., 2003).

Studies by our laboratory showed that the plasma level of leptin was significantly decreased in calorie restricted mice and exercised mice with paired feeding, but not in exercised mice with *ad libitum* feeding (Xie et al., 2007). Interestingly, we found that leptin in subcutaneous fat cells was not affected by weight control treatment (Xie et al., 2007). All the above evidence suggests that leptin is important in mediating the cancer protective effects of weight control. Further research is needed to characterize the specific role of leptin in cancer development.

Adiponectin

Adiponectin is also an adipocytokine that is secreted in adipose tissue and plays an important role in obesity-related disorders. The gene of adiponectin is located on diabetes susceptibility locus chromosome 3q27 (Sonnenberg et al., 2004; Takahashi et al., 2000). Adiponectin was found to account for 0.01% of total plasma protein in human serum (Arita et al., 2002). It exists in several forms: trimers, hexamers, high molecular weight multimers (HMW), or globular form. HMW form was suspected to be the most bioactive form (Richards et al., 2006; Fisher et al., 2005).

Two adiponectin receptors have been identified. The signaling downstream of adiponectin receptors is still under investigation. Miyazaki et al. (2005) found that different forms of adiponectin have distinct biological effects, which may be through differential activation of downstream signaling.

Some evidence has showed that adiponectin is an insulin sensitizing hormone and may process anti-diabetic activities (Kantartzis et al., 2005). The blood level of adiponectin was found to be lower in obese people compared to controls. Adiponectin was able to neutralize LPS activity and has anti-inflammation activities(Tsuchihashi et al., 2006; von Eynatten et al., 2006). In addition, adiponectin was also found to be a modulator of lipid metabolism and might have

preventive effect on cardiovascular disease. Kim et al. (2006) found that an increase of adiponectin concentrations or the maintenance of the higher levels was negatively associated with cardiovascular risk factors in nondiabetic CAD male patients, independent of adiposity and smoking status (Kim et al., 2006).

The potential anticancer properties of adiponectin have been investigated both in epidemiological study and animal models. There are three case control studies showed that low serum adiponectin levels were associated with an increase risk of breast cancer in women (Miyoshi et al., 2003; Chen et al., 2005; Kang et al., 2005). In breast cancer patient, people who have low serum adiponectin levels tended to have more aggressive tumors (Miyoshi et al., 2003). The inverse relationship between serum adiponectin and endometrial cancer risk was also identified by two case control studies in Italy and Grace, respectively (Dal et al., 2004; Petridou et al., 2003). Adiponectin was found to be lower in prostate cancer patient compared to healthy controls where the levels were negatively correlated with histologic grade and disease stage (Goktas et al., 2005). Studies by Ishikawa et al. (2005) showed that in gastric cancer patients, their plasma adiponectin levels were significantly lower than healthy controls. In addition, the plasma adiponectin was negatively associated with tumor size, depth of invasion and tumor stage in undifferentiated gastric cancer (Ishikawa et al., 2005). A prospective nested case-control study conducted by Wei et al. (2005) observed that men with low plasma adiponectin levels had a higher risk of colon cancer than men with higher adiponectin. However, one study reported adiponectin was not associated with colorectal cancer (Lukanova et al., 2006). Overall, studies in human subjects provided some evidence that adiponectin could protect against certain type of cancer.

The cancer preventive effect of adiponectin may partially explained by its ability in modulating the biology of tumor cells. Studies by Yokota et al., (2000) found that adiponectin suppressed the growth of myelocyte cells, induced apoptosis in myelotye leukemia cells, and inhibited TNF-alpha production. Adiponectin was found to inhibit breast cancer MDA-MB-231 and MCF-7 cells proliferation and induce cell cycle arrest and apoptosis in these cells (Kang et al., 2005b; Dieudonne et al., 2006). Bub et al., (2006) reported that adiponectin suppressed the growth of prostate cancer cells. In colon cancer cells, however, Ogunwobi et al. (2006) demonstrated that adiponectin was a promoter of colon cancer HT29 cells.

Studies showed that plasma adiponectin level was negatively associated with obesity, glucose and lipid levels, and insulin resistance (Bluher et al., 2006). Weight control through dietary calorie restriction and/or exercise seem to elevate plasma adiponectin, however, the results are not very conclusive. Studies showed calorie restricted rats had a high level of plasma adiponectin with reduced blood glucose, plasma insulin, and triglyceride levels when compared with ad libitum fed controls (Zhu et al., 2004). However, in a human study, the serum concentration of adiponection was not found to change in people after three weeks calorie restriction (Barnholt et al., 2005). For the effect of exercise, Jamurtas et al. (2006) showed that plasma adiponectin was not changed in people up to 48 hours post-acute exercis. Oberbach et al. (2006) reported that after four weeks of physical training, adiponectin levels was significantly increased in people who had type 2 diabetes. The changing of adiponectin levels was correlated with enhanced insulin sensitivity (Oberbach et al., 2006). Other adipocytokins, such as resistin and omentin, may also play a role in weight control-mediated cancer preventive effects, but their cellular and physiological function are still not clear (McTernan et al., 2006; Yang et al., 2006).

Other hormones related to cancer prevention by weight control

Insulin

Insulin is an important hormone that regulates blood glucose level. In the liver, it promotes glycogen synthesis by stimulating glycogen synthase and inhibiting glycogen phosphorylase. In muscle and fat tissues, insulin induces uptake of glucose via increasing GLUT4 expression. Insulin is also functioned as a moderate mitogen. After binding to its receptor, insulin may activate signaling pathways via phosphorylation of the insulin-receptor stubstarate-1, Akt, mitogen activated protein (MAP) kinase, and PI3K kinase (Rose et al., 1998; Finlayson et al., 2003). Therefore, insulin has been found to induce the growth of both normal and cancerous cells (Watkins L et al., 1990; Koenuma, M. et al., 1989; Bjork et al., 1993). Insulin also promotes the bioactivity of IGF-1 via either increasing the number of growth hormone receptors in the liver or reducing hepatic secretion of IGFBP1, which binds and inhibits the activity of IGF-1 (Underwood et al., 1994; Powell et al., 1991).

Obesity or lack of physical activity is found to be a major factor inducing insulin resistance and further hyperinsulineamina. Epidemiological studies showed that increased plasma insulin was associated with a high risk of cancer (Kaaks R et al., 2000; Nilsen, TI et al., 2001). Dietary calorie restriction and/or regular exercise has been linked with a decreased plasma insulin in several studies (Zhu et al., 2005, Friedenreich et al. 2002, Giovannucci et al., 2001). It is noted that weight control via decreasing calorie intake or increasing energy expenditure can regulate glucose homeostasis and increase insulin sensitivity.

Glucocorticoids

Glucocorticoid hormones are a class of steroid hormones. The major function of these hormones are involved in regulation of glucose metabolism, such as stimulation of

gluconeogenesis in the liver, inhibition of glucose uptake in the muscle and adipose tissue, stimulation of fat breakdown in adipose tissue, and mobilization of amino acids from extrahepatic tissues. Glucocorticoids are also important in fetal development and have anti-inflammatory and immunosuppressive effects.

Glucocorticoid hormones act though binding to intercellular glucocorticosteroid receptor. After binding with the hormone, the new formed receptor-ligand complex dissociates with heat shock proteins and then translocates into the nucleus, where it binds again to glucocorticoid response elements (GRE) and acts as a transcription factor. Glucocorticoid receptor usually works as a negative transcription factor, and it has been shown to inhibit the transcription of almost all immune system-related genes. In some cases, activated glucocorticoid receptor may interfere with other transcription factors, such as AP-1 and NFκB (Stöcklin et al., 1996; Subramaniam et al., 2003) that are crucial in the regulation of a number of genes involved in inflammation, differentiation, cell proliferation, apoptosis, oncogenesis, and other biological processes (Karin and Chang, 2001; Shaulian and Karin, 2001; Ghosh and Karin, 2002; Karin et al., 2002).

In addition, glucocorticoid steroids are potential tumor inhibitors. Administration of hydrocortisone in the diet showed preventive effect on the promoting phase of skin carcinogenesis in the mice (Trainin, 1963). There are a number of studies shown that glucocorticoid steroids are elevated in calorie restricted animals (Pashko and Schwartz, 1992; Yaktine et al., 1998). Adrenalectomy was found to decrease plasma corticosterone levels and abrogate the preventive effect of dietary restriction on skin tumor development (Pashko and Schwartz, 1992; Stewart et al., 2005). Similar results were also observed in lung carcinogenesis but not in mammary gland tumors (Pashko and Schwartz, 1996; Jiang et al., 2004). When

administrating corticosterone in adrenalectomized mice, the cancer preventive effects of calorie restriction on skin carcinogenesis were restored as shown by our previous publication from the Birt lab (Stewart et al., 2005). Overall, the published data indicate glucocorticoids may be critical mediators in cancer prevention by calorie restriction.

Molecular Targets of cancer prevention by weight control

Effects on cellular processes

It is well known that cancer arises due to the loss of a normal growth control. In normal tissues, cell growth and cell death are highly regulated and balanced. In cancer, this regulation is disrupted, which is either from increased cell proliferation or loss of programmed cell death, or both.

The effect of calorie restriction on cell proliferation has been investigated in numerous studies. Lok et al. (1990) reported that 25% of calorie restriction decreased cell proliferation by 72% in mammary gland and 30-60% in skin, esophagus, bladder, and GI tract of female Swiss Webster mice. Pashko and Schwartz (1992) showed that 27% of food restriction suppressed TPA-induced epidermal [^{3}H]-thymidine incorporation. In C57BL/6 $\times$C3HF$_1$ mice, a murine strain that develops liver tumor spontaneously, 40% of dietary restriction was found to decrease cell proliferation significantly in the liver (James et al, 1994). Dunn et al. (1997) demonstrated that 20% of calorie restriction significantly inhibited BrdU incorporation in the bladders of p53 knock out mice. Restoration of IGF-1 brought the cell proliferation back to the level of the control mice (Dunn et al., 1997). Comparing to *ad libitum* feeding, 30% of dietary calorie restriction significantly inhibited cell proliferation in carcinogen treated mouse skin (Fischer et al., 1998). Using a heavy water labeling, Hsieh et al. (2005) investigated a time-course of the effects of calorie restriction on cell proliferation rates in female C57BL/6J mice. It showed the

proliferation rates of mammary epithelial cells and T cells were markedly reduced within 2 weeks with calorie restriction regimen when compared to that of ad libitum fed mice. Two weeks after refeeding, the cell proliferation rates rebounded to the basal level (Hsieh et al., 2005). We found that the percentage of PCNA in skin epithelial cells was significantly lower in 20% of calorie restricted mice than ad libitum fed mice, as shown by immunohistochemistry staining (Xie et al., 2007). The percentage of the splenocyte in S phase was significantly reduced by 40% of calorie restriction in p53 knock out mice as well as wild type mice, as shown by Hursting et al. (1994). Studies in Thompson lab showed that cell cycle regulators, i.e., cyclin D1, cyclin E, cyclin-dependent kinase (CDK)-2, and CDK-4, were decreased by 40% of calorie restriction in rat mammal carcinomas, while cyclin-dependent kinase inhibitors (CKI), i.e., Kip1/p27 and Cip1/p21, increased (Zhu et al, 2003; Jiang et al, 2004). Overall, the effects of calorie restriction on cell proliferation are clear and reproducible in the animal models. For physical activity, studies in our lab found the cell proliferation rates of exercised mice with paired feeding had a lower rate than sedentary mice, but exercise with ad libitum feeding actually enhanced proliferative rates in epidermal cells, suggesting exercise alone without dietary calorie limitation might promote cellular proliferation and result in inconsistent impact on cancer protection (Xie et al., 2007).

Programmed cell death or apoptosis is highly regulated by a series of arranged morphological and biochemical events (Adams, 2003). It is important for maintenance of tissue homeostatsis, embryo development, and immune defense. Defects in apoptosis are thought to play an important role in cancer development (Gerl and Vaux, 2005; Lossi et al., 2005). Induction of apoptosis was observed in both normal liver and putative preneoplastic foci induced by hepatomitogen cyproerone acetate in dietary calorie restricted rats (Grasl-Kraupp et al, 1994).

Dietary restriction was also found to induce apoptosis in the liver of C57BL/6 ×$C3HF_1$ mice (James et al, 1994). Increased apoptosis was observed in the bladder preneoplasia of p-cresidine-treated p53-deficient mice by dietary calorie restriction (Dunn et al., 1997). In mammary gland, calorie restriction induced apoptosis in both premaglignant and malignant pathologies (Thompson et al., 2004). Thompson et al., reported that apoptosis regulatory molecules, i.e., Bcl-2, Bcl-xl, and XIAP, decreased and Bax and Apaf-1 increased significantly in the mammary carcinomas of calorie restricted rats when compared with that of the control rats (Thompson et al, 2004). They also reported that the activities of both caspases-9 and caspases-3 were significantly induced and Akt phosphorylation was depressed by calorie restriction. The authors proposed that an induction of apoptosis by calorie restriction might be associated with its inhibitory effect on IGF-1 signaling. As for the physical activity, the research on apoptosis is sparse. Studies from our lab showed that caspase-3 activity but not caspase-3 protein increased significantly in epidermis of dietary calorie restricted and treadmill exercised mice in comparison with the sedentary controls (Xie et al., 2007).

Collectively, all these data above indicate that modulation of cellular processes including inhibition of cell proliferation and restoration of apoptosis, is a molecular target in weight control for cancer prevention. Figure 1.1 shows a proposed mechanism by which weight control may inhibit cancer development via inhibiting the cross-talk between hormone-dependent and TPA-promoted signaling pathways, resulting in modulating cellular proliferation and anti-apoptosis. Weight loss changes circulated growth factor and/or hormone levels such as IGF-1, leptin, adiponectin, and glucocorticoids, which thus inactivate TPA-induced signaling through hormone or growth factor-dependent cascades, e.g., Ras-MAPK and PI3K-Akt pathways. Finally, it may

lead to an inhibition of TPA-induced cellular proliferation and elimination of IGF-1- persuaded anti-apoptosis.

Reduction of oxidative stress

Oxidative stress may injure cellular DNA, protein and lipids in the tissue. It is thus associated with ageing and many chronic diseases. In carcinogenesis, reactive oxygen and nitrogen species can attack DNA directly and induce DNA mutations. Oxidative stress also occurs by the reactive products of a peroxidation from various macromolecules, such as lipid peroxidation that may lead to protein and DNA modification. Cumulative evidence has been shown that long term calorie restriction in rodents extend maximum life span and decrease oxidative damage to DNA and proteins (reviewed by Credilla et al., 2005). Qu et al. (2000) found that 60% of calorie restriction completely abolished the increased oxidative damage in cloribrate-induced mouse liver (Qu et al., 2000)

Other possible effects

Calorie restriction may also interfere with the expression balance between oncogene and tumor suppressor gene directly. “Oncogenes refer to genes whose activation can contribute to the development of cancer” (Osborne et al., 2004). They are mutated versions from pro-oncogenes, which function in cell proliferation and differentiation. Over-expression of oncogenes usually causes out of control cellular growth. One of the most known oncogenes is Ras (Retrovirus-associated DNA sequences) family. Ras plays an important role in cell proliferation and can inactive tumor suppressors and promote cancer development (Fernandes et al, 1995). “Tumor suppressor genes refer to those genes whose loss of function results in the promotion of malignancy” (Osborne et al., 2004). Typically, a normal function of tumor suppressor genes is to inhibit cellular proliferation. Mutations of these genes usually result in a loss of their growth

inhibition ability, which in turn may favors of cellular proliferation. Some examples of well known tumor suppressor genes include p53, retinoblastoma susceptibility gene, Wilms' tumors, neurofibromatosis type-1, and familial adenomatosis polyposis coli, etc. Previous studies have demonstrated that food restriction may induce an over-expression of tumor suppressor gene p53 (Fernandes G et al, 1995). In the Brown-Norway rats fed with calorie restricted diet or ad libitum diet, Hass et al. (1993) found that pancreatic acinar cells from calorie restricted animals had a lower growth rate and less N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced transformation. Calorie restriction derived cells showed decreased c-Ha-ras gene expression, lower rate of mutation of p53 tumor suppressor gene, and increased genomic methylations of DNA (Hass et al., 1993).

DNA repair occurs in the normal mammalian cells to repair DNA damage caused by multiple factors including oxidative stress. It reported that dietary calorie restriction enhanced DNA repair ability against DNA damage caused by UV exposure (Lipman et al, 1989; Weraarchakul et al, 1989). Hursting et al. (1999) suggested that both calorie restriction and exercise could induce DNA repair pathway, therefore block the early stage of carcinogenesis. However, 30% of calorie restriction failed to activate DNA repair pathways and inhibit tumor development in the DNA mismatch deficient mice (Tsao et al, 2002).

As we know more about the protective mechanisms of weight control, it is becoming apparent that it is not only one single mechanism involved. Most likely, it is a combination of multiple factors and multiple signaling pathways involved. Hundreds of biological molecules may cooperate in this network complex. Therefore, traditional molecular biology techniques seem inadequate to meet the requirements to gain a broader and deeper overview of the mechanisms. Fortunately, recently developed technologies named “-omics” may provide us a

chance to take a global view of these biological processes. Microarray study is the first step to obtain a gene expression profile, together with proteomics and lipidomics may generate a clear picture of a profiling response to the weight loss treatment.

"-Omics" studies of Weight Control in skin carciogenesis

Transcriptomic (microarray, gene expression profiling)

Transcriptome is a study of the entire range of transcripts produced by a given organism. The development of microarray technology provided a useful acreening tool in the assesment of gene expression profiles in a large scale or even the whole genome. Microarray analysis is very different from traditional gene expression methods such as Northern blot, nuclear protection assay, expressed sequence tags (ESTs), and RT-PCR which are used extensively in the study of individual gene expression. Microarray analysis include spotted cDNA, Affymetrix Genechip oligo array and tissue microarray. It has been widely used in the study of gene function, identification of tumor-specific molecular markers, genotyping, and gene discovery.

Microarry has been used in the study of skin carcinogenesis in several labs. Using a mouse cDNA microarray, Schlingemann et al., screened the gene expression in tumor promoter TPA induced mice skin. In this study, female C57BL/6 mice was treated with 10 nmol TPA in 100 μl acetone for six hours, with control mice treated with acetone only (Schlingemann et al., 2003). The author used a cDNA microarray which has 5000 murine genes. Comparing to vehicle treated samples, 89 genes were found to be differentially expressed in mice skin treated with a single dose of TPA for six hours. The author identified 54 genes up regulated by TPA and 35 genes down regulated by TPA (Schlingemann et al., 2003). Among the TPA induced genes, it was found that Sprr1a, JunB, Il4rα and Gp38 also highly expressed in papilloma and squamous

cell carcinoma, which implied that these genes may play an important role in the process of skin tumorgenesis. In a similar study, Wei et al., (2003) analyzed the gene expression profile of keratinocyte progenitor cells from TPA-treated Tg.AC mice using Atlas^{TM} Mouse 1.2 Microarray. The Tg.AC transgenic mouse carries activated v-Ha-ras oncogene and is more sensitive to tumor promoters than wild type mice. The mice were treated with 5ug of TPA in 200 μl acetone on back skin twice a week for two weeks. Among the 1176 genes analyzed, 11 genes were found changed significantly after the treatment of TPA compared to control. Nine genes were up regulated by TPA, they are: Galetin 7, nucleoside diphosphate kinase B (LGALS7), cytoskeletal epidermal keratin(NDK B), deleted in split hand/split foot 1 gene (Dss1), DNA double-strand break repair RAD21 homolog (HR21SP), transcription termination factor 1 (TTF1), Thymosin β4, Calpactin 1ight chain, and 40 S ribosomal protein SA. Two genes were down regulated by TPA, they are Apoliporotein E precursor APO-E) and type 1 cytoskeletal keratin 15 (CK15) (Wei et al., 2003). The gene profile of mice skin after TPA promotion was also investigated by Riggs et al.(2005), using a cDNA microarray containing about 8000 genes. The back skin of female DBA/2J mice (TPA susceptible stain) and C57BL/6J mice (TPA resistant strain) was treated with 3.2 nmol TPA in 200ul acetone two times a week for two weeks (Riggs et al., 2005). The results showed more than 450 genes were significantly changed by TPA treatment in either mouse stain, with 44 genes showed differential expression upon TPA application between TPA sensitive mice and TPA resistant mice. The author also identified genes that map within TPA promotion susceptibility loci and differentially expressed in the epidermis of C57BL/6J mice and DBA/2J mice. The genes are Gsta4, Cd109, 1190002N15Rik, Ppgb, Nmes1 (MGC58382), Serpinb2, Chi3l1, Mapkapk2, Prdx6, Npl and Sema4g. Three genes, Gsta4, Nmes1 (MGC58382), and Serpinb2, were thought to play an important role in the

susceptibility to TPA promotion (Riggs et al., 2005). The identification of genes that are responsive to TPA treatment provided us a better understanding of the process of skin carcinogenesis.

In addition to skin carcinogenesis, microarray analysis has also been used in the study of the effect of weight control on other tissues. Higami et al., (2004, 2006) examined the gene expression in epididymal white adipose tissue of C57Bl6 mice using high oligonuleotied microarray, which has more than 11,000 genes. Compared to control fed mice, about 345 genes were changed in energy restricted mice, 109 of them are up regulated. The author also identified that energy restriction up-regulated genes mostly function in mitochondrial energy metabolism, and the genes involving in inflammation, cytoskeleton, extracellular matrix and angiognesis were down-regulated (Higami et al 2006). Han et al., reviewed the impact of dietary restriction on gene expression based on 25 recent studies that have used microarray (Han and Hickey, 2005). The author summarized the gene expression profile of several species after dietary restriction. They found that the overall effect of dietary restriction on metabolism, stress and immune response, cell growth and transcription regulation were similar among different species (Han and Hickey, 2005).

Classical analysis of microarray data compares gene expression at the level of individual genes and identifying the significantly differentially expressed ones (Smyth, 2004). As discussed above, the investigators usually list the groups of up-regulated and down-regulated genes. Some other methods, such as northern blot, real time RT-PCR, and in situ hybridization are usually used to confirm the gene expression identified from microarray. In order to further interpret the biological function of these lists of genes, GO (gene ontology), a worldwide database that defines genes to various functional categories, were introduced and widely used (Ashbruner et

al., 2000). GO has three organized branches in describing gene products, and they are biological process, cellular component and molecular functions. The three ontologies are separate and independent. Now, the most common analysis using GO is the gene set enrichment analysis (Mootha et al., 2003), in which it tests whether a group of genes (such as significantly changed genes come from microarray) is significantly associated with a certain biological states.

Although the development of microarray technologies has enabled the study of gene expression profile in a high throughput manner, however, microarray analysis can not provide information regarding the translational regulation of genes and/or post-translational modifications of proteins. Proteomics approach provides a useful tool to study most expressed proteins, including their isoforms and post-translational modifications such as glycosylation, phosphorylation and proteolytic cleavage.

Proteomics

In addition to gene transcription profiles, protein expression profiles have also been a focus of biology research. The Proteome is the final outcome of genome expression comprises all the proteins present in a cell at a particular time. Proteomics, the global overview of proteome, is to investigate the expressions, modifications, interactions and regulation of proteins in organisms comprehensively. Through a proteomics approach, researchers are able to determine the expression of thousands of proteins in a single sample simultaneously.

The most classical and widely used proteomics technique is 2D PAGE (two-dimensional polyacrylamide gel electroporesis) coupled with peptide MS. 2D PAGE have been used to separation protein since 1970s. Basically, proteins are first separated by isoelectric focusing (IEF) based on their PI. Then in the second dimension, proteins are separated using SDS-PAGE

according to their PI and molecular weights. After separation, proteins are stained with dyes, such Coomassie blue, fluorescence dye or silver. Several software programs have been developed to quantitative analysis the image of 2D gels and characterize the abundance of a certain protein spot, such as DeCyde and Melani. Usually, in gel digestion will be performed, which is either by trypsin or by cyanogens bromide. At last, the generated peptides are analyzed by MALTI-TOF MS. In order to decrease gel to gel variance, 2D DIGE (two-dimensional difference gel electroporesis) technology was developed and used a lot (Marouga et al., 2005). With this method, protein samples are labeled with cyanine (Cy) dye on lysine residues before separated by IEF and SDS PAGE. Proteins samples with different Cy dyes (Cy2, Cy3 or Cy5) can then be mixed and run on the sample 2D gel. With 2D DIGE, the spot matching are simpler and more accurate. It also greatly reduced the variance between gels, as the usually two to three samples are running on the same gel.

A major limitation for the 2DE approach is that coverage is restricted to the most abundant proteins. Integral membrane protein and some transmembrane signaling proteins are also not identified by 2D proteomics. It maybe due to the protein in solubility constraints and subsequent difficulties encountered during extraction (Huang et al., 2003; Santoni et al., 2000). Another reason could be that the concentrations of integral membrane proteins and signaling proteins are lower than that needed and require affinity-based purificate to amplify signal prior to running the 2D gel.

In addition to 2DE, proteins may also be separated by ion-exchange or liquid chromatography. With multidimensional liquid chromatography approach, protein samples are digested with trypsin and then the complex protein peptides is separated using mulltidimensional liquid chromatography separation techniques. The most commonly used techniques are strong

cation exchange chromatography for separation of the first dimension and reversed phase column for second dimension. Nanospray or nanoflow flowrates are usually used to improve sensitivity. Previous liquid chromatography separation, specific sample labeling using stable isotope affinity tagging will improve the quantitative analysis of paired protein samples (Gygi et al., 2002). Isotope-coded affinity tags (ICAT) and isobaric tag-based methodology for peptide relative quantification (iTRAQ) are a widely used approach. With the iTRAQ approach, as many as four different protein samples can be labeled with isobaric regents respectively and mixed together to be analyzed by mulltidimensional LC MS/MS. Another promising quantitative proteomics strategy is stable isotope labeling by amino acids in cell culture (SILAC) (Waanders et al., 2007). SILAC makes all proteins from the cells distinguishable by metabolic incorporation of non-radioactive heavy isotopes in the whole proteome of desired cells. It also allows mixing of two cell populations for combined additional sample manipulation, which make it more reliable when compare different treatment (Blagoev et al., 2006).

In addition to the separation-based and mass spectrometry-based proteomics approaches, antibody-based microarrays are a rapidly emerging technology (Kopf and Zharhary, 2007). The common procedure of antibody microarrays includes: at first antibodies are printed on a solid plate. Then, protein sample, mixture of proteins, is applied on the plate. The interaction between antibody and its specific antigen can be then be detected. The antibody microarry approach is becoming an important proteomics research tool, especially on known proteins.

Using 2D PAGE and proteomics, Huang's group investigated and published the proteome map of BALB/c murine skin (Huang et al., 2003; 2005). By 2D gel analysis, the author identified more than 200 spots shown on the gel, and 44 spots were identified by TOF MASS. The author

identified proteins that enriched in epidermis are HSP27, vimentin, keratin 10, 14, 15, α-actin, 40S ribosomal protein SA, prohibitin, Cu/Zn superoxide dismutase and galecin-1.

Overall, the advancement of 2DE, mass spectrometric techniques, sequence data base correlation and antibody array has enabled the identification of proteins in complex mixture. Proteomics is helpful for further understanding of the molecular basis of cancer progress and revealing more clues on the potential cancer preventive effect of weight control by dietary caloric restriction and/or exercise.

Lipidomics

Greater than 1000 molecular species of lipids are found in eukaryotic membranes. Lipids include a broad range of molecules, which are classified into fatty acids, phospholipids, sterols, sphigolipids, terpenes and so on. Lipid components of plasma membranes play essential roles in constructing the permeability barrier of cells and organelles. In addition, lipids are also used to relocate membrane protein during protein sorting and signal transduction. Therefore, it is suggested that lipidomics be classified into two categories: architecture membrane lipidomics and mediator lipidomics (Serhan, et al., 2006). Investigation of the potential roles of lipids in the regulation and control of cellular function is very important in the understanding of normal physiology and disease processes.

Traditionally, the analysis of lipids only focused on a particular class of lipids. With the development of mass spectrometry, now it is able to quantitatively analyze a broad range of lipid species in a single platform. As reviewed by Welti et al., two methods are now mostly used in the analysis of lipid profiling (Welti and Wang, 2004). One is Bruger method, which utilizes the precursor and neutral-loss scanning capability of a tandem mass spectrometer (Bruger et al., 1997). The other method is by utilizing the scanning capabilities of single quadrupole mass

spectrometry (Han et al., 2003). Both of the methods can conduct the global analysis of lipids by using a very small amount of tissue (about 1mg dry weight).

With the help of lipidomics center in our university, the membrane lipids of skin samples from both sedentary and exercised mice were profiled. By using electrospray ionization mass spectrometry-based lipidomics approach, a total of 338 phospholipid species from 12 categories were identified. Ouyang et al. (manuscript), in our lab found 57 phopholipid species were significantly changed by exercise. Compared to sedentary controls, most phosphatidylinositol (PI), ether phosphatidylcholine (ePC), and some lysophosphatidylcholine (lysoPC) molecular species decreased significantly with exercise with in pair fed mice. Some phosphatidylcholine (PC) and lysophosphatidylethanolamine (lysoPE) molecular species containing long-chain polyunsaturated fatty acids were higher in exercised pair-fed mice than in the sedentary controls, which suggested that exercise with food control led to a higher level of omega-3 fatty acid. In contrast to pair fed exercised mice, exercise with ad libitum-fed mice showed little impact on lipid profiles (Ouyang et al., manuscript).

Other studies that used very recently

In addition to microarray, proteomics and lipidomics, other high throughput methods have also been developed recently. For example, a chromatin immunoprecipitation (ChIP)-based methods was now used to identify protein-DNA interaction profile DamID (Orian, 2006). Epigenomics, the study of the epigenetic changes on a genome wide scale, was also developed recently (Plass, 2002). Epigenetic is heritable changes that do not depend on changes in a DNA sequence. Epigenetic changes, such as DNA methylation and chromatin modification, were found to be involved in gene regulation of both tumor suppressor genes and oncogenes and it was important in cancer development (Baylin, 2005a). DNA methylation is the covalent addition

of a methyl group at the 5' carbon of cytosine, which results in 5-methylcytosine. Genes are silenced or inactivated when the DNA in the promoter region of the genes is methylated (Bird, 2002). Aberrant DNA methylation has been linked to atherosclerosis, cancer and other diseases (Scarano et al., 2005). Compared to normal cells, cancerous cells tend to be globally hypomythylated and CpG island herpermyhtylation (Baylin, 2005b). Studies showed that some cancer preventive effects by dietary factors may relate to DNA methylation patterns. Nutrients that are involved in one carbon metabolism, such as folate, vitamin B12, vitamin B5, methionine and choline, will influence methyl group supply and therefore affect DNA methylation patterns (Davis and Uthus, 2004). Caloric restriction was also found to have effect on DNA methylation on mouse liver (Miyamura et al., 1993). Hass et al. examined primary culture of pancreatic acinar cells from caloric restriction fed Brown-Norway rats and found that caloric restriction was able to increase genomic methylaiton of ras DNA (Hass et al., 1993). A high throughput method for determination of DNA methylation profiles was developed by Wilson et al., (2006) recently, in which the author used methylated DNA immunoprecipitation (MeDIP) for isolating methyl cytosine rich fragments and array based comparative genomics hybridization (array CGH). By using MeDIP-array CGH, the DNA methylation profiles are able to be obtained simultaneously at the genome wide level.

Glycomics is the study of the glycome, the whole set of glycans produced in a single organism. Glycosylation is the enzyme-catalyzed covalent attachment of a carbohydrate to a polypeptide, lipid, polynucleotide, carbohydrate, or other organic compound. Gycosylation usually is catalyzed by glycosyltransferases, utilizing specific sugar nucleotide donor substrates (Powell and Varki, 1994). Glycosylation is a protein post-translational modification process. Glycan profile was found to be changed in cancer (Miyamoto, 2006). Compare to normal cells,

malignant cells either loss of expression or excessive expression of certain structures, such as the persistence of incomplete or truncated structures, the accumulation of precursors, and the appearance of novel structures. Studies showed that certain glycan biosynthetic pathways are frequently correlated with malignant transformation and tumor progression. For example, increased integrin and cadigrin glycosylation was found to favorite tumor progression and metastasis (Pocheć et al., 2003; Brooks et al., 2008).

Overall, the cancer preventive effects of weight control via decreasing caloric intake and/or physical activities have been studied for a long time. However, the exact mechanism is still poorly understood. The recently developed omics technologies, such as microarray, proteomics, lipidomics, provide us far reaching tools to evaluate overall response related to disease risk, which appears worthwhile for future studies.

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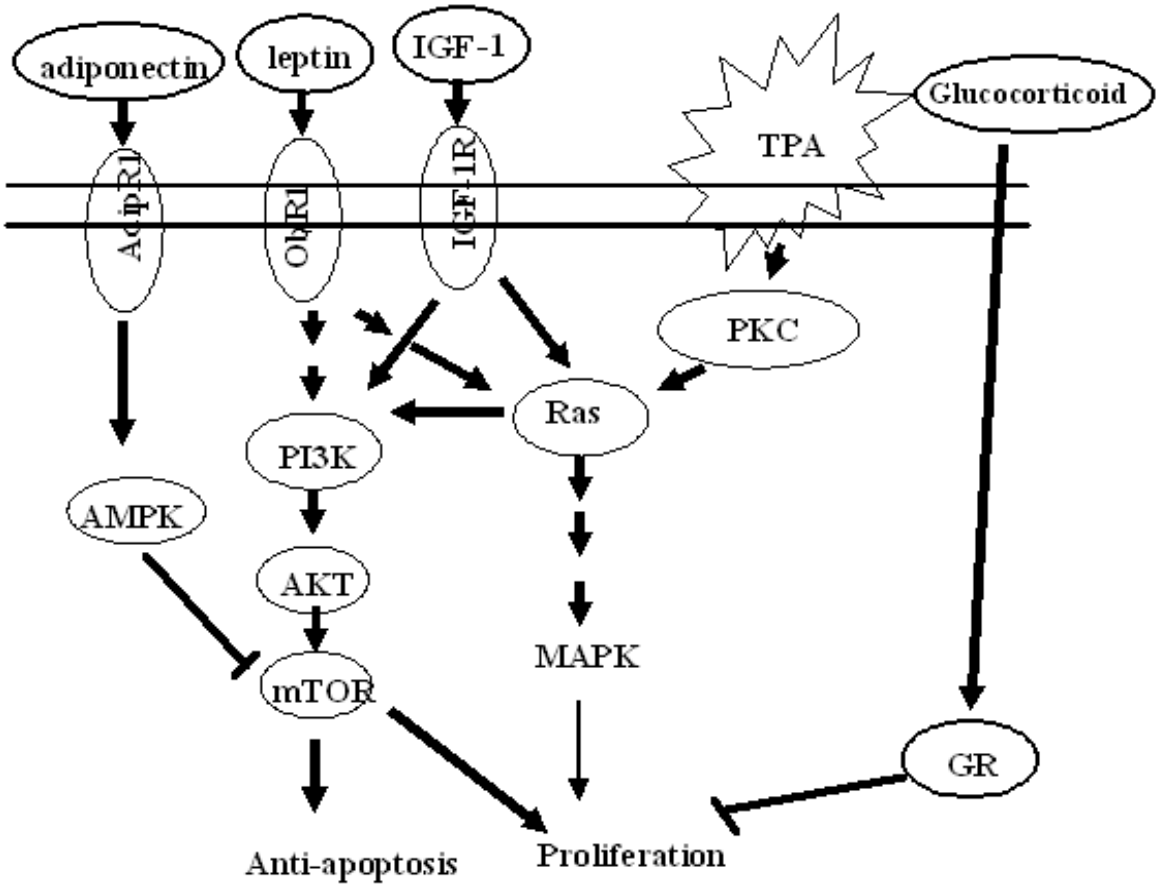
Figures and Tables

Figure 1-1 A proposed mechanism by weight control. Weight control may inhibit cancer development via inhibiting the cross-talk between hormone-dependent and TPA-promoted signaling pathways, resulting in modulating cellular proliferation and anti-apoptosis.



CHAPTER 2 - IGF-1 Restoration Partially Reversed PI3K and Related Phospholipids Reduction by Weight Control via Treadmill Exercise in Mice Skin Tissue

Abstract

Studies in animal models demonstrated that weight control along with physical activity has cancer preventing effect. By using the TPA-induced cancer promotion model, we and others have found that the IGF-1-dependent signaling was reduced in exercised mice with limited caloric intake. To further confirm the role of IGF-1, we restored IGF-1 in SENCAR mice that were pair fed with the sedentary control and exercised by treadmill at 13.4 m/min for 90 min/day, 5 days/week, for 10 weeks (PF+Exe). IGF-1 was restored by i.p. injection at 10 μg/kg body weight twice per week in the last two weeks. As expected, both body weight and plasma IGF-1 levels were significantly reduced in PF+Exe mice when compared with controls. The circulation level of IGF-1 was not significantly changed by IGF-1 restoration probably due to its fast degradation as observed in pharmacokinetic study. Western blot analysis showed two hours after TPA treatment, PI3K expression PF+Exe group was significantly decreased. The reduced PI3K was partially reversed by IGF-1 restoration. However, IGF-1 restoration had no effect on IGF-1 receptor. A total of 338 phospholipids from 12 lipid species in the skin tissues were detected and the percentage of phosphatidylinositol 38:4 was significantly reduced in PF+Exe mice but increased in IGF-1-restored mice. Furthermore, IGF-1 injection significantly enhanced some lysophosphatidylcholine species such as 20:3, 20:4, and 20:5 in PF+Exe group. Overall, our findings indicated that the PI3K pathway and the phospholipids that are related to signaling pathway, cell proliferation, and/or cell death were selectively reduced by PF+Exe treatment, with IGF-1 restoration apparently able to reverse, at least in part, the impact of weight control by physical exercise and moderately caloric restriction.

Key words: dietary calorie restriction, IGF-1, SENCAR mice, PI3K, H-ras, IGF-1 receptor, phospholipids profile

Introduction

The health benefit of physical activity/exercise, a form of movement using skeletal muscles, has been known for many decades. Accumulated evidence both in human studies and animal models has shown that physical activity is helpful in decreasing cancer risk. Researchers found that moderate exercise such as brisk walking for 3 to 4 hours per week is effective to prevent colon cancer (Martinez et al., 1997). It was also reported by the Women's Health Initiative that walking about 30 minutes per day could reduce 20 percent of breast cancer risk in postmenopausal women (McTierman et al., 2003). A recent comprehensive review of human studies showed that the evidence is convincing for physical activity to prevent colon cancer, probable for breast cancer (post menopause) and endometrium cancer, while the evidence for lung, pancreas and breast cancer (pre menopause) is limited (The *Second Expert Report on Food, Nutrition, Physical Activity and the Prevention of Cancer*, http://www.aicr.org). Studies in animal models, mostly from colon and breast cancer model, suggested that physical activities might inhibit carcinogenesis, although the effects was less consistent or potent as a calorie restriction approach (Rogers et al., 2008). Michna et al. found that voluntary running wheel exercise inhibited UVB-induced skin tumorigenesis in SKH-1 hairless mice. Their study showed that exercise delayed the appearance of tumors, and both the number of tumors per mouse and the tumor volume per mouse was decreased compared to mice not using the running wheel (Michna et al., 2006). Using forced treadmill exercise, our lab found that exercise with pair feeding, was effective in controlling body weight and selectively abrogating tumor promoter induced PI3K-Akt pathway (Xie et al., 2007).

A number of studies have been performed with the goal of elucidating the exact mechanism of cancer prevention by weigh control. Although until now, no definite conclusions

have been put further. Several hypotheses have been mentioned, such as relative oxygen species deletion, DNA repair, apoptosis regulation, oncogene inhibition and hormone alteration. The hormone-related signaling alterations, especially for insulin, IGF-1 and leptin, seem to be key factors in weight-control dependent cancer prevention (Jiang and Wang, manuscript). IGF-1, a critical mediator modulating growth hormone and growth, is known to stimulate cell proliferation, inhibit apoptosis and induce tumorigenesis (Werner et al., 2000). Heterotetrameric tyrosine kinase receptor, such as IGF-1 receptor, which has the similar structure and function to insulin receptor, can modulate the physiological function of IGF-1 (Butler et al., 1998; LeRoith et al., 1995). Once the IGF-1 receptor is activated, it induces the phosphorylation of many intracellular proteins that then activate various signaling pathways such as the phosphatidylinositide 3 kinase (PI3K)/Akt pathways which leads to anti-apoptosis and the mitogen-activated protein (MAP) kinase which inhibit differentiation (Foncea et al., 1997). Prospective cohort studies have revealed that people with higher concentrations of IGF-I have an increased risk of developing several types of cancers including prostate cancer, premenopausal breast cancer, and colon cancer (Chan et al., 1998; Ma et al., 1999).

Studies showed that constitutive expression of IGF-1 and activation of IGF-1 receptor signaling pathways in basal epithelial cells seems to play an important role in skin cancer development. The mRNA level of IGF-1 and IGF-1 receptor in dermal and epidermal of mouse skin was found to be significantly increased in the skin papillomas and carcinomas (Rho et al 1996). It was found that HK1.IGF transgenic mice, in which IGF-1 is over expressed in epidermis driven by human keratin 1 promoter, were more sensitive to tumor promoters such as TPA, chrysarobin, okadaic acid initiated by DMBA than wild type mice (Bol et al., 1997; Wilker et al., 1999). In addition, squamous papillomas and carcinomas were found to developed

spontaneously in BK5.IGF-1 transgenic mice with over expression of IGF-1 in the basal layer of skin epidermis (DiGiovanni et al., 2000). PI3K/Akt pathway was found to play an important role in IGF-1 medicated tumor promotion. Inhibition of PI3K activity significantly blocked epidermal proliferation, as well as skin tumor development in DMBA initiated IGF-1 transgenic mice (Wilker et al., 2005). Previous studies in our lab found that exercise with paired feeding decrease the expression of PI3K, phosphoserine Akt, and p42/p44-MAPK (Xie L et al., 2007). The results implied that IGF-1 and PI3K pathway plays an important role in cancer prevention in weight controlled mice.

Phospholipids are a class of lipids that all contain a diacyglyceride, a phosphate group, and a simple organic molecule. They are major component of all biological membranes. In addition to be the structural building blocks for a cell or organelle in the membrane, many phospholipids serve as precursors for different signaling pathways (www.lipidmaps.org). Studies have shown that imbalance in the lipids network will lead to disease progression in cancer, cardiovascular disease and chronic inflammation (Wymann et al., 2008). Among the phospholipids, phosphotidylinostiols (PIs) and its phosphorylated derivates, phosphotidylinotiols phosphates (referred as phosphotinositides), have been found to play an important role in cellular signaling and intracellular trafficking (Krauss et al., 2007). Phosphotidylinositols and phosphotidnositides can be phosphorylated by phosphatidylinostiol kinases, such as phosphatidylinostiol 3-kinases (PI3K), which is activated by a variety of extra cellular signals such as growth factors and tumor promoters. PI(3,4,5)P3, the major product of PI3K, is able to bind and activate Akt, thus activating many down stream signaling proteins that regulate cell survival and cell cycle progress. Elevated levels of PI(3,4,5)P3 and the upregulation of Akt are found to be oncogenic and promote the transition to malignancy (Cantley 2002; Engelman et al.,

2006; Manning and Cantly, 2007). Some lysophospholipids, formed by hydrolysis of phospholipids by phospholipase A2, have also been recognized as important cell signaling molecular in recent years. For example, lysophosphatidylcholine (LysoPC) may activate phospholipase C and release diacyglyercols and inositol triphosphate, which leads to activation of protein kinase C and intracellular Ca^{2+}. Consequently, it results in activating the mitogen-activated protein kinase (MAPK) signaling pathway (Xu et al., 2002).

Recent development of matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization tandem mass spectrometry has allowed for rapid and more sensitive detection of a variety of lipid species using very small amounts of samples (Welti and Wang, 2004; Wenk, 2005). Previous study in our lab analyzed the mice skin phospholipids profile by using electrospray ionization tandem mass spectrometry (ESI-MS/MS). Total 338 phospholipids were able to be detected in the mouse skin samples. We found that 57 phopholipid species were significantly changed by exercise comparing to sedentary controls, most phosphatidylinositol (PI), and some lysophosphatidylcholine (lysoPC) molecular species decreased significantly in exercise with pair feeding mice (Ouyang et al., manuscript).

In this study, we tried to confirm the requirement of IGF-1 dependent signaling reduction in skin cancer prevention of weight control by restoring IGF in treadmill exercised mice. We observed that body weight, plasma IGF-1 level, and PI3K protein experssion were significantly decreased in pair-fed exercised mice compared with the controls. We also found that the reduced PI3K level was partially reversed by IGF-1 restoration in exercise, pair-feeding mice. Our lipidomics study showed that in the mice skin tissues, the percentage of phosphatidylinositol 38:4 was significantly reduced in PF+Exe mice but increased in IGF-1-restored mice. Furthermore, IGF-1 injection significantly enhanced some lysophosphatidylcholine species such

as 20:3, 20:4, and 20:5 in PF+Exe group in comparison with the control. The results implied that the PI3K pathway and the phospholipids that related to the above signaling pathway were selectively reduced by weight control via physical exercise and moderately caloric restriction, and IGF-1 restoration seems be able to partially reverse these impacts.

Materials and methods

Animals and treatments

Female SENCAR mice (NIH, Frederick, MD) arriving at about 6 weeks of age were assigned to one of the three treatment groups for 10 weeks after a two-week adaptation and training period: ad libitum-fed sedentary control with AIN-93 diet (Control Group, n=8), pair-fed at the amount as the control and treadmill exercise at 13.4 m/min for 90 min/d, 5 d/wk (PF+Exe group, n=12). Mice were kept in separated cages individually in a conditionally controlled animal room with about 40% humidity and 12h light/12h dark cycle. The body weight of each mouse was recorded twice per week. In the last two weeks, IGF-1 (Novozymes GroPep, Australia) was administrated via intraperitoneal injection at 10 ug/g body weight to five PF+Exe mice twice per week. For the remaining seven PF+Exe mice and control mice, they were injected with the saline vehicle (sham). After ten weeks formal exercise, mice were shaved on the dorsal skin. Twenty-four hours after the final injection, mice were applied TPA topically at the dose of 3.2nmol/200µl acetone for two hours, then sacrificed by the accepted animal use protocal. The dorsal skin tissue were snap-frozen in liquid nitrogen and saved at -80°C for further analysis

Pharmacokinetics study for plasma level of IGF-1: SENCAR mice were obtained from NIH (Frederick, MD) at six weeks old of age. After one week of adaptation, the mice were

treated with IGF-1 by i.p injection at the dose 10μg/g body weight, and terminated at 0, 1, 2, 6, 12, and 24 hours after injection respectively.

Assessment of plasma IGF-1 levels

Blood was immediately collected in heparin-coated tubes after decapitation and centrifuged at 1,000g for ten minutes at 4℃ to isolate plasma. IGF-1 levels were measured by IGF-1 (Direct) RIA kit (ALPCO, NH).

Assessment of protein levels by western blotting analysis

Mice skin tissue was smashed into powder in liquid nitrogen, mixed with Mg^{2+} lysis/wash buffer (MLB, 25mM HEPES, 150mM NaCl, 1% lgepal CA-630, 10mM $MgCl_2$, 1mM EDTA, 2% glycerol, 10ug/ml aprotenin and 10ug/ml leupeptin) and then homogenized. The lysate was then centrifuged at 4`℃ with speed 12,000g for 15 minutes. The supernatant was collected and the protein concentration was measured by Bio-Rad protein assay (Bio-Rad, Hercules, CA). 2X Laemmli reducing sample buffer (0.125mM Tris-HCl, 4% SDS, 1ml Glycerol, 4%-βmercaptoethanol and 0.02% bromophenyl blue) was used to mix with and denature the proteins. The proteins were separated by 10% SDS-PAGE gel and transferred to nitrocellulose membrane using Trans-Blot SD SEMI-DRY Transfer Cell (Bio-Rad, Hercules, CA) with 15 V for 20 minutes. The membrane was blocked with 5% blocking buffer (5% dry milk in PBST containing 0.5‰ Tween 20) at 4℃ for 2 hours, and incubated with first antibody (PI3K (110kD), IGF-1R (85kD), H-ras (21kD) and β-actin (internal control, 43kD) (Santa Cruz Biotechnology Inc., Santa Cruz, CA)) respectively overnight. After first antibody incubation, the membrane was washed with PBST (0.5‰ Tween 20) three times with five minutes each time, and incubated with anti-goat or anti-mouse HRP-conjugation secondary antibody for 45 minutes (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Finally the bands were visualized and

quantified by the FluorChemTM 8800 Advanced Imaging System (Alpha Innotech, San Leandro, CA).

Lipid extraction and lipid profiling

Skin samples, about 0.2 to 0.4 gram, were ground after freezing with liquid nitrogen. Two volulmes of solvent (chloroform: methanol 1:2 v/v + 0.01% butylated hydroxytoluene) were added to 1 part tissue (v/w) and shaken well. Then the mixture was centrifuged at 1,000 rpm for 15 minutes at room tempreture, the lower layer was collected. The supernant was then mixed with 1 ml of chloroform, shaken well, and centrifuged to collect lower layer. The remaining supernant was mixed with chloroform and separated to collect lower layer one more time. The lower layers were combined and then washed with 300 µL 1.0 M KCl and then with 300 µL water. The extracts were delivered to the Kansas Lipidomics Research Center for phospholipids profiling. A "triple" quadrupole tandem mass spectrometer (Micromass Ultima, Micromass Ltd, Manchester, UK) was used for the analysis. The acyl group identification were carried out as described previously (Welti and Wang, 2004). Data processing was performed using Biosystems Analyst software. Lipids in each class were quantified by comparison to an internal standard.

Statistic analysis

One-way ANOVA was used to analyze the body weight, IGF-1 levels and the protein expressions. Difference in phospholipid levels among the three treatment groups were compared using a one-way ANOVA, F-test for significance followed by pair comparisons. Data are expressed as means ± S.E (standard error). The levels of significance of all statistical tests are set at the 5% level. All the statistical analyses were performed using SAS 9.1.

Results

Impact of exercise with pair feeding on body weight

The body weight of control and PF+Exe mice are shown in figure 2-1. Compared to control mice, the body weight of PF+Exe mice was significantly lower in the last two weeks of exercise training ($p=0.0128$). The body weight of control mice kept increasing during the experimental period, which began with 32.2 (±1.4) gram and ended with 36.5 (±1.4) gram ($p=0.017$). The body weight of PF+Exe mice remained similar during the experimental time, beginning with 31.9 (±1.3) gram and ending with 32.4 (±0.8) gram, with no significant changes during the whole experimental period.

Effect of IGF-1 injection on plasma IGF-1 in PF+Exe mice

The plasma IGF-1 levels of each group are shown in figure 2-2. After ten weeks of treadmill exercise, the circulation levels of IGF-1 were significantly decreased in exercise with pair-fed group compared to sedentary control mice. However, the IGF-1 levels were very similar in PF+Exe mice with or without IGF-1 injection. The pharmacokinetic study of IGF-1 injection was also studied and the results are shown in figure 2-3. The plasma IGF-1 showed highest at one hour after injection. It kept decreasing, and reached to the original level around 24 hours post injection.

Impact of exercise with pair feeding and IGF-1 restoration on IGF-1 receptor and PI3K protein expression

The IGF-1 receptor and PI3K protein expression were detected by western blot analysis. The ratio of IGF-1 receptor and PI3K band density to β-actin is shown in Table 2-1. For IGF-1 receptor, the data shows no significant difference among all the three treatment groups: control, PF+Exe and PF+Exe with IGF-1 injection. However, the expression of PI3K was significantly

decreased in PF+Exe mice compared to control. The IGF-1 injection group showed a partial increase in PI3K expression.

Impact of exercise with pair feeding and IGF-1 restoration on phospholipids profile

Figure 2-4 shows phosphoinositol (PI) profiling in mice skin. The major component of PI is PI 38:4, which takes cover 90% of total PI. The data shows that PF+Exe significantly decreased PI 38:4 in the skin tissue. Restoration of IGF-1 in PF+Exe mice increased the level of PI 38:4 partially. Figure 2-5 shows lysoPC and lysoPE profile. Restoration of IGF-1 increased PC 20:3, PC 20:4 and PC20:5 compared to control and PF+Exe. Most of the lysoPC species have a lower level in PF+Exe mice compared to control. However, due to the large variance, the results are not significant at the 0.05 level. Figure 2-5 (B) shows IGF-1 injections decreased lysoPE 20:4 and 22:5 in PF+Exe mice. Figure 2-6 shows the profiling of ether phospholipids. PF+Exe increased some species, such as 36:1 ePC, 38:2 ePE, 40:4 ePE, 38:2 ePS, and injection of IGF-1 seems have no effects on these increasing.

Discussion

Previous studies in our lab found that the circulation levels of IGF-1, some protein expressions in IGF-1 related signaling pathways, phosphoinositol and some lysophospholipids were decreased in weight controlled mice via treadmill exercise with paired feeding. To further confirm the significant role of IGF-1 in skin cancer prevention by weight control, the current study restored IGF-1 by i.p. injection and investigated the variations of plasma IGF-1, correlated protein expression and skin phospholipids profile.

Weight control treatments for ten weeks in PF+Exe and control groups had significantly different body weights. The results are consistant with previous finding in our lab. It confirmed

that exercise with limited food intake is able to prevent weight gain (Xie et al., 2007; Ouyang et al., manuscript).

The results showed that plasma IGF-1 was significantly reduced in PF+Exe mice comparing to sedentary control. Reducing of plasma IGF-1 by weight control has been found in a number of studies. Serum IGF-1 was found to be decreased significantly as early as the first week in female Sprague-Dawley rats after beginning of calorie restriction treatment (Ruggeri et al., 1989). Comparing to their respective *ad libitum* fed control, serum IGF-1 is found to be about half in 40% calorie restricted rats and 74% in 20% calorie restricted p53 deficient mice (Hursting et al 1993; Dunn et al., 1997; Zhu et al., 2005). The effect of exercise alone on plasma IGF-1 is difficult to make definite statements. It was found that exercise decreased IGF-1 level in children and adolescents. But for adults, the plasma IGF-1 levels were not decreased either by short bout exercise or physical training (Kaaks et al., 2004). Contrary to short bout exercise, studies showed that plasma concentrations of IGF-1 were significantly lower in endurance runners than age and sex matched sedentary controls (Fontana et al., 2006). In rodent model, both of our and other labs found that exercise with ad libitum feeding was not sufficient to decrease plasma IGF-1 levels (Xie et al., 2007; Bravenboer et al., 2001). When the exercised mice were fed with a same amount as their sedentary counterpart, plasma levels of IGF-1 were modestly but significantly reduced. Modest reduction of IGF-1 by exercise with paired fed has also been found previously in our lab (Xie et al., 2007).

In order to further investigate the requirement of IGF-1 reduction in skin cancer prevention by weight control, we injected human recombinant IGF-1 into treadmill exercised mice. The results showed that the plasma levels of IGF-1 in IGF-1 injected mice were similar to those injected with only saline vehicle. Pharmacokinetics showed that after injection of IGF-1,

peak plasma IGF-1 levels occurred within one hour and it almost reached to basal level around 24 hours. Since the exercised mice were terminated one day after last IGF-1 injection, it is no surprising that we observed similar IGF-1 levels in PF+Exe mice with IGF-1 injection or with vehicle only.

Although restoration of IGF-1 didn't show significant impact on plasma level of exercised mice one day after injection, acute elevation of plasma IGF-1 after mutilple injections still partially reversed the reduced PI3K protein expression. Pair fed exercise was found to selectively block PI3K-Akt pathway in TPA-induced mice skin, which includes reduction of Pik3ca gene expression and PI3K protein expression and decrease of Akt phosphorylation (Xie et al., 2007). It is interesting that both PF+Exe and IGF-1 treatment had no effects on IGF-1 receptor protein expression. The results suggest that circulating IGF-1 rather than IGF-1 receptor is the key target that modulated by weight control via exercise and limited food intake in skin cancer prevention.

In mammals, and particularly in mice tissues, phospholipid PIs was demonstrated to consist mainly of sn1-18:0, sn2-20:4 species (Augert G et al., 1989; Postl et al., 2004). Consistently, the major molecular specie in mice skin was identified as 38:4 PI in our study. Similar as our previous findings, exercise with pair feeding significantly decreased PI 38:4 (Ouyang et al., manuscript). The decreasing of PI 38:4 together with reduction of PI3K protein expression suggests that the major product of PI3K, phosphatidylinositol-3, 4, 5-trisphosphate (PIP3), might also be reduced. As a consequence, the activity of downstream signaling proteins such as Akt was also decreased since their activation requires PIP3 binding as seen in our previous study. Restoration of IGF-1 partially abolished the reduction of PI 38:4 in pair fed exercised mice. It is anticipated that down stream signal, such as decreased Akt activity may also

be reversed. Therefore, further studies to check Akt activity, apoptosis and cell proliferation marker appear to be warranted.

In addition to PI, previous study also found that most of the lysoPCs were significantly decreased in exercise with pair feeding CD-1 mice, which is similar as what we found in SENCAR mice (Ouyang et al., manuscript). Using Affymatrix microarray, our lab found that phospholipase A2 (PLA_2) was decreased by exercise with pair feeding. PLA_2 is an enzyme that hydrolyzes the sn-2-acyl bond of phospholipids of cell membrane and lipoproteins, which producing free fatty acids and lysophospholipids. The reduction of PLA_2 may help to explain the decreasing of lyso PCs in PF+Exe group. Lysophospholips have been found to have functions in cell signaling. LysoPC can bind to specific receptors, such as G protein coupled receptor 1 and 4, and then activate the specific phospholipase C. The process will induce the releasing of diacylglycerols and inositol triphosphate with resultant increases in intracellular Ca^{2+} and activation of protein kinase C. It also activates the mitogen-activated protein kinase in certain cell types (Prokazova et al., 1988; Xu 2002). Changes of lysoPC have been characterized in inflammation related disease. It was found that total lysoPC and the ratio of lysoPC to PC were significantly higher in the plasma of ovarian cancer patient (Okita et al., 1997). The molecular percentage of palmitoyl- and stearoyl-lysoPC, and the ratios of palmitoyl-lysoPC to linoleoyl-lysoPC were also elevated in these patients (Okita et al., 1997). In multiple myeloma patients, serum lysoPC was significantly elevated comparing normal controls. The author also found higher ratios of palmitoyl-lysoPC to linoleoyl-lysoPC in the patients (Sasagawa et al., 1999). In our study, the ratio of lysoPC to PC is 0.14 for control, 0.09 for PF+Exe and 0.17 for PF+Exe mice with IGF-1 injection (data are not shown). Overall, reduced level of lysoPC and ratio of lysoPC to PC by exercise with pair feeding may decrease the cell damage, suppress cell

proliferation and also be beneficial with respect to cancer prevention. Restoration of IGF-1 increased lysoPC to that of the control mice level. The results implied that lysophospholipids may play a role in IGF-1 mediated signaling in skin carcinogenesis.

We obersved IGF-1 restoration only partially increased the reduced PI3K protein expression and PI 38:4 level. In addition, IGF-1 restoration seems have no effect on ether phospholipid profile, which was found to be changed by pair fed exercise. All the facts indicate that not only IGF-1 but some other hormones or unknown factors are also involved in weight control mediated skin cancer prevention. It is known that insulin and leptin could also activate PI3K-Akt signaling. Future studies with focus on the combined effects of IGF-1 and other hormones, such as leptin, on PI profile and PI3K signaling may help to expain the results currently obtained.

In the present study, IGF-1 was restored by multiple i.p.injections. Restoration of IGF-1 can also be achieved by using osmotic minipumps. Hursting et al., (1993) infused human recombinant IGF-1 into 40% calorie restriction rats by osmotic minipump. They found that IGF-1 infusion reversed the plasma IGF-1 in DCR mice to similar level as in ad libitum fed rats. Resotration IGF-1 also increased cell proliferation activity and enhanced mitogen responsiveness in dietary restriction treated rats (Hursting et al., 1993). Also via osmotic minipumps, restoration of IGF-1 in 20% calorie restricted p53 deficient mice increased cell proliferation and inhibited apoptosis dramatically, although the infusion did not change tumor incidence (Dunn et al., 1997). Plasma IGF-1 was found to be significantly increased in the calorie restricted mice with IGF-1 treatment. Restoration of IGf-1 through osmotic minipum approach seems work better to maintain an expected high IGF-1 level in the animals. However, the minipums may induce inflammation in the animals. In our lab, we also tried to restore IGF-1 in PF+Exe using

mipnipums. It will be interesting to compare the IGF-1 plasma levels and its effect on PI3K related signaling using both of the two restoration methods.

In summary, we investigated the specific role of IGF-1 in skin cancer prevention by weight control. We determined the plasma IGF-1, correlated protein expression and skin phospholipids profile in exercise with pair feeding mice with or with IGF-1 restoration, and compared to that of sedentary control. The study showed that PI3K protein expression, PI 38:4 and some lysoPCs were reduced by weight control via exercise with limited calorie intake and the reduction was reversed by IGF-1 injection partially. The results provide us a better understanding of the IGF-1 related signaling in weight control mediated skin cancer prevention.

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Figures and Tables

Table 2-1 Impact of IGF-1 restoration on IGF-1 receptor and PI3K protein expression by western blot analysis. The numbers show the mean and standard error of the ratio of IGF-1 receptor or PI3K band density to β-actin. Each treatment group has 5 to 7 replicates. Different letters shows different significant levels, a>b, P<0.05. The data shows that there was no statistical difference between groups for IGF-1 receptor. PI3K expression was significantly decreased in PF+Exe group, but that reduction was partially reversed by IGF-1 restoration.

	control	PF+Exe	PF+Exe+IGF-1
IGF-1 receptor	2.07 (0.65)	1.23 (0.32)	1.82 (0.32)
PI3-K	3.62 (0.91)[a]	1.22 (0.33)[b]	1.93 (0.62)[a,b]

Figure 2-1 Body weights of the SENCAR mice were reduced significantly by exercise with pair-feeding (PF+Exe). Results are means ± SE, n =8-12, *P ≤ 0.05 vs. the controls. The first two weeks are adaptation and training period. The arrow indicates the day that the physical exercise was started.

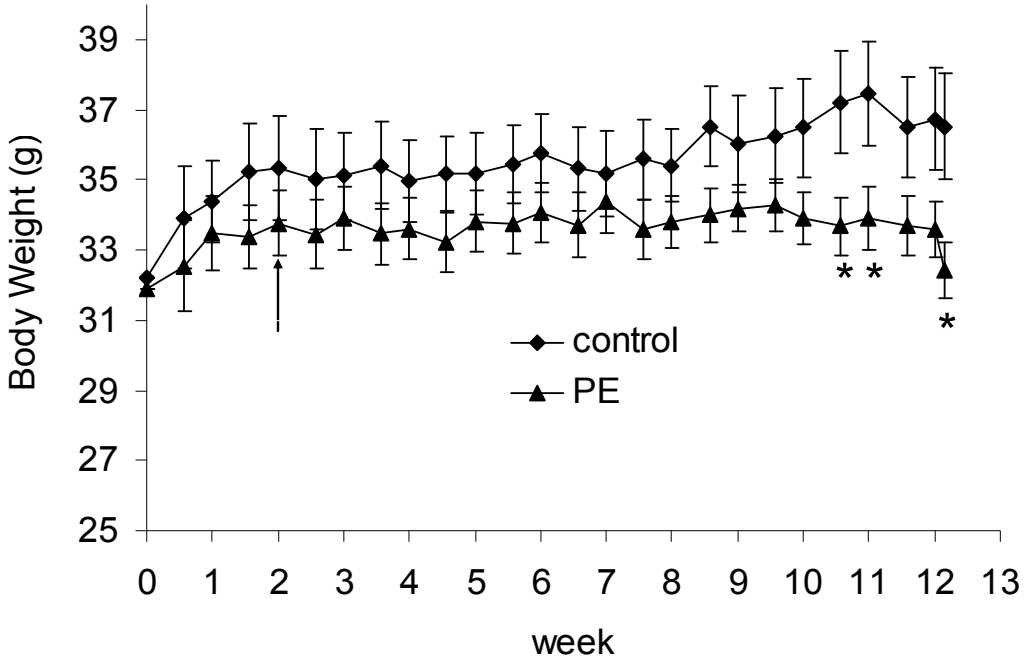


Figure 2-2 Plasma IGF-1 levels of SENCAR mice in weight control study. Results are means ± SE, n =5-8. Bars with different alphabetic letters differ significantly, P ≤ 0.05.

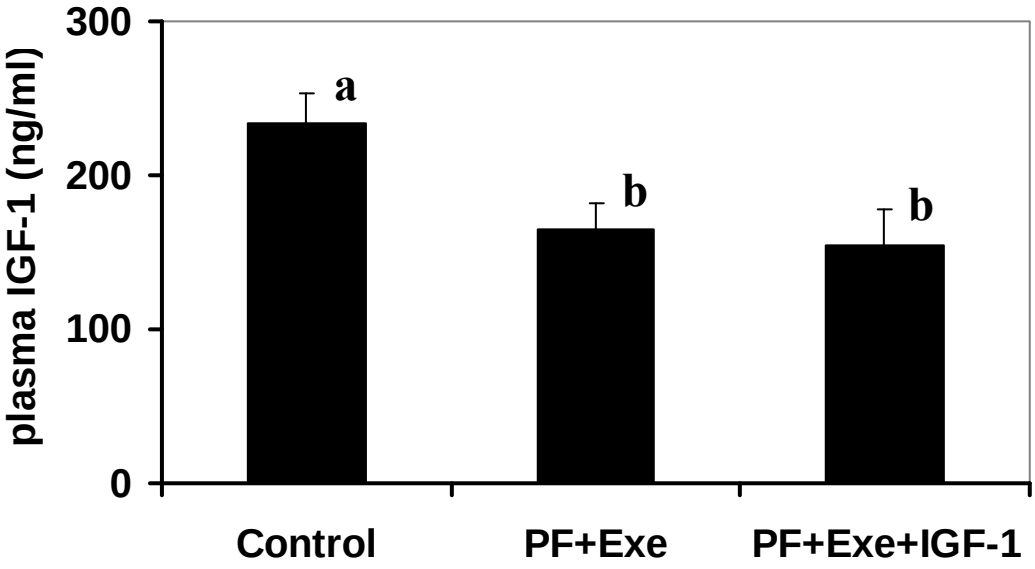


Figure 2-3 Pharmacokinetic study of IGF-1 injection. The peak plasma IGF-1 levels were achieved within one hr and almost reached to basal level at 24 hrs after i.p. injection. Means ± SE (n =7 for 0 hr, n=3 for 1 , 2, and 6 hr; n=2 for 12 and 24 hr).

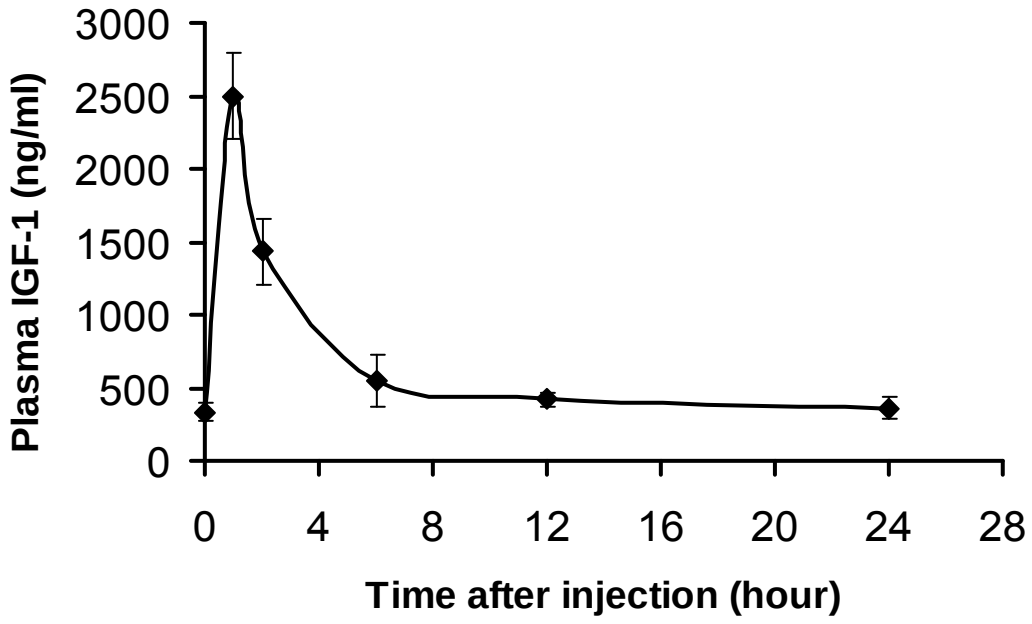


Figure 2-4 Effects of pair fed exercise with or without IGF-1 restoration on phosphoinositol profile in skin tissues. Results are means ± SE, n = 5-7. The bars with different alphabetic letters differ significantly (a>b, P < 0.05).

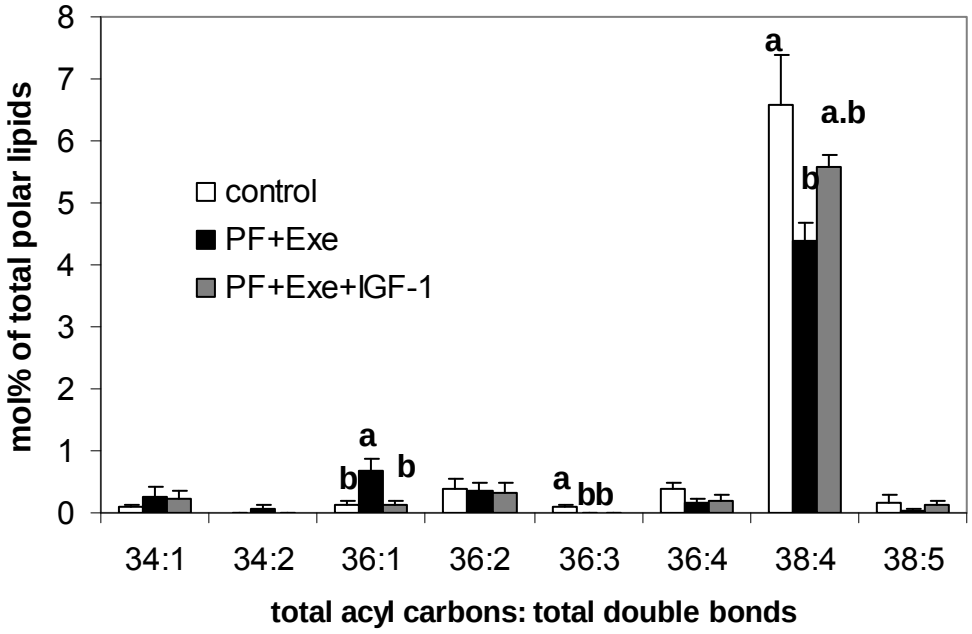
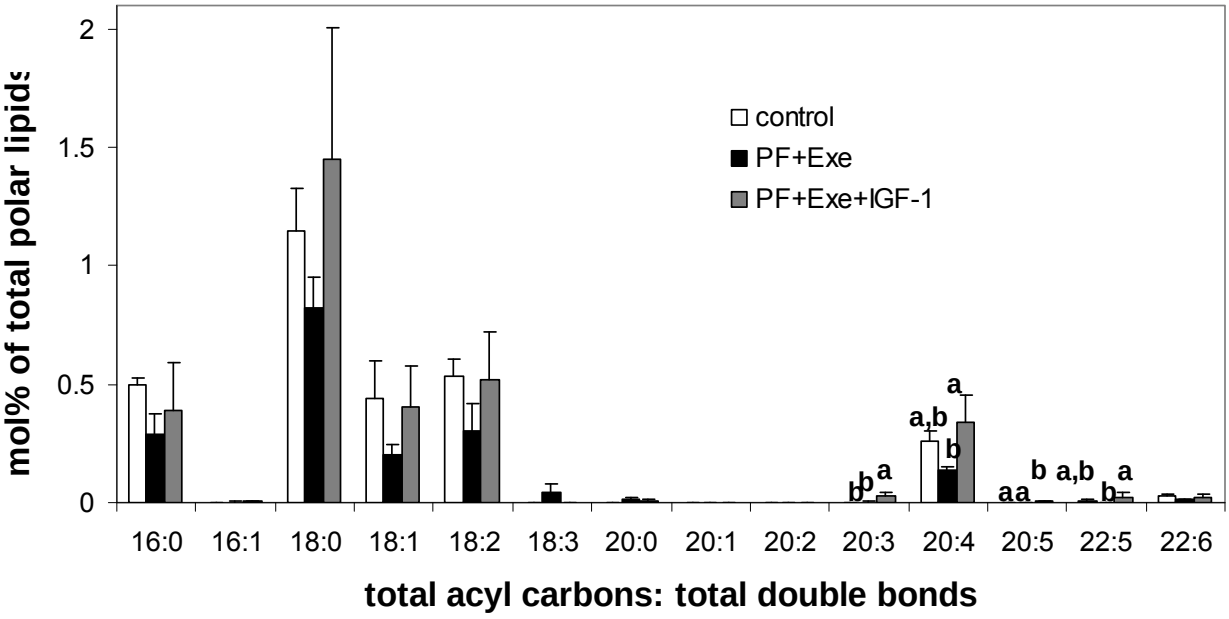


Figure 2-5 Effects of pair fed exercise with or without IGF-1 restoration on A) lysophosphatidylcholine (lysoPC), B) lysophosphatidylethanolamine (lysoPE), in mice skin tissues. Results are means ± SE, n = 5-7. The bars with different alphabetic letters differ significantly (a>b, P < 0.05).

A) lysoPC



B) lysoPE

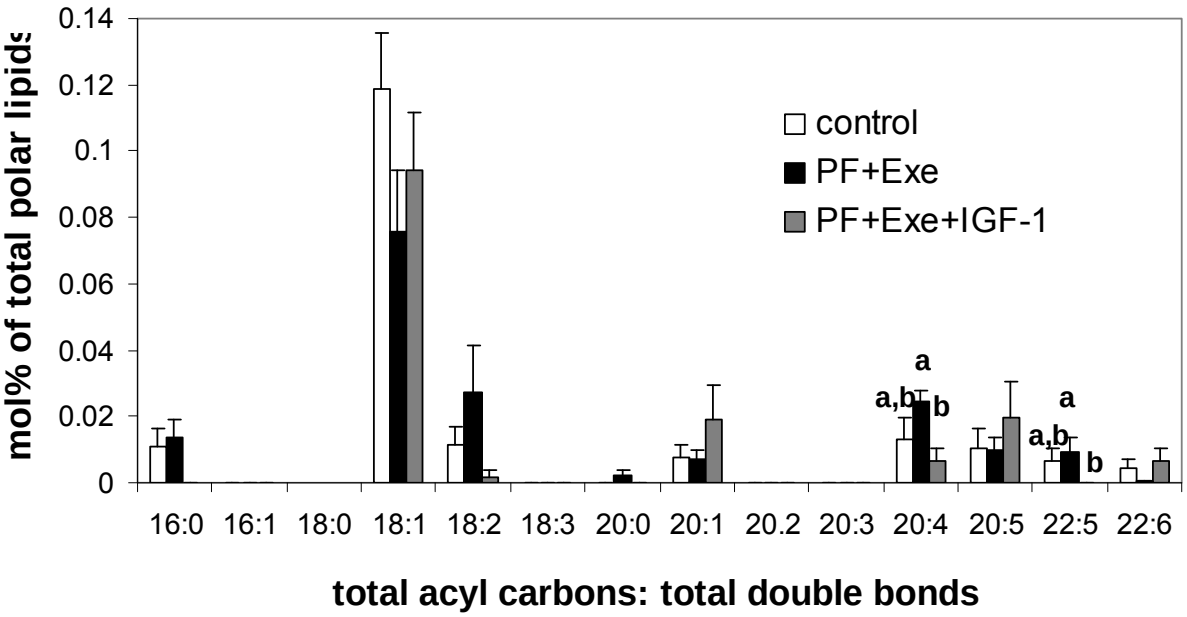
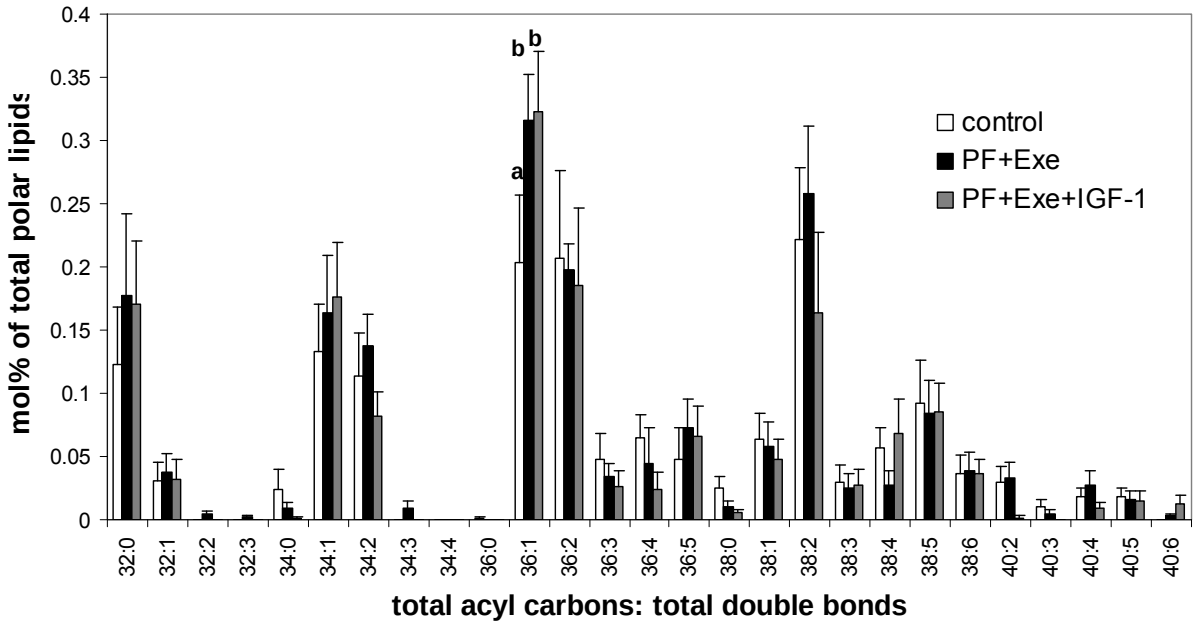
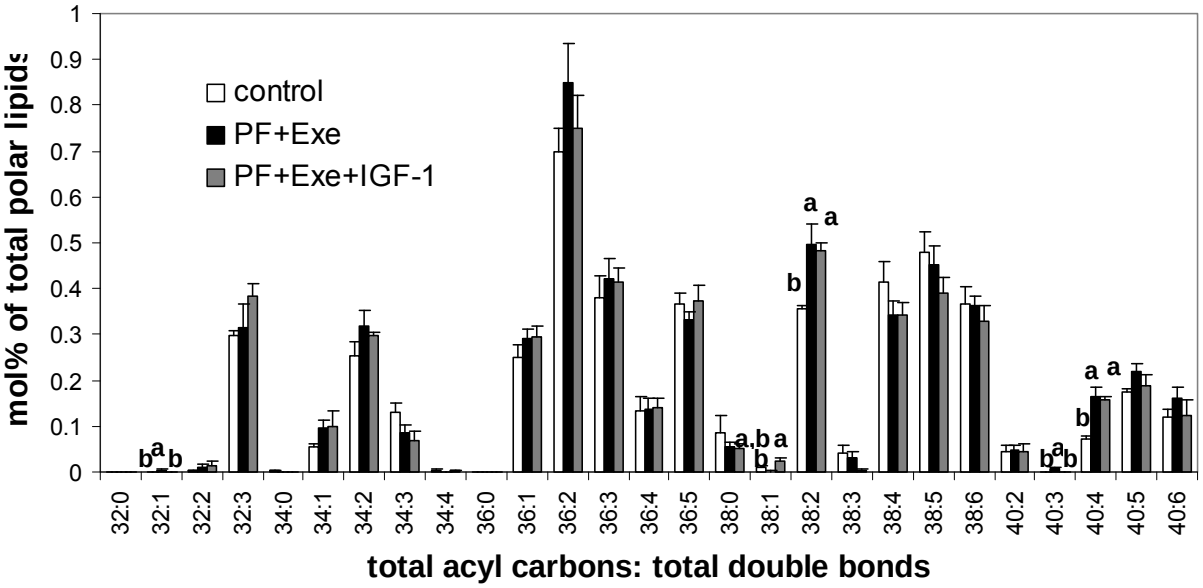

mol% of total polar lipids
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
control
PF+Exe
PF+Exe+IGF-1
a
a,b
b
a
a,b
b
16:0
16:1
18:0
18:1
18:2
18:3
20:0
20:1
20:2
20:3
20:4
20:5
22:5
22:6
total acyl carbons: total double bonds

Figure 2-6 Effects of pair fed exercise with or without IGF-1 restoration on A) ether phosphatidylcholine (ePC), B) ether phosphatidylethanolamine (ePE) and C) ether phosphatidylserine(ePS) in mice skin tissues. Results are means ± SE, n = 5-7. The bars with different alphabetic letters differ significantly (a>b, P < 0.05).

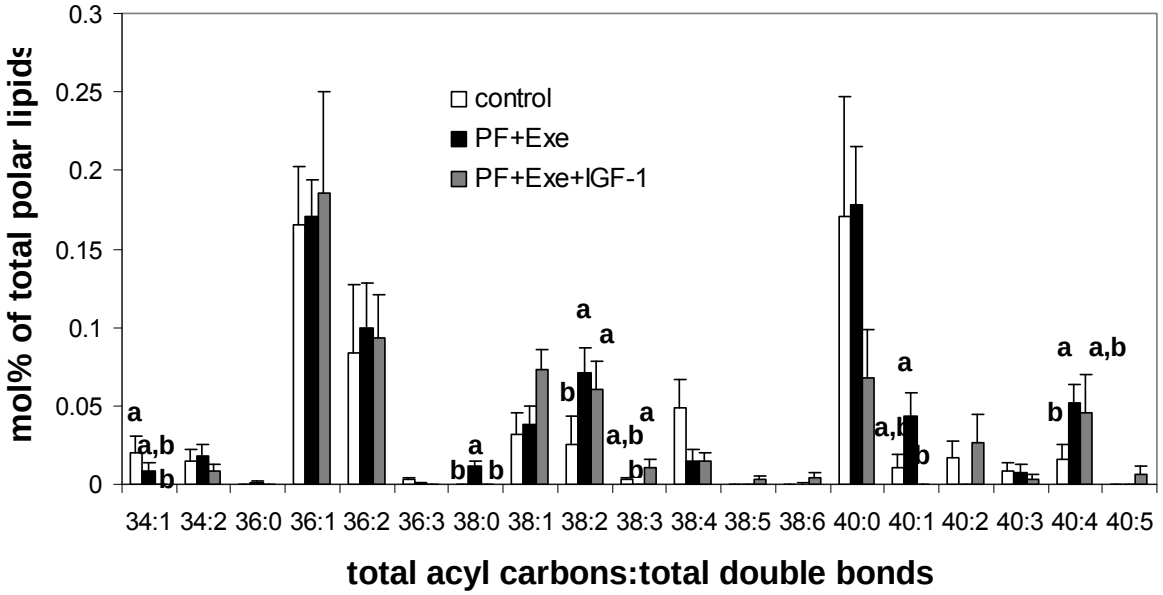
A) ePC



B) ePE


mol% of total polar lipids
control
PF+Exe
PF+Exe+IGF-1
total acyl carbons: total double bonds
32:0
32:1
32:2
32:3
34:0
34:1
34:2
34:3
34:4
36:0
36:1
36:2
36:3
36:4
36:5
38:0
38:1
38:2
38:3
38:4
38:5
38:6
40:2
40:3
40:4
40:5
40:6

C) ePS


mol% of total polar lipids
0.3
0.25
0.2
0.15
0.1
0.05
0
control
PF+Exe
PF+Exe+IGF-1
a
a,b
b
a
b
b
a
b
a
a
a,b
b
a
b
a
a,b
b
a
a,b
34:1
34:2
36:0
36:1
36:2
36:3
38:0
38:1
38:2
38:3
38:4
38:5
38:6
40:0
40:1
40:2
40:3
40:4
40:5
total acyl carbons:total double bonds

CHAPTER 3 - Weight Control via Calorie Restriction and/or Exercise Changed the DAG and TAG profiling in Muring Skin

Abstract

Studies in animal models have demonstrated that weight control via calorie restriction and/or physical activities prevent against cancer. To understand the underlying mechanisms, this study investigated the effect of weight control via treadmill exercise or calorie restriction on the triglyeride and diacylglyeride profiles in plasma and TPA-induced mouse skin tissues. CD-1 mice were randomly assigned into one of four treatment groups for ten weeks: ad libitum sedentary control (control), 20% calorie restriction (CR), ad libitum fed treadmill exercise at 13.4 meter/min for 60 minutes per day, five day a week (AL+Exe) and exercise but pair-fed at the amount as the sedentary control (PF+Exe). At the end of the treatment, the mice were treated with TPA topically and terminated at two and twelve hours post application. Plasma and dorsal skin tissue were collected and analyzed. Comparing to control animals, both pair fed exercise and caloric restriction significantly decreased the body weight, while AL+Exe has no significant effects. PF+Exe and DCR also significantly decreased TAG subtotals in the plasma and skin tissue. PF+Exe significantly decreased plasma total DAGs, and both PF+Exe and DCR changed the DAG profile in plasma and skin tissue. The results indicate a significant impact of weight loss upon TAG and DAG profiles, which may provide the mechanistic understanding of weight control for cancer prevention.

Key words: dietary calorie restriction, exercise, weight control, CD-1 mice, DAG, TAG

Introduction

It has been found that body weight control may decrease the risks of various kinds of cancers including the colon, endometrium, breast (postmenopausal), esophagus, and kidney (Vainio H et al. 2002). The recent published Second Expert Report on Food, Nutrition, Physical Activity and the Prevention of Cancer showed that obesity, weight gain, and also overweight short of obesity increase the risk of a number of cancers. The panel stressed that the prevention of cancer must also deal with the prevention of overweight, obesity and weight gain (WCRF/IAIRC, 2007). Weight control tends to become an important strategy against cancer and other chronic disease. Body weight control is carried out by the balance of energy, which is tightly associated with dietary caloric intake and/or physical activity (energy expenditure). The cancer preventive effect of calorie restriction is known for almost a hundred years. It has been studied extensively in various animal models and in various organs. The health benefit of physical activity (exercise) is also known for a very long time. It is agreed that physical activity is effective in protecting colon, breast (posmenopause) and endometrum cancer, while, for other cancers such as prostate, endometrial cancers, no definitive conclusions have been made about the prevention effect by physical activity ((WCRF/IARC, 2007).

Diacylglyerols (DAGs), which is also called diacyglyerides, are trihyric alcohol glycerol molecules linked through ester bonds to two long chain fatty acids in position 1 and 2. The DAGs are minor components in most tissues; however, they are important intermediate in lipid metabolism and key elements in cell signaling that regulate many cellular processes (Goni and Alonso, 1999). In cell membrane, DAGs can modify bilayer properties through facilitating membrane fusion/fission even and affecting intracellular vesicular trafficking (Goni and Alonso, 1999; Barona et al., 2005). DAGs are intermediates in the synthesis and degradation of most

phospholipids, such as hosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS), and triacylglycerols (TAGs). DAGs can be converted into PE or PC by choline/ethanolamine phosphotransferase (CEPT1) and choline phosphotransferase (CPT1) (Henneberry et al., 2002). DAGs can be etherified into triacylglycerols by adding a new fatty acid in the free position of the glycerol moiety (Coleman et al., 2004). In addition, DAGs can also be hydroxylized into PA, which then can form PI, phosphatidylglycerol and cardiolipin. It was found that sn-1,2-DAG, but not sn-2,3 DAG and sn-1,3 DAGs, play an important role in cell signaling. Actually, sn-1,2-DAG has been studied extensively as a lipid second massager. Simulated by external signals, such as IGF-1 EGF, plasma membrane PI will produce two second messengers, inositol-1,4,5-IP_3 and sn-1,2-DAGs (Michell 1975; Berridge 1987). It is well known that sn-1,2-DAGs can bind and activate protein kinase C (PKC), which plays an important role in cell proliferation and differentiation (Nishizuka 1992). In addition to PKC, sn-1,2 DAGs are also found to activate proteins such as chimaerins, Unc-13, protein kinase D and some mammalian homologues of transient receptor potential proteins (Caloca et al., 1999; Nurrish et al., 1999; Baron and Malhotra 2002; Hofmann et al., 1999). More recently, studies showed that sn-1,2-DAGs may also modulate Rho and Ras proteins (Takai et al., 2001; Topham 2006). DAGs are found to be impacted by certain disease stage and weight control. For example, studies by Birt lab showed that calorie restriction increased sn-1,2-DAGs per cell in skin epidermis (Kris et al., 1994).

Triacylglyerols (TAGs) consist of trihyric alcohol glyercol esterified with long chain fatty acids. For most TAG species, saturated fatty acids are usually found mainly in sn-1 position. Sn-2 is more likely to have unsaturated fatty acids, especially linoleic acid. For sn-3 position, it is found to prefer longer chain fatty acids (lipid library, www.lipidlibrary.co.uk). The

major roles of TAGs include storage of energy, reposition of fatty acids and being precursors of phospholipid biosynthesis. TAGs can also be hydrolyzed into DAGs, an important second messenger and a precursor of the major phospholipids as disccused above. Critical enzymes involved in fatty acid and tryiacylglerol biosynthesis are regulated by different hormonal, developmental and nutritional conditions (Sul et al., 1998; Coleman et al., 2004). Deceasing plasma TAGs by weight control via exercise and/or caloric restriction have been observed by many researchers. Wang et al. found that 30% caloric restriction decreased plasma TAGs and fatty acids concentrations, as well as triglyceride accumulation in the liver (Wang et al., 2006; Wang et al., 2007). The plasma TAGs lower effect by exercise has been found many years (Cullinane et al., 1982; Haskell et al., 1984). Studies showed that exercise may increase lipoprotein lipase activity in skeletal muscle and adipose tissue, and decrease hepatic triacylglyerols synthesis (Haskell et al., 1984).

Analyses of plasma or tissue TAGs and DAGs have been studied for many years. Previous measurements of TAGs or DAGs are focused on either total quantity of TAG or DAG in the cells, or fatty acids composition in TAGs or DAGs rather than unique TAG or DAG molecular species. The recent developments of tandem mass spectrometry using electrospray ionization and atmospheric pressure chemical ionization have made the global analysis and quantification of TAG and DAG profiles possible (Han et al., 2001; Murphy et al., 2007).

Our current research, for the first time, studied the impact of weight control on TAG and DAG lipid profile in the plasma and TPA treated mice skin tissue. Considering the significant role of DAGs in PI3K/Akt pathway and PKC/MAPK pathway, our findings regarding the effects of weight control on individual DAG species in TPA promoted mice skin tissue, may provide

insight into the mechanism of weight control on cancer prevention. Better understanding the relationship of body weight status and lipid profile would lead to novel approves for biomarker identification in early diagnostics of cancer, and cancer prevention.

Materials and methods

Animals and treatments

Female CD-1 mice arrived at eight weeks old were random divided into four groups: ad libitum feeding and sedentary (control), exercise with ad libitum feeding (AL+Exe), exercise with pair feeding (PF+Exe) and dietary caloric restriction (DCR). Ad libitum feeding groups (control and AL+Exe) were allowed to freely obtain the basal diet (AIN-93) while the pair-fed exercise group was fed daily the same amount as the sedentary mice. DCR group were fed with a 20% DCR diet for 8 weeks. The 20% DCR diet was formulated and provided by the Harlan Teklad (Madison, WI). The DCR diet consist the same amount of protein and essential micronutrients as the basal AIN-93 diet, but 20% less total calorie from carbohydrates and fat comparing to the basal diet. The amount of food given to DCR and PF+Exe group was calculated based on the previous week food consumption of control mice. In the experiment, water was provided ad libitum. A speed adjustable rodent treadmill (Boston Gears, Boston, MA) was used for mice exercise. After 2 weeks training, the exercise groups ran on the treadmill at 13.4 m/min, 60 min/day and 5 days a week for 10 weeks. All mice were housed individually at 75 ± 1ºF and 60% relative humidity with 12 hr light / 12 hr dark cycle. Body weight and food consumption were recorded every week.

At the end of the experiment, the mice were treated with 6.4 nmol 12-O-tetradecanoylphorbol-13-acetate (TPA) in 200μL acetone on the dorsal skin, which were shaved

two days before treatment. Mice were sacrificed two hours after TPA treatment. The dorsal skin samples were snap-frozen in liquid nitrogen and kept at -70 ºC until further analyses.

Lipid extraction

Skin samples, about 0.2 to 0.4 gram were ground with liquid nitrogen. Two volumes of solvent [chloroform: methanol 1:2 v/v + 0.01% butylated hydroxytoluene (BHT)] were added to 1 part tissue (v/w) and shaken well. Then the mixture was supplemented with 1.25 volumes each of chloroform and water and centrifuged at 200g. The lower layer, which contains the lipids, was collected. Then, twice, 1.25 volumes of chloroform were added to the tissue, the samples were centrifuged and the lower layer was collected. The combined lower layers were washed with 300 µL 1.0 M KCl, then with 300 µL water and transferred to a new tube. The extracts of skin lipids were dried down under a stream of nitrogen and re-dissolved in 1000 µL of chloroform transferred to 2 mL pre cleaned glass vials and stored at -70^{o}C until analysis.

Mass spectrometric analysis of neutral lipids

5 uL of lipid extract as well as the appropriate internal standard (4.7 nmol of di-15:0 DAG or 3.1 nmol of tri-17:1 TAG) were combined with solvents chloroform-methanol-300 mM ammonium acetate in water in the ratio 300:665:35 and a final volume was 1 mL. Unfractionated lipid extracts were then introduced by continuous infusion into the ESI source on a triple quadrupole MS/MS system (API 4000; Applied Biosystems/MDS Sciex, Ontario, Canada). Samples were introduced using an autosampler (LC Mini PAL; CTC Analytics AG, Zwingen, Switzerland) fitted with the required injection loop for the acquisition time and presented to the ESI needle at 30 µL/min.

The analysis of the samples by mass spectrometry was realized at the Kansas Lipidomics Research Center (Division of Biology, Kansas State University), using a modified method which

was first developed by Murphy et al. (2007). Ammoniated adducts of DAGs and TAGs undergo characteristic loss of single neutral species, as RCOOH+NH3, for each fatty acyl present in the molecules. DAG and TAG molecules were detected by a combination of several neutral loss scans. The neutral losses to monitor are selected according to the known composition in fatty acids found in mouse lipids. The following neutral losses were scanned for detecting DAG and TAG molecules: 14:0 (neutral loss (NL) of 245), 15:0 (NL 259, internal standard), 16:0 (NL 273), 16:1 (NL 271), 18:0 (NL 301), 18:1 (NL 299), 18:2 (NL 297), 18:3 (NL 295), 20:3 (NL 323), 20:4 (NL 321).

The following instrumental parameters were used: collision gas pressure at 2 (arbitrary units); collision energy, with nitrogen in the collision cell, at 20 V; declustering potential at 100 V; entrance potential at 14 V; potential at 14 V; source temperature (heated nebulizer) at 100°C; interface heater was on; +5.5 kV or –4.5 kV was applied to the electrospray capillary; curtain gas set at 20 (arbitrary units); and the two ion source gases were set at 45 (arbitrary units). Each neutral loss spectrum was scanned from m/z 500 to 950 over 4 s, and then the scan was repeated 30 (TAGs) to 37 (DAGs) times (2.0-2.5 min total time).

The background of each spectrum was subtracted, the data were smoothed, and the peak areas were integrated using a custom script and Applied Biosystems Analyst software. Isotopic overlap corrections were applied, and the DAG and TAG lipids were identified using in-house EXCEL templates and quantified in comparison with the internal standards.

Statistic analysis

One-way ANOVA was used to analyze the body weight. The data from two hours after TPA treatment was combined with tweleve hour's data, as no significant effect was found between the two different time points. Difference in DAG or TAG levels among the four

treatment groups were compared using a one-way ANOVA, F-test for significance followed by pair comparisons. Data are expressed as means ± S.E. The levels of significance of all statistical tests are set at the 5% level. That is, a statistical test is declared significant if its p-value is less than 0.05. Principal component analysis was applied to analyze the similarity of the four treatment groups. All the statistical analyses were performed using SAS 9.1.

Results

Impact of weight control via calorie restriction and/or exercise on body weight

The body weight of control, AL+Exe, PF+Exe and DCR mice are shown in figure 3-1. Adult CD-1 mice in sedentary control group gained weight slowly throughout the whole experiment period. Their body weight is 22.2 gram in the beginning and 27.6 gram in the end. There is no significant difference between AL+Exe mice and sedentary control. Both PF+Exe and DCR significantly decreased body weight compared to control and AL+Exe.

Impact of weight control via calorie restriction and/or exercise on plasma TAG and DAG in CD-1 mice

Figure 3-2 showed the profile of subtotal TAG in the plasma of CD-1 mice. For TAGs in each category, there is no significant difference between control and AL+Exe. However, both PF+Exe and DCR significantly decrease TAG containing fatty acid 16:0, 16:1, 18:0, 18:1, 18:2, 18:3. Only DCR significantly decreased TAGs have fatty acid 20:4. The total plasma DAGs are showed in Figure 3-3. PF+Exe significantly decreased total plasma DAG comparing to control. Figure 3-4 shows the ten most abundant DAG species in the plasma. DAG that has 16:1 and 20:0 fatty acyl are most abundant molecular specie, whose concentration is about 0.2-0.3 nmol per μL in the plasma. The second most abundant DAG molecular specie is 20:0/18:3, which has a concentration about 0.1 nmol per μl in the plasma. Both DAG 16:1/20:0 and DAG 18:3/20:0

have no significant difference between all the groups. For DAGs 16:0/16:0, 16:0/18:1, 16:0/18:2, 18:1/18:1; 18:1/18:2, 18:2/18:2, they are significantly decreased in PF+Exe and DCR groups, but not in AL+Exe group comparing to control.

Impact of weight control via calorie restriction and/or exercise on TAG and DAG profile in skin tissue

The effect of weight control on TAG profile is shown in Figure 3-5. For the subtotal TAGs that have fatty acyl 14:0, 16:1, 18:0, 18:2, there is no significant difference between all the groups. Comparing to control, only the subtotal TAGs that have 18:3 was significantly decreased in DCR group. For subtotal DAGs that have fatty acyl 18:1, 20:3, 20:4, DCR is significantly lower than AL+Exe.

Total DAGs in skin tissue are shown in Figure 3-6. No group is significant difference with control. AL+Exe is showed to have most total DAGs in mouse skin, and its amount is significant higher than DCR group. Figure 3-7 shows the mole percentage of individual DAG molecular specie to total DAG in mice skin. The data showed that DAG 18:1/18:1, 18:1/16:0, 18:2/16:0, 18:2/18:1are the most popular species in mice skin, which each of them takes about 20% of total DAG. As no DAG molecular specie in AL+Exe group is significant from control, the data shows that AL+Exe and control have very similar DAG. Comparing to control, DAG 16:0/20:2, 18:0/20:3, 18:0/20:4, 18:1/20:2, 18:1/20:4, 16:0/16:0 are significantly higher in DCR group. For DAGs 16:0/18:2, 18:1/18:2, 18:2/18:2, both PF+Exe and DCR significantly decreased their distribution.

Principal component analysis of the TAG and DAG species of plasma and skin stissue

Principle component analysis (PCA) was used to assess the difference of lipid profile among weight control treatment. Figure 3-8 shows the score plot for plasma TAG (A), plasma

DAG (B), skin TAG (C) and skin DAG (D). For plasma TAG, PC1 represents 58.8 % and PC2 represents 9.5% of the variance. The plot shows PF+Exe, AL+Exe and DCR are close together, and all of the three are separated from control. For plasma DAG, PC1 explains of 56.2% and PC2 explains 13.1% of the variance. The plot shows PF+Exe and DCR have similar pattern of PCA scores, while AL+Exe and control are similar. PC1 represents 46.2% and PC2 represents 12.2% of the variance for skin TAG, and PC1 represents 24.8% and PC2 represents 13.2% of the variance for skin DAG. Both skin TAG and skin DAG plots shows PF+Exe, AL+Exe and control are closed, and DCR is very different from the other three.

Discussions

This study found that weight control via exercise with paired feeding or calorie restriction decreased body weight, resulting a significant impact on TAG and DAG profiles in both plasma and skin tissues.

The study showed that exercise with pair feeding and 20% dietary caloric restriction significantly decreased body weights compared to sedentary control mice, while *ad libitum* feeding had no signficant effects. The results are consistent with our previous findings (Xie et al., 2007; Ouyang et al., manuscript) and others in APC^{Min} mice and SKH mice (Mehl et al., 2005; Michna et al., 2006). The treadmill exercise, 13.4 meters /min for 60 minutes a day, is considered moderate intensity. As the mice are fed ad libitum, it is possible that their calorie intake also slightly increased. Therefore, exercise without consideration of dietary intake maybe can not produce sufficient negative energy balance, at least not as much as 20% of total energy intake, for significant body weight loss. When the food consumption of the exercised mice was adjusted to that of sedentary control, the body weight was significantly decreased. Using a similar pair feeding strategy, Hursting lab found that voluntary wheel running mice with restricted food

consumption had a significant lower body weight and decreased intestinal polyp development (Colbert et al., 2006). The data suggested that to maintain body weight through exercise, dietary intake should be considered as an important factor.

For plasma TAG, we found that AL+Exe did not change subtotal TAGs significantly compared to control neither in plasma nor in the skin. PF+Exe significantly decreased TAGs in plasma, but not in the skin. 20% DCR decreased TAGs both in plasma and skin. The results from TAG and DAG may help to explain what we found previously that PF+Exe and DCR, but not AL+Exe, significantly decreased the percentage of fat as analyzed by DAX (Xie et al., 2007). The TAG lowering effect by exercise and DCR has been found for many years. One hour high intensity exercise has been found to reduce total plasma TAG up to 22% in human (Cullinane et al., 1982). It was suggested that the plasma TAG lowering effect by exercise depends on energy expenditure (Petitt and Cureton, 2003), and only negative energy balance was effectively in decreasing total plasma TAG (Burton et al., 2008). Although in the current study, we did not measure total TAGs. The subtotal TAG profile still confirmed that plamas TAG are highly correlated with body weight, body fat composition and energy balance. In addition, we should notice that for total plasma TAGs containing 20:3 fatty acids, which are polyunstrurated fatty acids, its concentration is highest in PF+Exe group. Exercised mice seem have a higher plasma total 20:3 level than sedentary counpartment. It indicates this group of TAG might be increased by exercise treatment. Further study to elucidate the particular impact of exercise on TAGs contraining 20:3 fatty acids in the plasma may help to better understanding of the impact of exercise on human health.

For total DAG in the plasma, we found that PF+Exe has the lowest concentration among the four groups, and its level is significant less than sedentary control. In mice skin, Kris et al.

(1994), found that calorie restriction increased total sn-1,2-DAGs in mice skin. In our study, however, the total skin DAGs in calorie restricted mice and exercised and pair-fed mice were not significantly different with controls. The discrepancy of the results may due to the different methods used. Kris et al. determined total DAGs by using labeling DAG with DAG kinase (Kris et al., 1994). What they detected was total DAG per cell. In this study, we measured individual DAG species by using MS and the total DAG extracted was calculated by adding up all the DAG species. In fact, what we obtained was total nmol DAG per mg of fresh weight tissue.

DAG, especially sn-1,2-DAG, as a second signal messenger has been well known. It may binds and activates proteins, such as PKC, and activates signals leading to cell growth and proliferation (Nishizuka 1992). The talk between PKC and its activator DAG is found to be very complicated. So far, at least 11 PKC isozymes have been identified and they are classified into three groups: classical PKCs (cPKCs: PKCα, PKCβI, PKCβII and PKCγ), novel PKCs (nPKCs: PKCδ, PKCε, PKCη and PKCθ) and atypical PKCs (aPKCs: PKCζ and PKCλ/ι).cPKCs can be activated by calcium and DAG, nPKCs is only DAG dependent, and aPKCs are not responsive to either calcium or DAG. It was found that different DAG species may have different affinity in binding of PKC isomers. As we know, the combination of two acyl chains would generate a diverse of DAG profiles among about fifty structurally different molecular species. Each fatty acyl could be saturated, mono-unsaturated, di-unsaturated, or polyunsaturated (Hodgkin et al., 1998; Wakelam 1998). DAGs converted from PI species usually compose a larger amount of polyunsaturated acyl combinations, such as 18:0/20:4 DAG, while DAGs originated from PC species usually have mono unsaturated and saturated acyl chains (Holub and Kuksis, 1978). Shinomua et al., found that saturated and trans-unsaturated free fatty acids can not bind and activate cPKC, but cis-unsaturated fatty acids such as oleic (18:1 ω9), linoleic (18:2 ω6),

linolenic (18:3 ω3) and arachidonic acid (20:4 ω6) can enhance cPKC phosphorylation and activation (Shinomura et al., 1991). Studies showed that DAGs with polyunsaturated fatty acyl are more active in PKC binding, while saturated DAGs seems to be poor PKC activators (Marignani et al., 1996; Schachter et al., 1996; Torrecillas et al., 2001). The PKC activation ability in lipid vesicles *in vitro* was found to be 18:0/22:6 > 18:0/20:5 > 18:0/20:4 > 18:1/18:1. In addition, same DAG specie may also selectively activate PKCs. For example, DAG (18:0/20:4) was able to activate PKCα, PKCβ. However, it was less active for PKCδ (Deacon et al., 2002). In SENCAR mice skin, PKCα and PKCζ protein expression was significantly reduced by calorie restriction (Birt 1995), and PKCβ and PKCγ was not detected (Dlugosz et al., 1992). Caloric restriction was also found to reduce PKC activity while phorbol dibutyrate showed a lower binding ability to epidermal cells (Birt 1995). It is interesting that we found some of the DAGs such as DAGs 16:0/20:2, 18:0/20:3, 18:0/20:4, 18:1/20:2, 18:1/20:4, or 16:0/16:0 were significantly increased by weight control (PF+Exe and DCR) and some DAGs such as 16:0/18:2, 16:1/18:2, 18:1/18:2, 18:2/18:2 were significantly decreased in both PF+Exe and DCR mice. The results seems to be opposite as what we expected, as DAGs containing polyunstaturated fatty acids such as 18:0/20:4 and 18:1/20:4 are elevated. It should be noticed that the DAGs we observed in the skin tissue could contain both signaling DAG and non-signaling DAG (D'Santos et a., 1999). The impact of weight control on DAG and PKC interaction will be more clear if the activated DAG and non-activated, background DAG could be separated.

We have found that most PI species, especially the major PI species with 38:4 fatty species were reduced by weight control. PI turnover was also found to be increased by DCR (Birt 1995). The decreases of PI species and increase of PI turnover may explain the increase of certain DAG species, especially the one containing polyunsaturated acyls. Further analysis the

relationship of phospholipids, such as PI and PC, with DAG and TAG in the skin tissue may provide a better idea on the lipid-mediated signal transduction system in the process of skin carcer prevention by weight control.

The results from principle component analysis for plasma TAG showed that the first two components of AL+Exe are separated well from that of control. It indicates exercise with *ad libitum* feeding do have some impact on plasma TAG profile, although it does not have significant effects on individual group TAGs. The plasma TAG profile could be use as a marker for weight control via exercise and/or dietary calorie restrition. Similary, plasma DAG seems to be a good marker for weight loss, as PE and DCR were well separated from AE and control.

Our study, for the first time, investigated the effect of weight control on TAG and DAG profile in the plasma and TPA treated skin tissue of CD-1 mice. We observed that weight control, via pair fed exercise or caloric restriction was effective to prevent body weight gain, which may due the negative energy balance. Weight control was also found to have significant effects on DAG profile, especially in TPA treated skin tissues. The results suggest that DAG-related signal transduction system, which is important in skin carcinogenesis, are mediated by weight control. The studies provide insight into the mechanism of weight control on cancer prevention.

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Figures and Tables

Figure 3-1 Body weights of CD-1 mice were reduced significantly by dietary calorie restriction (DCR) and exercise with pair-feeding (PF+Exe). The first two weeks are adaptation and training period. The arrow indicates the day that the dietary calorie restriction and physical exercise started. Results are means ± SE, n =13-17. Different letters show statistically significant difference (a>b>c)

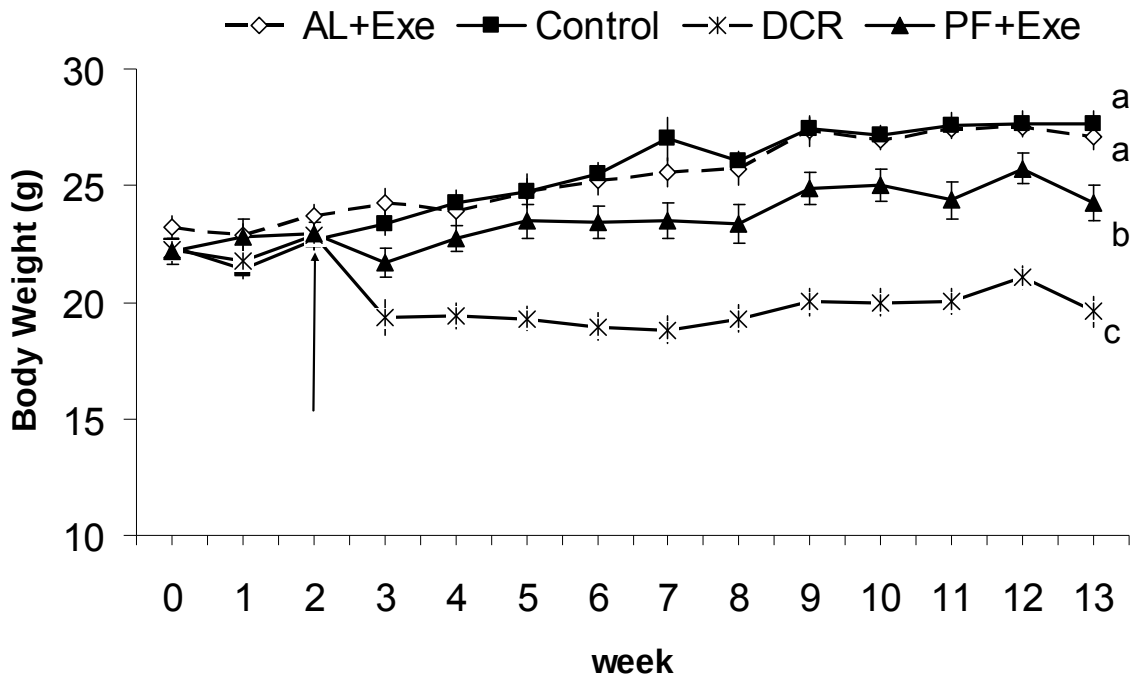


Figure 3-2 Plasma TAG of CD-1 mice in weight control study, results are means ± SE, n =13-17. Bars with different letters differ significantly, a>b, P ≤ 0.05.

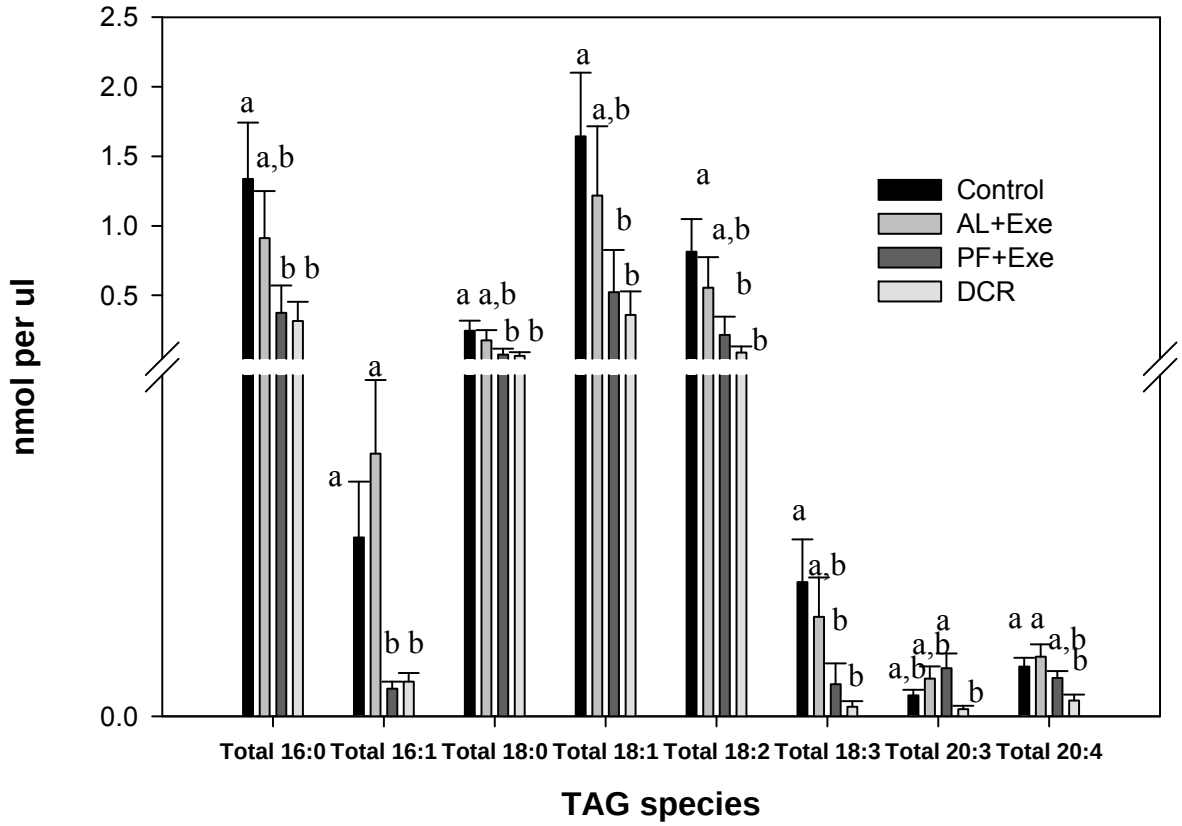


Figure 3-3 Total plasma DAG of CD-1 mice in weight control study, results are means ± SE, n =13-17. Bars with different alphabetic letters differ significantly, a>b, P ≤ 0.05.

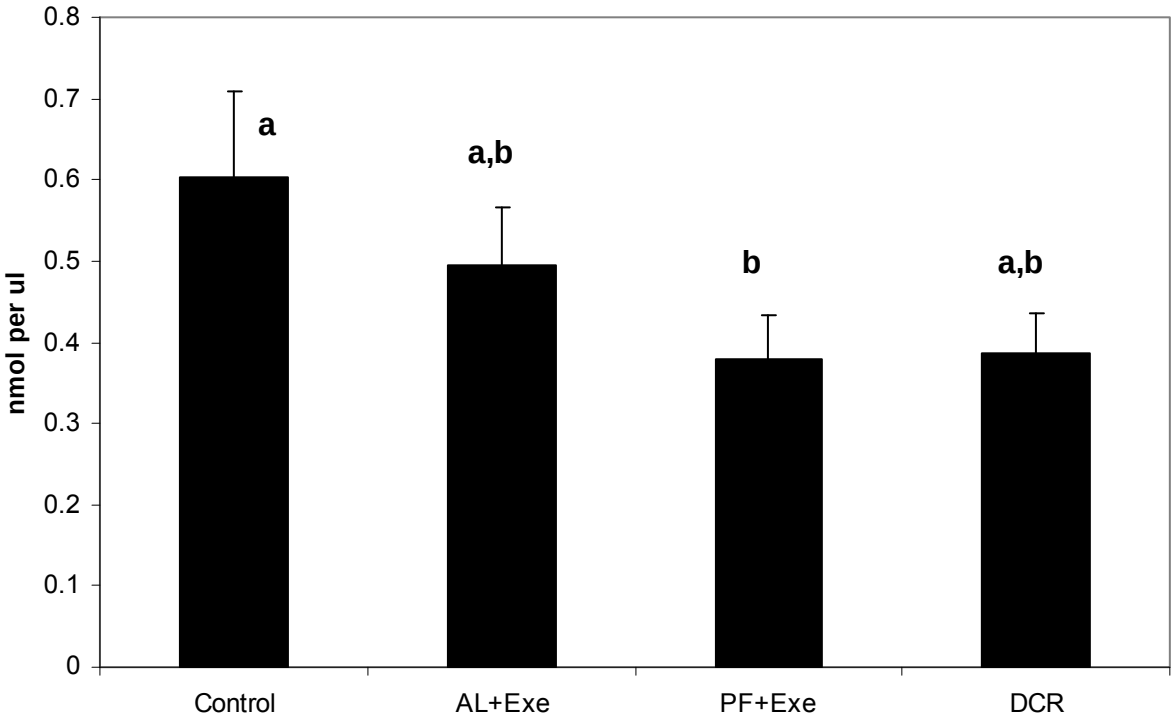


Figure 3-4 Top 10 most abundant species of DAG species in the plasma of CD-1 mice in weight control study, results are means ± SE, n =13-17. Bars with different alphabetic letters differ significantly, P ≤ 0.05.

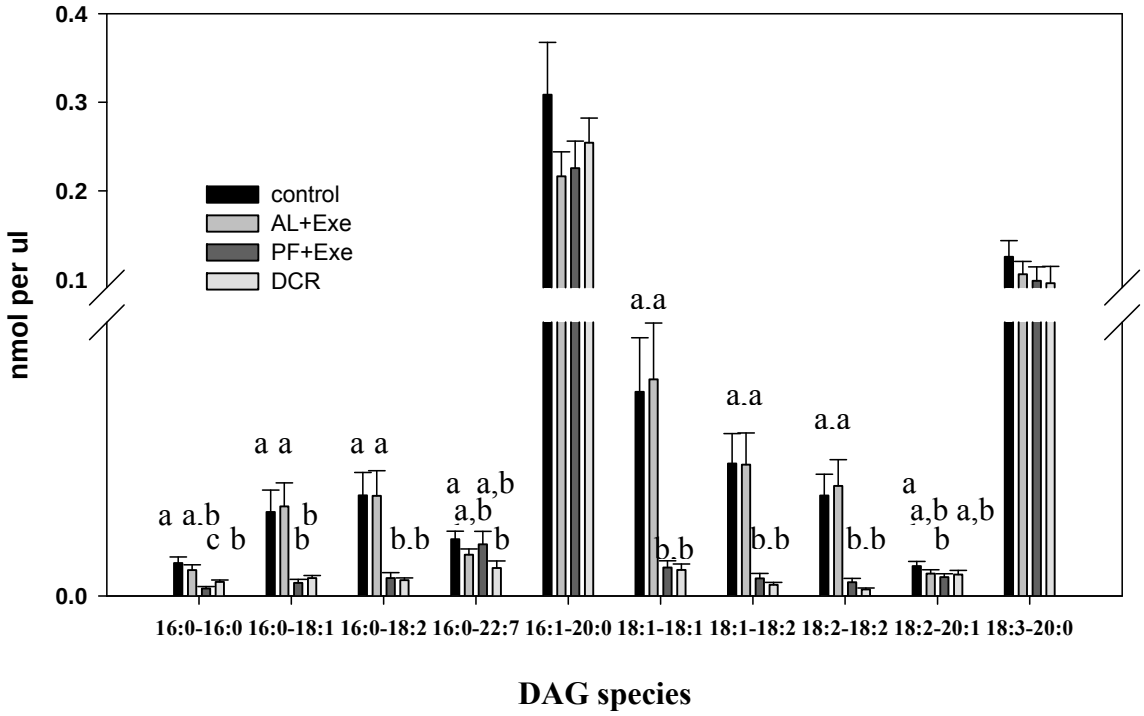


Figure 3-5 Tissue TAG of CD-1 mice in weight control study, results are means ± SE, n =10-13. Bars with different alphabetic letters differ significantly, a>b, P ≤ 0.05.

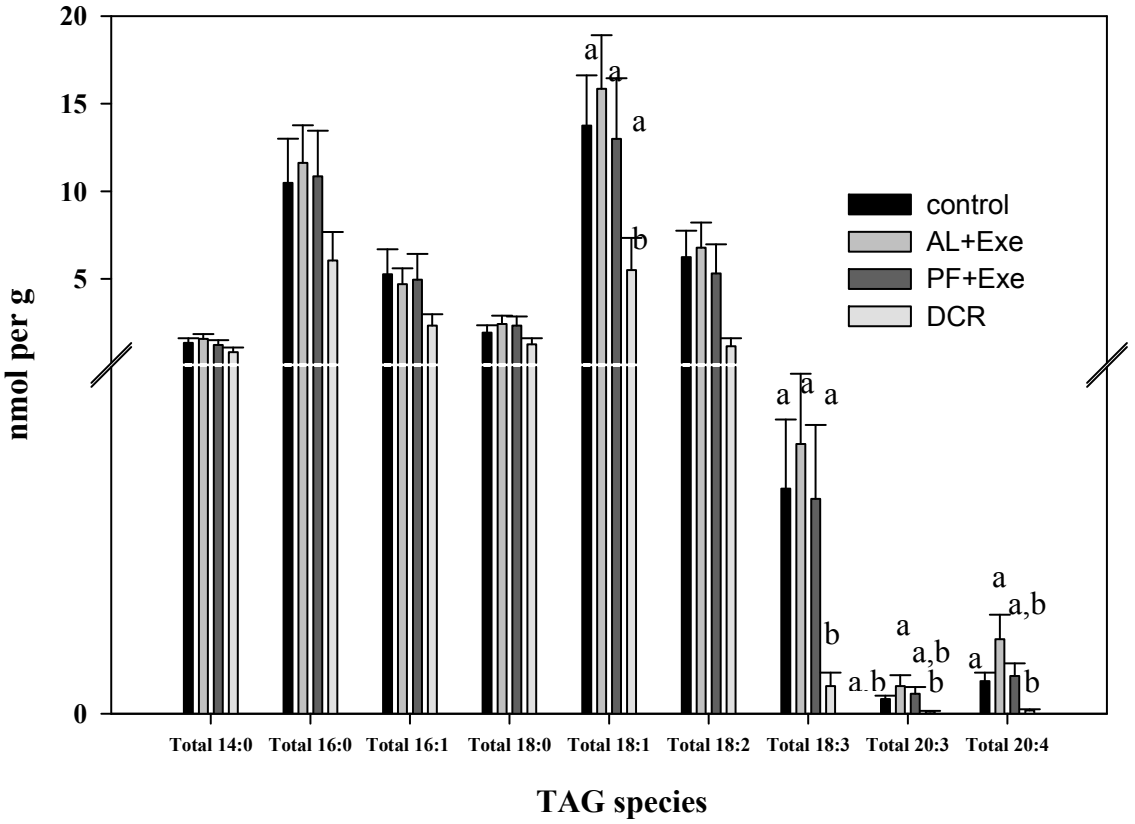


Figure 3-6 Total tissue DAG of CD-1 mice in weight control study, results are means ± SE, n =10-13. Bars with different alphabetic letters differ significantly, a>b, P ≤ 0.05.

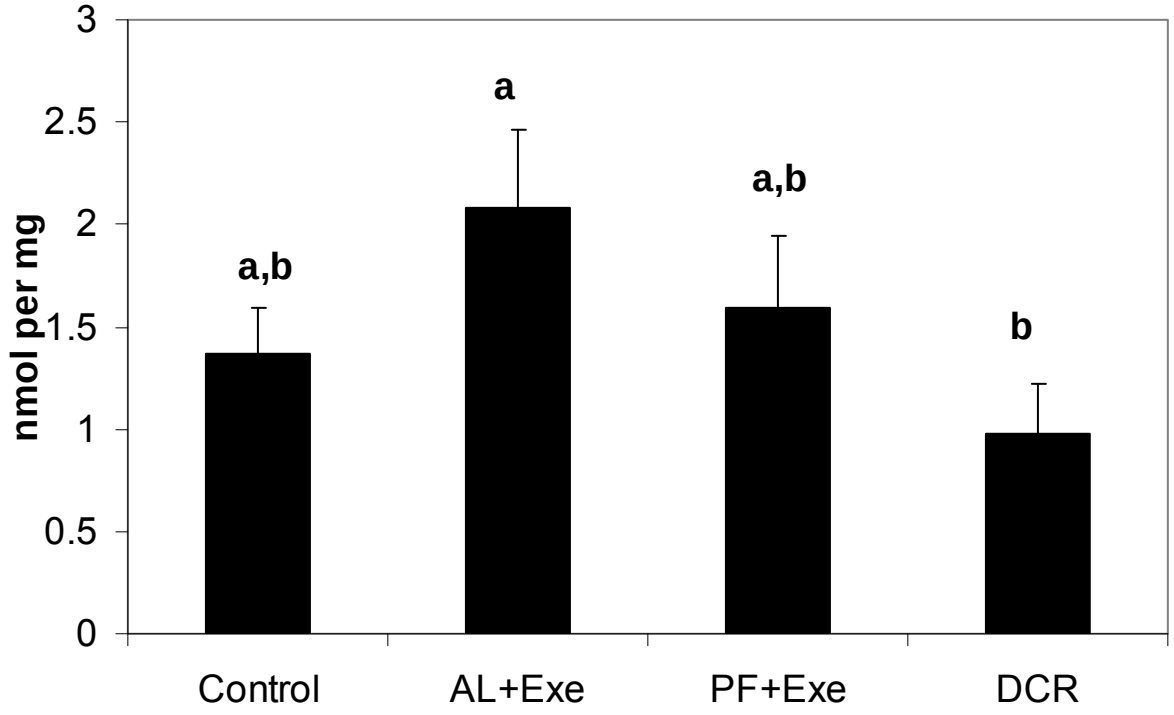
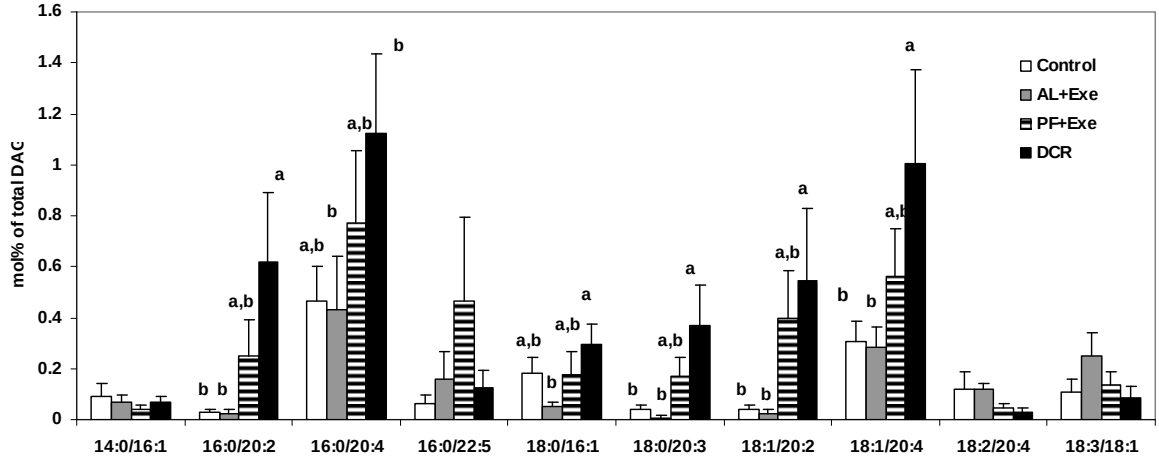


Figure 3-7 Tissue DAG of CD-1 mice in weight control study, results are means ± SE, n =10-13. Bars with different letters differ significantly, P ≤ 0.05.



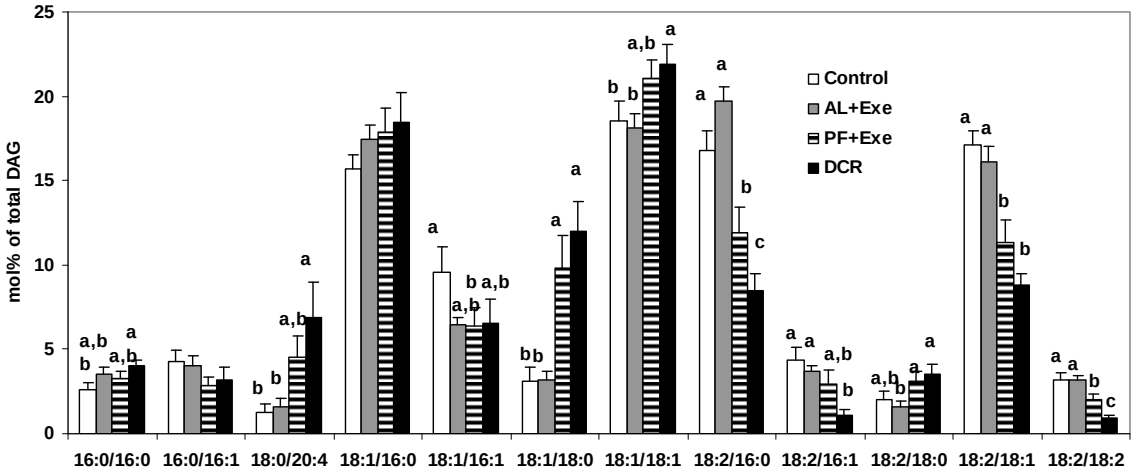
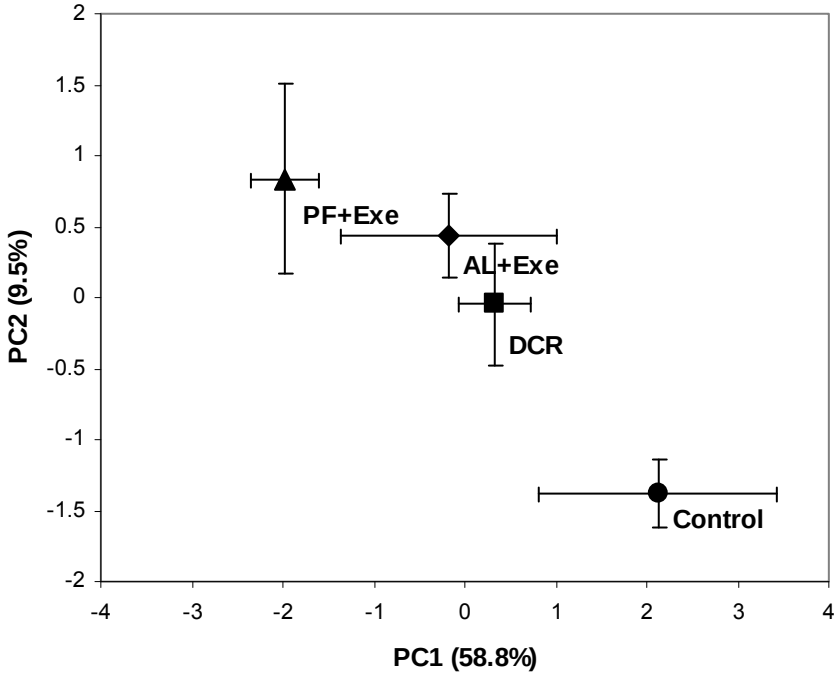
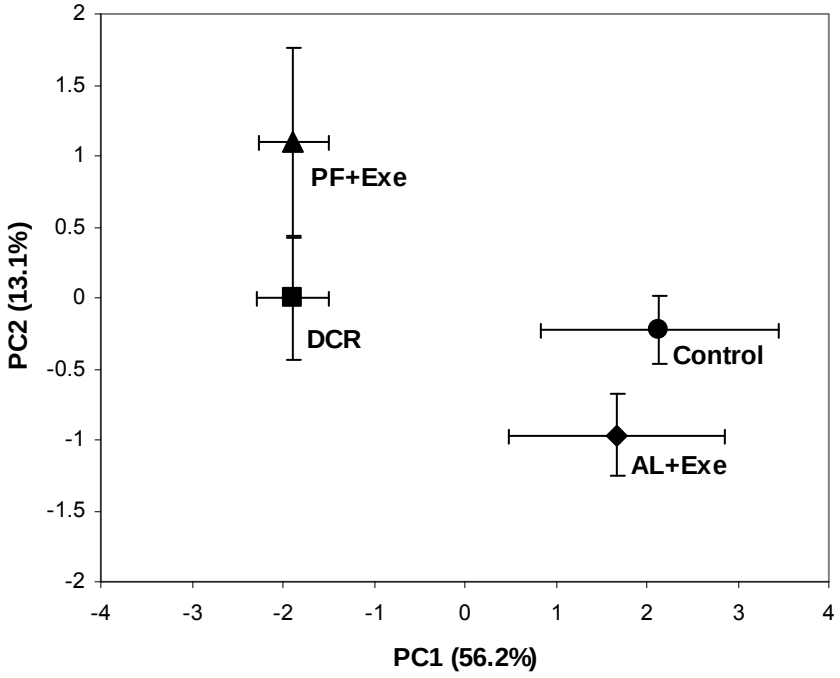


Figure 3-8 Principal component analysis (PCA) of the first two principal components of (A) plasma TAG, (B) plasma DAG, (C) skin TAG, (D) skin DAG of the four treatment groups.

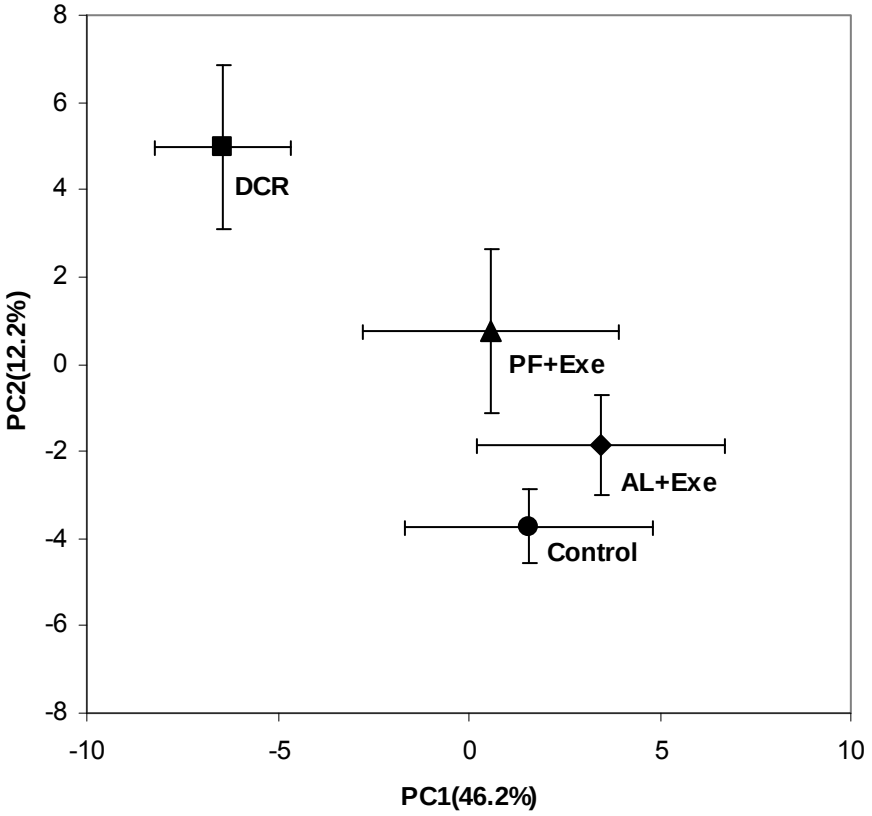
(A) Plasma TAG



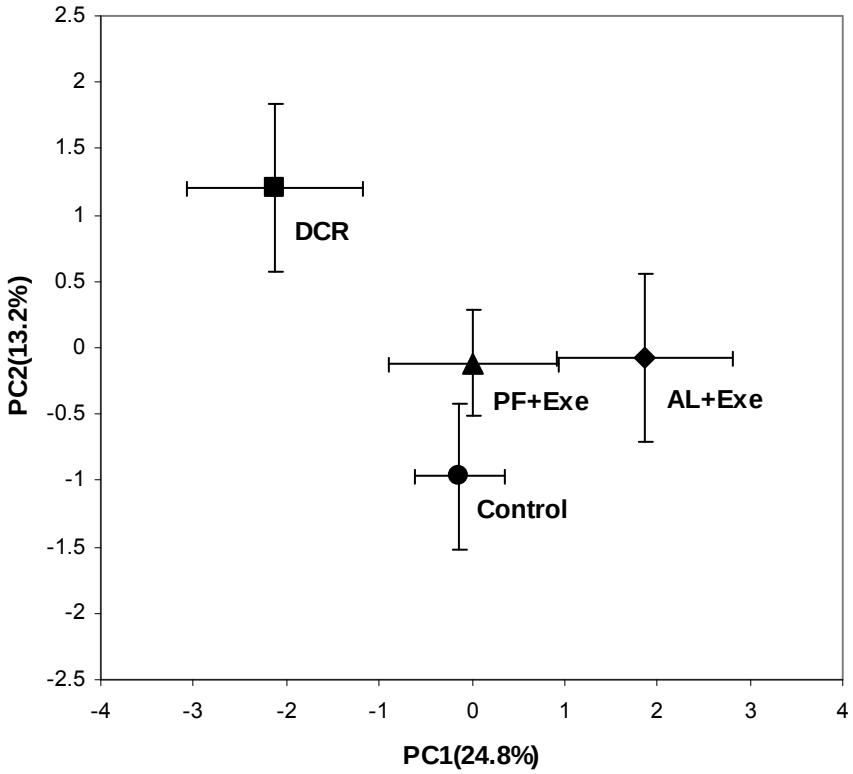
(B) Plasma DAG


PF+Exe
DCR
Control
AL+Exe
PC2 (13.1%)
PC1 (56.2%)
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-4
-3
-2
-1
0
1
2
3
4

(C) Skin TAG



(D) Skin DAG


2.5
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-4
-3
-2
-1
0
1
2
3
4
PC2(13.2%)
PC1(24.8%)
DCR
PF+Exe
AL+Exe
Control

CHAPTER 4 - Profile of Gene and Protein Expression in the Skin Tissues of Weight Controlled Mice

Abstract

Experimental evidence has demonstrated that weight control via dietary caloric restriction and/or exercise may reduce cancer risk. However, the underlying mechanisms are not clear. In this study, SENCAR mice were treated for ten weeks in one of the four groups: ad libitum-fed sedentary control, ad libitum-fed exercise (AL+Exe), exercise but pair-fed at the amount of control (PF+Exe), and 20% of dietary calorie restriction (DCR). Two hours after topical TPA treatment, the skin tissues were analyzed by Affymetrix for gene expression and DIGE for proteomics, respectively. Among 39,000 transcripts, 152, 223 and 784 probe sets were found significantly changed by DCR, PF+Exe, and AL+Exe, respectively. PF+Exe and DCR showed similar impact on signaling pathways-related gene expression as analyzed by GenMAPP. Braf and Akt1 appeared to be the key modulator in MAPK pathway and insulin signaling pathway respectively in response to weight control via DCR or PF+Exe. Of the total 86 proteins identified, about 24 proteins were significantly changed by DCR. Although we could not ambitiously clarify all the cancer-related mechanistic targets in the scope of this study, overview of all the gene and protein alteration profiles may provide intrinsic mechanisms of cancer prevention by weight control in the future studies.

Introduction

During the past twenty years, the prevalence of overweight and obesity is keep growing (CDC, 2006). Obesity is associated with increased risk of several chronic diseases, such as diabetes, cancer, and cardiovascular disease. Studies showed moderate-to-strong evidence for positive association between overweight/adiposity and higher risk of cancer in colon, kidney, esophagus, endometrium, thyroid, and postmenopausal breast cancer (WCRF/IARC, 2007). Increased body weight was also found to be associated with higher mortality in cancer (Calle EE, 2003). Weight control via decreased dietary energy intake (caloric restriction) and/or increased energy expenditure (physical activity) has become an important strategy against cancer and other chronic disease. Caloric restriction is referred to decrease of energy intake without malnutrition. Since 1906, the cancer preventive effect of caloric restriction has been studied and confirmed in various animal models, such as primate and rodent and in various organs, such as mammary gland, prostate, colon and skin. The health benefit of physical activity (exercise) has been known for many decades. According to the review by IARC group, there was sufficient evidence showed physical activity was able to prevent colon and breast cancer (WCRF/IARC, 2007). Currently, the key question that intrigues many researchers and remained unanswered is: what is the mechanism that weight control acts on cancer prevention. Studies showed that both caloric restriction and exercise were able to modulate certain circulating hormones, such as insulin, IGF-1, glucocorticosteroid, adipocytokins, which are important in modulation of cell proliferation, cell death and normal functions (Jiang and Wang, manuscript). Caloric restriction may also enhance DNA repair and moderate oxidative damage to DNA, as well as reduce oncogene expression. In two stage skin carcinogenesis model, weight control was found to inhibit TPA promotion by blocking PKC-Raf-ERK signaling pathway and suppressing the activation of

transcription factor AP-1. Recent studies in our lab showed that IGF-1-dependent signaling pathway also played an important role in TPA-induced cancer promotion. DCR and/or exercise were able to decrease circular level of IGF-1 and block PI3K-Akt pathways and therefore inhibit TPA promotion in mouse skin (Xie et al., 2007). So far, all the previous studies are based on traditional molecular biology techniques, through which people can only identify the change of a single gene or protein at one time. However, it is well known that weight control is a very complicated biological process. It does not only target on a single gene or protein but have multiple targets through out of the genome. The process of cancer development is also complicated. In the two stage skin carcinogenesis model, the development of skin tumor includes changes in DNA conformation, levels of mRNA, amount of protein transcription and protein post translation modification. Hundreds of biological molecules cooperate in this network complex. Therefore, traditional molecular biology techniques failed to meet our requirement to gain a broader and deeper view into the mechanism of weight control on caner prevention.

Fortunately, recently developed “omics” technologies provide us a chance to take a more global view of these biological processes. The advent of high throughput technologies such as microarray analysis is the first step to obtain cell specific gene expression profile. Together with proteomics, lipidomics and glycomics, large amounts of data can be generated. System biology, as a tool to integrate the database, utilizes all the information for modeling of a biological process. Consequently, the challenging is to explore the best statistic methods and set up bioinformatics tools for data mining.

The transcriptome is a study of the entire range of transcripts produced by a given organism. The development of microarray technology provides a useful tool in the assessment of gene expression profile in a large scale or even the whole genome. Microarray analysis is quite

different from traditional gene expression methods such as Northern blot, nuclear protection assay, expressed sequence tags (ESTs), and RT-PCR which are used extensively in study the expression of individual genes. It has been widely used in the study of gene function, identification of tumor-specific molecular markers, genotyping, and gene discovery.

In addition to gene transcription profile, protein expression profile has also been a focus of biology research. The proteome is the final outcome of genome expression compring all the proteins present in a cell at a particular time. Proteomics, the global overview of proteome, is to investigate the expressions, modifications, interactions and regulation of proteins in organisms comprehensively. Through a proteomics approach, researchers are able to determine the expression of up to a few thousand proteins in a single sample simultaneously. The most widely used proteomics technique starts with 2D PAGE (two-dimensional polyacrylamide gel electrophoresis) coupled with tryptic peptide MS analysis. 2D PAGE have been used to separation protein since 1970s. Basically, proteins are first separated by isoelectric focusing based on their PI. Secondly, proteins are separated using SDS-PAGE according to their PI and molecular weights respectively. After separation, proteins are stained with dyes, such Coomassie blue, fluorescence dye or silver nitrate. Several software programs have been developed to quantitative analysis the image of 2D gels and characterize the abundance of a certain protein spot. Usually, in gel digestion will be performed, which is either by trypsin or by cyanogen bromide. Lastly, the generated peptides are analyzed by MALTI-TOF MS. In order to decrease gel to gel variance, 2D DIGE (two-dimensional difference gel electrophoresis) technology was developed and is currently used in many labs (Marouga et al., 2005). With this method, protein samples are labeled with one of several cyanine (Cy) dyes on lysine residues before being separated by IEF and SDS PAGE. Proteins samples with different Cy dyes (Cy2, Cy3 or Cy5)

can then be mixed and run together on the same 2D gel. With 2D DIGE, spot matching are simpler and more accurate. It also greatly reduces the variance between gels, since two to three samples are running on the same gel.

We use Affymatrix microarray analysis identified genes that are significantly changed by AL+Exe, PF+Exe, and DCR, respectively. We found PF+Exe and DCR showed similar impact on signaling pathways-related gene expression as analyzed by GO function analysis and GenMAPP. Using GeneMapp we identified Braf and Akt1 as the key modulator in MAPK and insulin signaling pathway. Using 2D DIGE proteomics approach, our lab studied the effect of calorie restriction on skin cancer prevention. The proteins that are significantly changed by DCR involves in glycoylsis, actin related cell modility, redox homostasis and cellular stress. The gene and protein expression file bring new targets that are important in weight control mediated skin cancer prevention.

Methods

Animal Studies

Female SENCAR mice arrived at eight weeks old were random divided into four groups: ad libitum feeding and sedentary (control), exercise with ad libitum feeding (AL+Exe), exercise with pair feeding (PF+Exe) and dietary caloric restriction (DCR). Ad libitum feeding groups (control and AL+Exe) were allowed to freely obtain the basal diet (AIN-93) while the pair-fed exercise group was fed daily the same amount as the sedentary mice. DCR group were fed with a 20% DCR diet for 8 wks. The 20% DCR diet was formulated and provided by the Harlan Teklad (Madison, WI). The DCR diet consist the same amount of protein and essential micronutrients as the basal AIN-93 diet, but 20% less total calorie from carbohydrates and fat comparing to the

basal diet. The amount of food given to DCR group was calculated based on the previous week's food consumption of control mice. In the experiment, water was provided ad libitum. A speed adjustable rodent treadmill (Boston Gears, Boston, MA) was used for mice exercise. After 2 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. All mice were housed individually at 75 ± 1ºF and 60% relative humidity with 12 hr light / 12 hr dark cycle. Body weight and food consumption were recorded every week.

At the end of the experiment, the mice were treated with 6.4 nmol 12-O-tetradecanoylphorbol-13-acetate (TPA) in 200 uL acetone on the dorsal skin, which are shaved two days before treatment. Mice were sacrificed two hours after TPA treatment. The dorsal skin samples were snap-frozen in liquid nitrogen and kept at -70 ºC until further analyses.

Protein Extraction and Purification

Mouse skin tissues were homogenized in extraction media (0.175 M Tris-HCl pH 8.8, 5% SDS, 15% glycerol, 0.3 M DTT, 3 mM PMSF) and centrifuged at 14000 × g for 30 minutes at 4 °C. The supernatant were collected and the protein concentration was determined by Protein RC DC assay (Bio-Rad, Hercules, CA). The protein lysis were purified by ReadyPrep 2-D cleanup kit (Bio-Rad, Hercules, CA) and reconstituted in protein sample buffer (7M urea, 2M thiourea, 4% CHAPS, 30mM Tris, pH=8.5). The concentrations of the purified protein samples were determined using protein RC DC assay (Bio-Rad, Hercules, CA) with BSA as protein stardard. The internal standard was composed by pooling equal amount of proteins from all four groups.

2D-Electrophoresis

Sample labeling with cyanine minimal dyes was carried out according to the manufacture's instruction (GE healthcare, Piscataway, NJ). Protein (25 μg) was used for Cydye labeling and the ratio of protein to Cydye is 1 μg protein: 5pmol Cydye. The internal standard was always labeled with cy2, and the samples were labeled with Cy3 and Cy5 alternatively.

The protein mixture containing 25 μg labeled internal standard, 25 μg labeled protein from each treatment group respectively or 250 μg unlabeled protein were mixed with rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 140mM DTT and 2% ampholytes pH 3-10) and used to dehydrate IPG (pH 3 to 10) strips for 14 hours. The isoelectric focusing was carried on a PROTEAN IEF Cell following manufacture's instruction (Bio-Rad, Hercules, CA). Following IEF focusing, the strips were equilibrated in 4 mL of equilibration solution I (50 mM TRIS-HCl, 6 M urea, 30% glycerol, 2% SDS, 2.5% DTT, pH=8.8) for 10 minutes and then in 4 mL of equilibration solution II (50 mM TRIS-HCl, 6 M urea, 30% glycerol, 2% SDS, 2% idoacetamide, pH=8.8) for 10 minutes. SDS PAGE was conducted using a precast 8-20% gradient gel (Bio-Rad, Hercules, CA). Electrophoresis condition was set at 200V for 60 -70 minutes until the dye front reached the bottom of the gel.

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 matching process, up to 20 landmarks was defined all over the gel. After 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.

Protein identification

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.

Microarray Analysis

For microarray data analysis, the images were quantified by using GeneChip operating software 1.0 (GCOS 1.0; Affymetrix). The raw image readings were analyzed using Simpleaffy package from BioConductor (http://www.bioconductor.org) and R 2.7.2. The data were normalized using either MAS or RMA algorithms. The genes that are differentially expressed between the DCR and control groups were identified by using pair wise comparison. The data were filtered by using 1.5 fold changes and significant changes p-value less than 0.05 as a cutoff.

Cytoscape v2.6.0 coupled with plug-in BiNGO v2.0 was used to mapping the predominant gene ontology categories of the differentially expressed genes (Maere et al., 2005). 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 weight control specific pathways.

Results

The expression of mRNA from the different mouse skin was determined by using Affymetrix microarray, and then the gene expression data was analyzed with simpleaffy package from BioConductor. Compared to control, 152 probe sets by AL+Exe, 223 probe sets by PF+Exe and 784 probe sets were significantly changed by DCR respectively. The scatter plots of mRNA gene expression are shown in Figure 4-1. The x-axis represents gene expression level of control nice. The y-axis represents gene expression level of AL+Exe, PF+Exe, and DCR mice respectively. The spots shown in the graph are probe sets filtered out using 1.5 folder change and p value less than 0.05 cut off. The 2 fold, 4 fold and 6 fold cut off lines are indicated in the plot.

The gene sets identified by microarray analysis that were significantly changed by weight control treatments were further categorized using GO annotations. The over represented GO categories were identified using BiNGO. Figure 4-2 showed the visualization of gene network by Cytoscape. The size of the circles is representative of the proportional protein abundance in the dataset. The color of the circle indicates the degree of statistical significance. The darker of the circle, the stronger indicates of difference. The pathway analysis by GenMapp showed that b-raf was significantly decreased by PF+Exe and DCR in MAPK pathway. The results of pathway analysis were shown in Figure 4-3.

Figure 4-4 shows a representative overlaid 2-D image of CyDye labeled 25 μg protein lysates of ad libtum feeding exercised mice (cy3) and 25 μg of DCR mice (cy5). The image showed the overlay of cy3 and cy5. We identified 120 protein spots totally (Tables 4-1 and 4-2). Twenty-seven protein spots were significantly changed by DCR comparing to control. Among them, 15 were down regulated and 12 were up regulated. The down regulated proteins were: PDZ and LIM domain protein 1, myosin light chain proteins, enolase 3, beta muscle, gelsolin-like capping protein, ATP synthase, Ugp2 protein, phosphoglycerate kinase, aconitase 2, and

adenylate kinase isoenzyme 1. The up regulated proteins were: 6-phophogluconolactonase, trisephosphate isomerase, Kininogen 1 precusor, albumin, ornithine aminotransferase, apolipoprotein A-I, heat shock protein; alpha-crystallin related, carbonic anhydrase 3, Flavin reductase (NADPH-dependent diaphaphatase), peroxiredoxin 6, and albumin. One carbonic anhydrase 3 protein spot was significantly increased by DCR, while two carbonic anhydrase 3 protein spots decreased by DCR.

Discussion

The gene profile in TPA promoted mouse skin tissues was measured by Affimattrix microarray and analyzed by BioConductor (Wilson et al., 2005). The impact of weight control by exercise with ad libtum feeding, exercise with paired feeding, or 20% dietary calorie restriction on the gene expression were compared.

We used BioConductor analyzed microarray data set, and obtained similar results to what was obtained by Genespring (Jia et al., 2007). DCR showed most extensive impact on gene expression profile, while AL+Exe was most similar to control. We also did functional overexperssion analysis by GO annotation to identify major biological processes potentially impacted by weight control treatment using BiNGO and visualized by Cytoscape. The most genes affected by weight control treatment were involved in cell growth/maintenance and cell communication categories. The results were similar as what we found previously (Jia et al., 2007).

Using GenMapp, we found that PF+Exe and DCR showed a similar impact on signaling pathways-related gene expression. It should be noted that Braf appeared to be the key modulator in MAPK pathway in response to weight control by DCR and PF+Exe. The Braf gene expression was significantly decrease by DCR and PF+Exe, but increased by AL+Exe compared to control.

Braf gene encodes a protein that belongs to the raf/mil family of serine/threonine protein kinases. Studies showed that B-Raf is an essential component in the Ras-dependent Raf-1 activation complex, and it plays a significant role in regulating the MAP kinase/ERKs signaling pathway, which affects cell proliferation and differentiation (Mizutani et al., 2001), Braf mutations was found to be associated with various cancers, such as non-Hodgkin lymphoma, colorectal cancer, malignant melanoma, thyroid carcinoma and lung cancer. For insulin signaling, Akt1 was significantly reduced in both DCR and PF+Exe groups when compared to the control (data are not shown).Akt1, which is also calle PKB, is one of the serine/threonine-specific protein kinases. Akt1 is one of downstream effectors of PI3K. It can be activated by binding to phosphoinositides, which are coverted from PI with the catalyzing of PI3K. In addition to PI3K, Akt1 maybe also be activated by cAMP-elevating agents through protein kinase A. Akt1 protein can bind and regulate many downstream signal proteins, such as NF-κB, Bcl-2 family proteins and murine double minute 2 (MDM2), which lead to promoting of cell survival and inducing of protein synthesis (Steelman et al., 2008). The down regulation of Braf and Akt1 gene expression further supported our previous findings that both PI3K pathway and MAPK pathway are blocked by weight control.

In the current study for pathway analysis, we are using pathways constructed by KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.ad.jp/kegg). The pathways are not specie and tissue specific. Pathway studio, also one of pathway analysis softwares, may have larger pathway database. Therefore, in the future, it is worth to try different pathway analysis tools, and compare the results. The study will bring a better understanding the impact of weight control on gene expression on the a globle view,

Using 2D DIGE proteomics, we identified 120 protein spots, which equal to 86 proteins on the 2D gel. Among these proteins idenfied, we found that 20 proteins was significantly changed. Our results showed that two enzymes that involved in glycolysis, i.g., enolase and phosphoglyerate kinase, decreased significantly in DCR-fed mice. Triosephosphate isomerase was enhanced in DCR group. The suppression of glycolysis process by dietary calorie restriction has been observed before by several labs (Hipkiss et al., 2006; MaCarter and Palmer 1992). Most glycolytic intermediates are aldehydes, which are potentially toxic because of their ability to glycate proteins, nucleic acids and amino-lipids. Inhibition of glycolysis may thus limit cell damage by these intermediate and have beneficial effects (Hipkiss 2007).

By using protomics approach, we also identified that two proteins related to cell motility, PDZ and LIM domain protein1, and gelolin like capping protein, were decreased by DCR. One is PDZ and LIM domain protein 1, which is also known as CLP 36. It is a protein contains an N-terminal PDZ domain and a C-terminal LIM domain. CLP 36 is found to act as adapters between kinases and the cytoskeleton. It associates to kinases via its LIM domain and cytoskeleton via its PDZ domain (Bauer et al., 2000). Studies have showed that CLP 36 binds to cellular α-actinin-1 and α-actinin-4 in non-muscle cell (Vallenius et al., 2000). Activation of α-actinin lead to rapid organization of actin into microspikes at the cell-cell junctions and resulted in increased cell separation. α-actinin-4 has been found to be associated with cell motility, endocytosis and cancer invasion (Honda et al., 1998). Studies by Guvakova et al., found that α-actinin was induced by IGF through IGF-1 receptor and PI3K (Guvakova et al., 2002). CLP 36 may play an important role in IGF-1 induced α-actinin activation. Reduction of CLP 36 by DCR observed in this study might block the connection of signal proteins and α-actinin. The other one is gelolin like capping protein (CapG). CapG is a one of the actin-binding proteins. It is crucial for the organization of

the actin cytoskeleton, and involved in cell signaling, receptor-mediated membrane ruffling, phagocytosis, and motility. Studies showed that CapG has oncogenic functions. Overexperssion of CapG was found to promote cancer cell to invade collagen through Ras-PI3K signaling pathway (De Corte et al., 2004). CapG was also found to be regulated by transcription factor AP-1, which response to the activation of a variety of oncogenic signal transduction cascades (Bahassi et al., 2004). Previous findings showed that DCR could inhibit AP-1 transactivation; therefore it is not surprising that we found CapG protein expression decreased significantly by DCR.

In this study, we found that the aldose reductase protein significantly decreased in DCR group compared to control. Aldose reductase is the enzyme that converts glucose to sorbitol through an NADPH dependent reaction. In addition to sugar metabolism, aldose reductase was also found to be involved in redox homeostasis and cell signaling (Del Corso et al., 2008). Over expression of aldose reductase have been found in many cancer cells. Studies showed TPA can induce aldose reductase gene expression and its protein activity. Inhibition of aldose reductase was found to reduce transactivation of transcription factor AP-1 and NFk-B, therefore reducing cell proliferation (Kang et al., 2005 and Pladzyk et al., 2006). The reduction of aldose reductase by DCR may help to explain its inhibitory effect on AP-1 transactivation and cell proliferation.

We also found peroxiredoxin 6 was elevated by DCR compared to control. Peroxiredoxin is an antioxidant enzyme that reduces and detoxifies hydrogen peroxide, peroxynitrite and a wide range of organic hydroperoxides (ROOH). In mammalian cells, the modulation of cytokine-induced hydrogen peroxide levels by peroxiredoxin may also mediate signaling pathways related to cell proliferation, differentiation and apoptosis (Wood et al., 2003). Peroxiredoxin 6 is a 1-cys enzyme which can reduce H_2O_2 and organic hydroperoxides by utilizing GSH as the reluctant

(Manevich et al., 2004). The elevation of peroxiredoxin 6 by DCR observed in this study may reduce the oxidative stress and prevent DNA damage, leading to an inhibition of carcinogenesis.

The effect of DCR on carbonic anhydrases is quite complex as revealed by proteomcis study. Carbonic anhydrases catalyze the reaction of water with carbon dioxide to generate carbonic acid. Comparing to other carbonic anhyrase isomers, carbonic anhyrase 3 is in the cytosol and has a relatively low carbon dioxide hydratase activity. Carbonic anhydrases 3 may also function as an oxyradical scavenger and protect cells from oxidative damage (Räisänen et al., 1999). Several carbonic anhydrases 3 spots were identified by the proteomics in the mice skin. The different spots maybe different protein isomers or proteins at different phosphorylate stage. Further studies including western blot and enzyme activity assay are warranted for understanding of the role of carbonic anhydrase 3 in caloric restricted medicated cancer prevention.

Furthermore, a stress protein, α-crystallin related heat shock protein, was found to be over-expressedin DCR-fed mice in this study, suggesting up-regulation of certain stress proteins that might protect cells against oxidative stress (Holliday, 2006).

The proteins identified so far, as listed in tables 4-1 and 4-2, are high abundance proteins. Using the corrent approach, we are unable to detect integral membrane proteins and tranmembrane signal proteins, which are important in mediating TPA induced skin carcinognesis process and we are more interested in. One possible of the possible reasons is the protein expression levels may be much lower than detection limit. Another possible reason is that the membrane associated proteins are more hydrophobic, and subsequently hardly to be extracted. Using membrane protein specific extraction protocol and/or affinity-based purification prior to Cydye staining and 2D gel running may improve the protein concentration in the sample and

therefore amply the protein spots for identification and quantification comparision.Overall, the current proteomics approach seems need to be modified in order to better study of signal protein expression profile in mice skin.

Overall, we investigated the impact of weight control via exercise and/or calorie restriction on the gene and protein expression profile by using the high throughput approaches, i.g., microarray and proteomics, respectively. For gene expression, we identified 152, 223 and 784 probe sets that were significantly changed by AL+Exe, PF+Exe, and DCR, respectively. For protein expression, we identified 86 proteins and found at least 20 proteins were significantly changed in DCR-fed mice when compared with the controls. Overview of all the gene and protein expression profiles and evaluation of individual change by weight control may provide us a global view of the effects of weight control, and help us to further understand the intrinsic mechanisms behind. Proteomics studies with focus on membrance associated membering protein are warranted.

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Figures and Tables

Table 4-1 Proteins spots that are significantly changed by DCR

Access No	P-value for ttest	Average ratio	protein spot name	effect of DCR
gi|78099787	0.0018	-1.89	PDZ and LIM domain protein 1	↓
gi|2829841	0.025	-1.62	myosin; light chain; phosphorylatable	↓
gi|91115	0.022	-1.57	myosin A2 catalytic light chain	↓
gi|6679651	0.0056	-1.49	Enolase 3 beta muscle	↓
gi|6679651	0.047	-1.41	Enolase 3; beta muscle	↓
gi|110227377	0.021	-1.36	gelsolin-like capping protein	↓
gi|10717134	0.012	-1.33	carbonic anhydrase 3	↓
gi|148677501	0.016	-1.32	ATP synthase; H+ transporting; mitochondrial F1 complex	↓
gi|6671746	0.028	-1.31	enolase 3; beta muscle	↓
gi|1351911	0.0023	-1.3	Aldose reductase	↓
gi|6680748	0.045	-1.3	ATP synthase; H+ transporting; mitochondria	↓
gi|20071838	0.012	-1.28	Ugp2 protein	↓
gi|70778976	0.017	-1.28	phosphoglycerate kinase	↓
gi|18079339	0.031	-1.21	aconitase 2	↓
gi|13959400	0.0088	-1.19	adenylate kinase isoenzyme 1	↓
gi|13384778	0.02	1.42	6-phophogluconolactonase	↑
gi|1864018	0.048	1.43	trisephosphate isomerase	↑
gi|12643495	0.049	1.48	Kininogen 1 precusor	↑
gi|26986064	0.015	1.55	albumin	↑
gi|8393866	0.0031	1.56	ornithine aminotransferase	↑
gi|10717134	0.017	1.57	carbonic anhydrase 3	↑
gi|15126646	0.025	1.58	apolipoprotein A-I	↑
gi|59958370	0.0089	1.65	heat shock protein; alpha-crystallin related	↑
gi|10717134	0.048	1.67	carbonic anhydrase 3	↑
gi|85541765	0.033	1.76	Flavin reductase (NADPH-dependent diaphaphatase)	↑
gi|6671549	0.0068	1.78	peroxiredoxin 6	↑
gi|26986064	0.02	1.93	albumin	↑

Table 4-2 Proteins spots that were identified but not significantly change by DCR

Access No	P-value for ttest	Average ratio	protein spot name
gi|21619364	0.051	1.22	Fibrinogen; B beta polypeptide
gi|109571	0.053	1.48	apoliprotein A-I precursor-mouse
gi|6671762	0.056	-1.34	creatine kinase; muscle
gi|6671762	0.068	1.46	Creatine Kinase
gi|56541074	0.078	1.54	Rho GDP dissociation inhibitor
gi|17046471	0.09	1.45	transferrin
gi|21410418	0.091	-1.43	Aldh6a1 protein
gi|146345383	0.092	1.23	carbonica anhyrase 2
gi|28436836	0.1	-1.14	trisephosphate isomerase 1
gi|18079339	0.11	-1.15	aconitase 2
gi|6671762	0.12	-1.23	creatine kinase; muscle
gi|51092293	0.13	-1.43	keratin 77
gi|6678413	0.13	1.11	triosephophate isomerase
gi|7305599	0.13	1.41	Transthyretin
gi|50156	0.14	1.15	beta-1-globin
gi|31543120	0.14	1.16	lectin; galactose binding
gi|18606172	0.15	1.29	transferrin
gi|3219774	0.17	1.28	peroxiredoxin 6
gi|12643495	0.17	1.6	kininogen-1 precursor
gi|7441489	0.19	-1.3	parvalbumin
gi|4760590	0.19	1.3	beta-1-globin
gi|13879226	0.21	-1.2	Rbm3 protein
gi|1304157	0.21	-1.18	78 kDa gluce-regulated protein precursor (GRP 78)
gi|10717134	0.22	-1.12	carbonic anhydrase 3
gi|20330802	0.23	1.62	transferrin
gi|49864	0.24	1.33	alpha-actin
gi|94395852	0.25	-1.11	similar to G3PDH
gi|26986064	0.25	1.46	albumin
gi|18152793	0.28	1.11	pyruvate dehyrdogenase (lipoamide beta)
gi|6671762	0.31	-1.13	creatine kinase; muscle
gi|17390379	0.31	-1.08	Sod2 protein
gi|33585570	0.32	-1.28	Myosin light chain; phosphorylatable
gi|148692928	0.33	-1.06	glutamate dehydrogenase 1
gi|31982861	0.34	1.55	carbonic anhydrase 3
gi|31982861	0.4	-1.06	carbonic anhydrase 3
gi|61402210	0.42	1.05	Apoa1 protein
gi|10943870	0.45	-1.05	aldo-keto reducatase family 1
gi|38020	0.45	1.08	sketetal muscle alpha-actin precursors
gi|33708	0.45	1.45	Monoglyceride lipase
gi|6671746	0.46	-1.19	cofilin 2; muscle

gi|94408011	0.48	-1.12	Predicted: similar to valosin isoform 1
gi|6671762	0.48	-1.09	creatine kinase; muscle
gi|31982856	0.48	-1.06	dihydrolipoamide dehydrogenase
gi|29789016	0.49	-1.03	myosin; light polypeptide 1
gi|54114937	0.49	1.07	Eno1 protein
gi|21359820	0.5	-1.28	myoglobin
gi|7106546	0.51	-1.19	14-3-3 protein sigma
gi|26986064	0.51	-1.09	albumin
gi|123242975	0.54	1.16	troponin I
gi|149266431	0.55	-1.18	similar to G3PDH
gi|54673814	0.64	-1.04	Enolase 1; alpha non-neuron
gi|6679078	0.67	-1.04	nucleoside-diphosphate kinase 2
gi|547679	0.68	1.03	heat shock protein beta-1
gi|31981826	0.69	1.03	electron transferrin flavoprotein; alpha polypeptide
gi|15126788	0.73	-1.03	Ferrtin heavy chain 1
gi|6753530	0.74	-1.11	cystallin; alpha B
gi|7949037	0.75	-1.04	Enoyl Coenzyme A hydrastase
gi|557414460	0.75	-1.01	DJ-1 protein
gi|31982861	0.76	-1.02	crabonic anydrase 3
gi|40254525	0.79	1.04	tropomyosin 3; gamma
gi|11875203	0.81	-1.02	tropomyosin beta
gi|29789016	0.86	1.03	myosin; light polypeptide 1
gi|31560030	0.89	1.02	tropomyosin alpha
gi|198845	0.9	1	lipocortin I
gi|226471	0.9	1.05	Cu/Zn superoxidae dismutase
gi|13542867	0.95	-1	nucleoside-diphosphate kinase 1
gi|1794160	0.96	-1	lamin C [Mus musculus domestics]
gi|21312444	0.97	1.01	glyoxalase domain containing 5
gi|6754450	0.97	1.03	fatty acid binding protein 5; epidermal
gi|6679439	0.98	1.01	peptidylprolyl isomerase A
gi|6678632	0.98	1.07	lectin; galactose binding

Figure 4-1 Scatter plot of gene expression in TPA treated skin tissues (A) AL+Exe Vs Control, (B) PF+Exe Vs Control and (C) DCR Vs Control

(A) **(B)** **(C)**

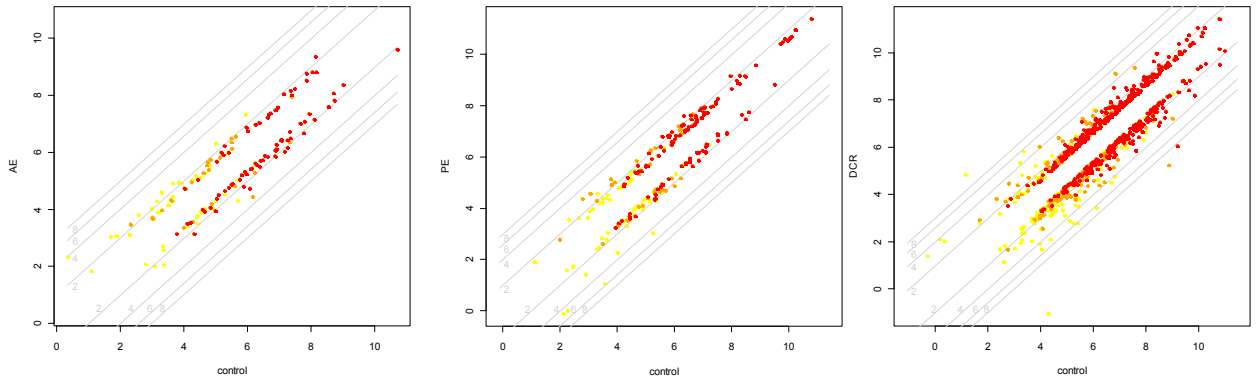
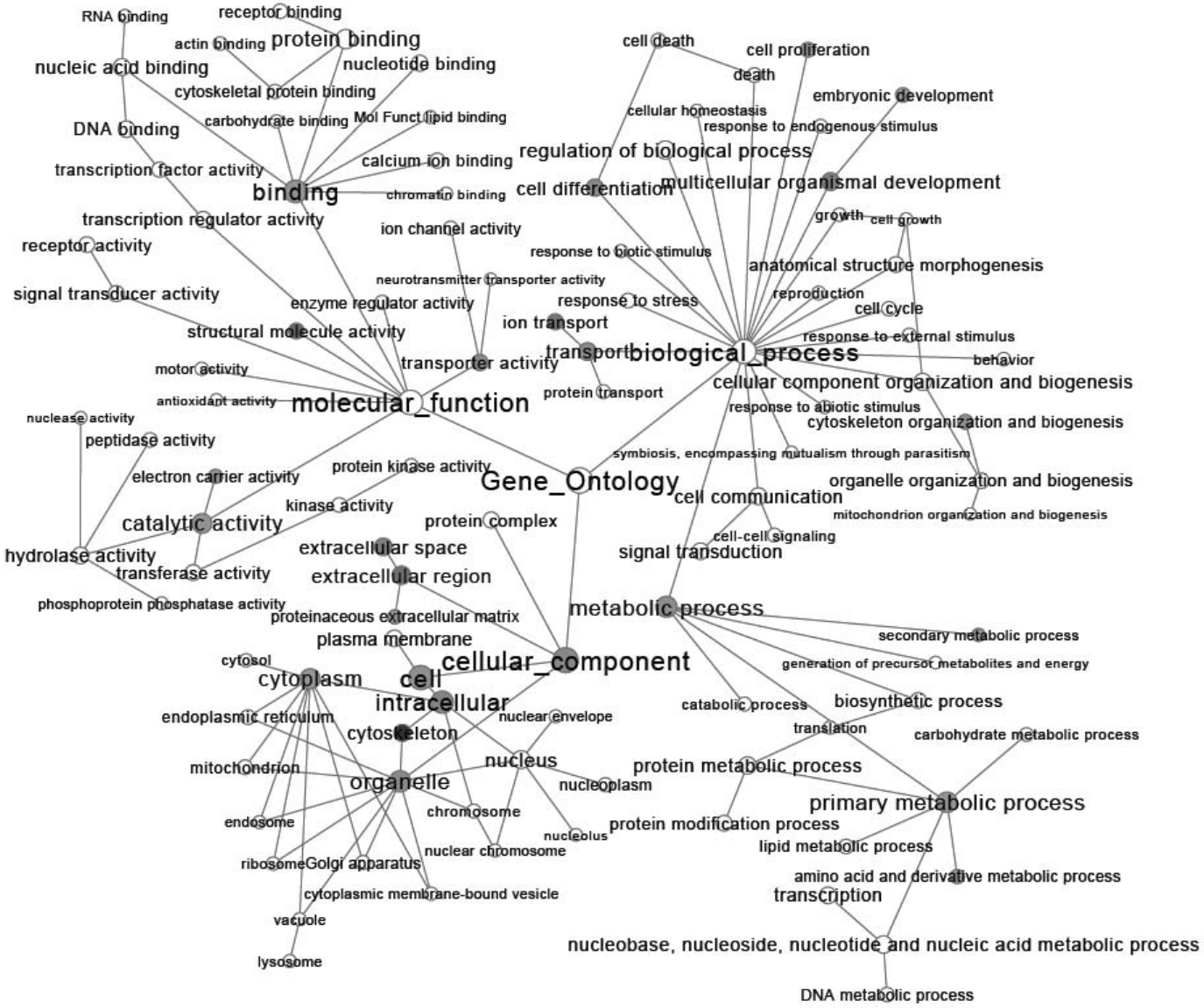
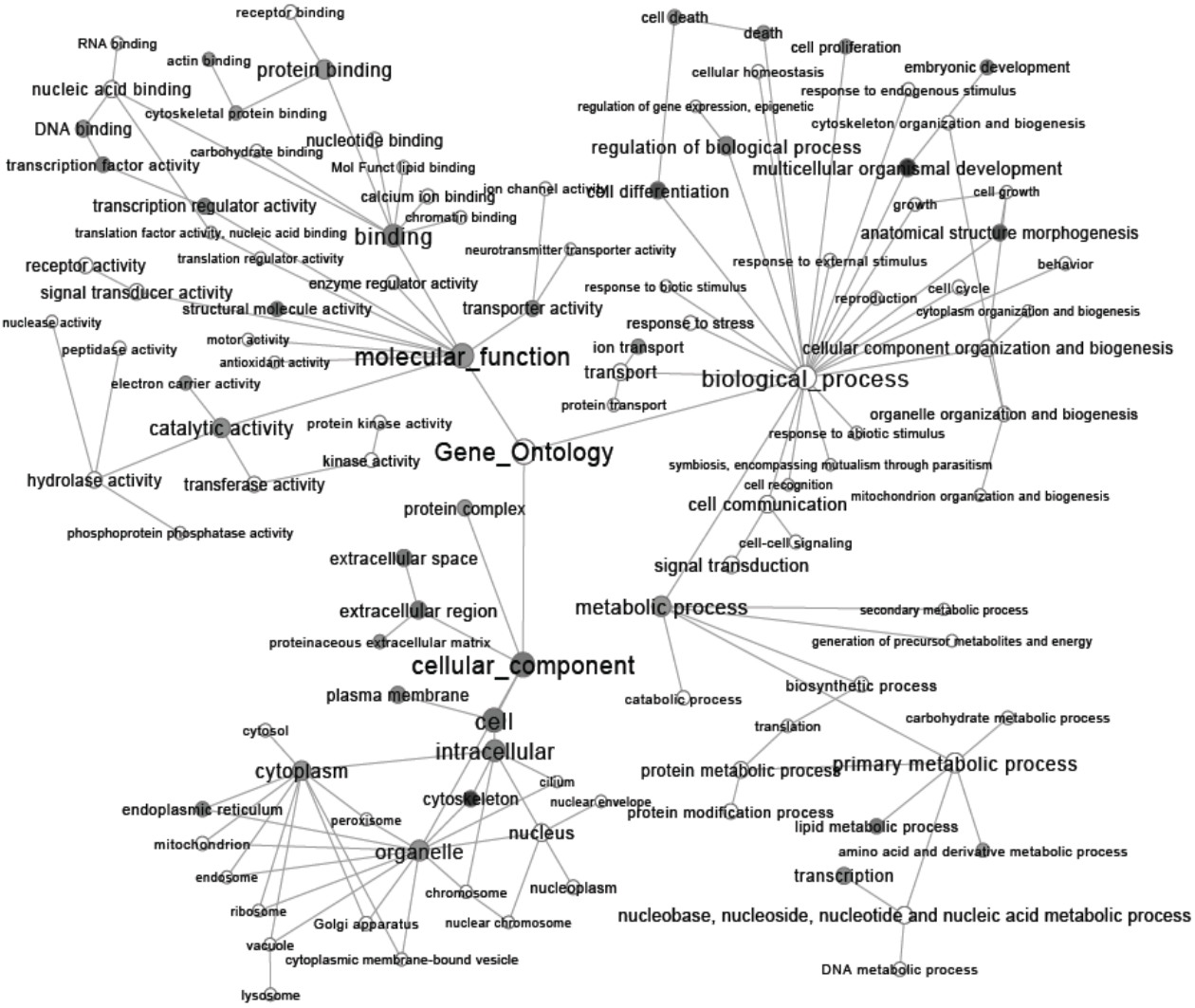


Figure 4-2 Gene ontology study in TPA treated skin tissues of AL+Exe (A), PF+Exe (B) and DCR (C) mice comparing to control mice.

(A) AL+Exe vs control



(B) PF+Exe vs control



(C) DCR vs Control

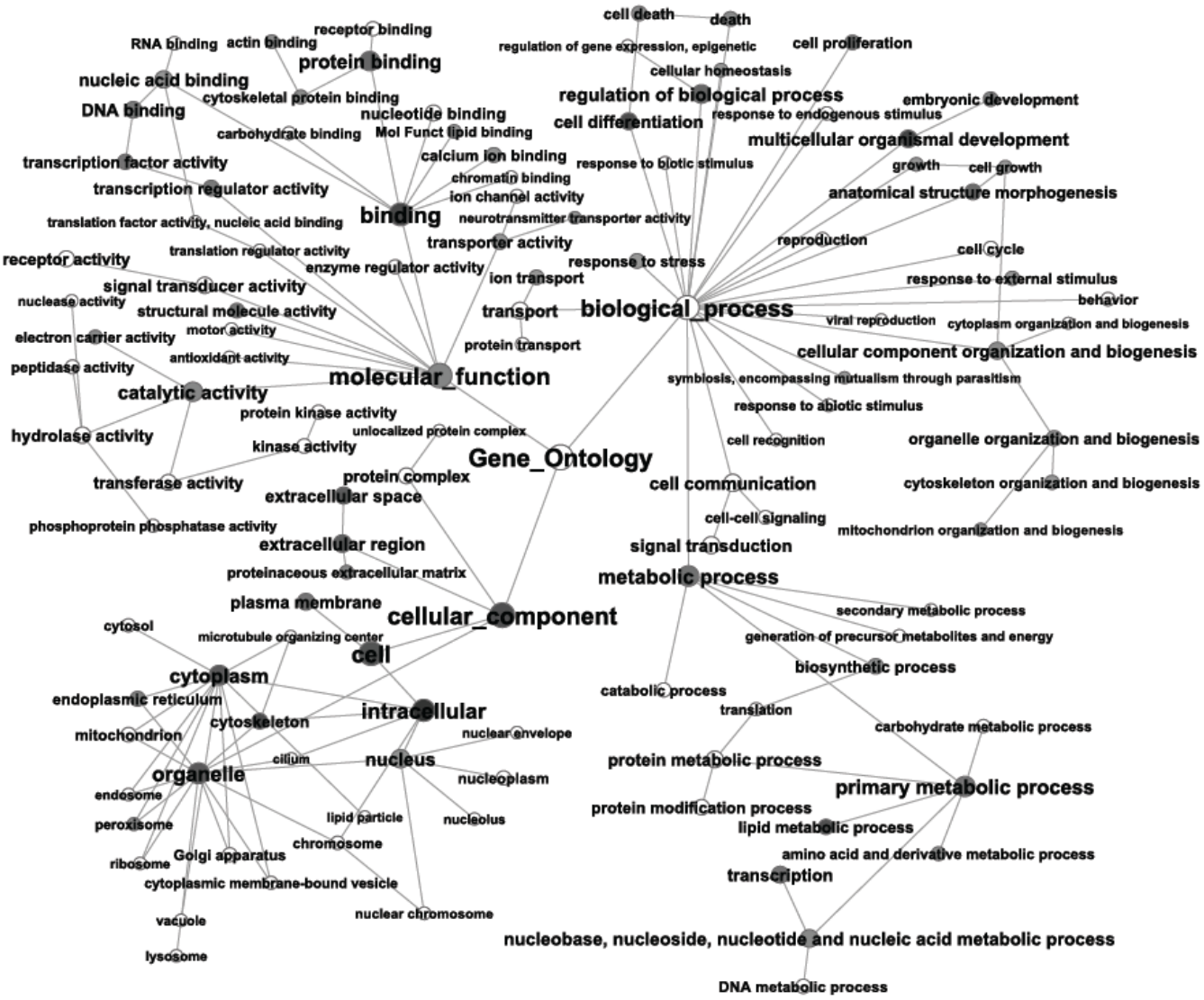
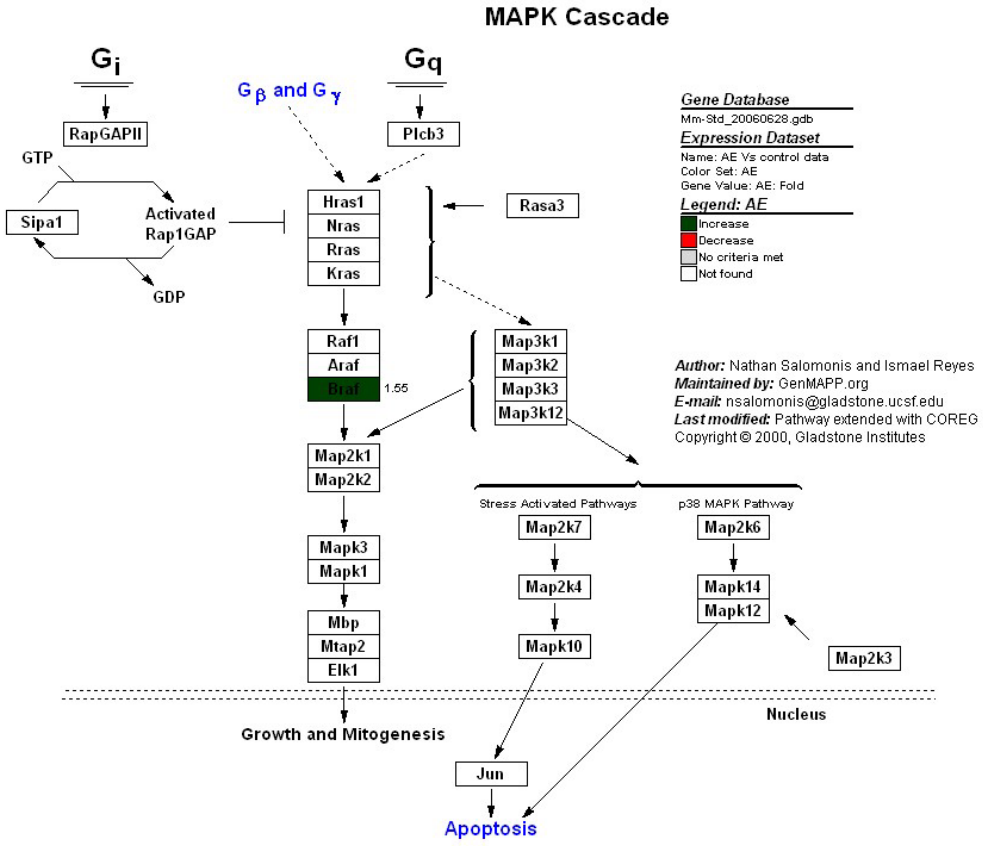
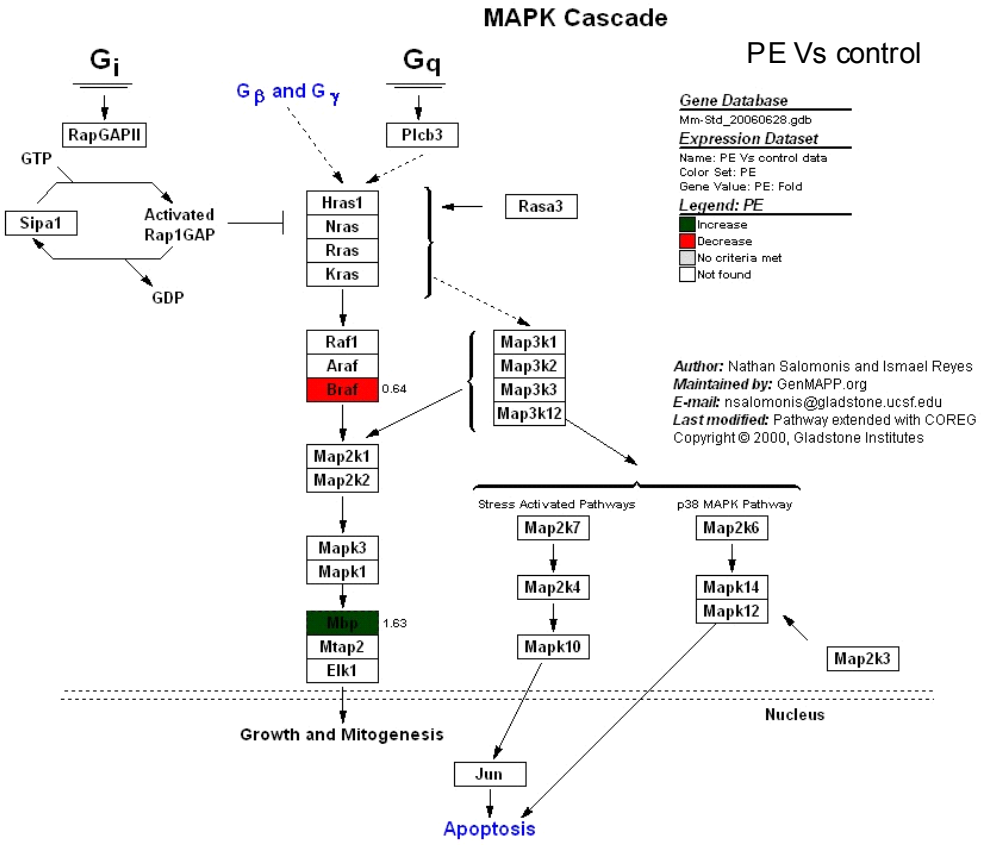


Figure 4-3 Pathway analysis by GenMapp in TPA treated skin tissues of control and DCR, PF+Exe and AL+Exe mice

(A) AL+Exe



(B) PF+Exe Vs Control



(C) DCR Vs Control

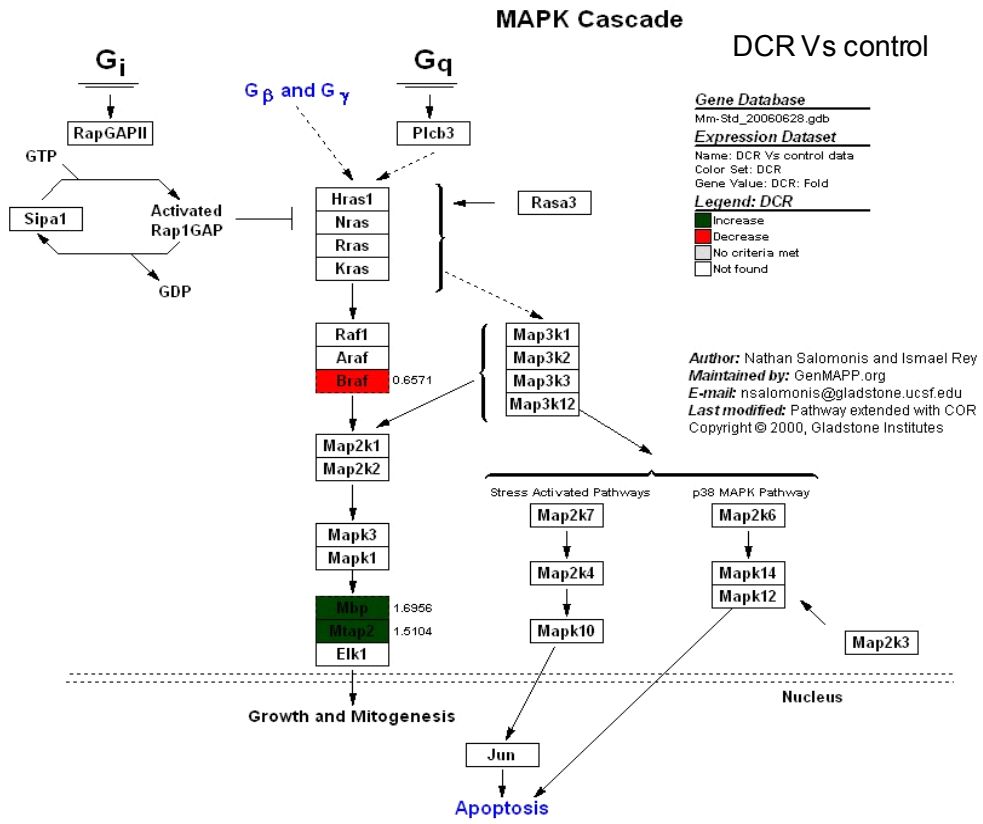
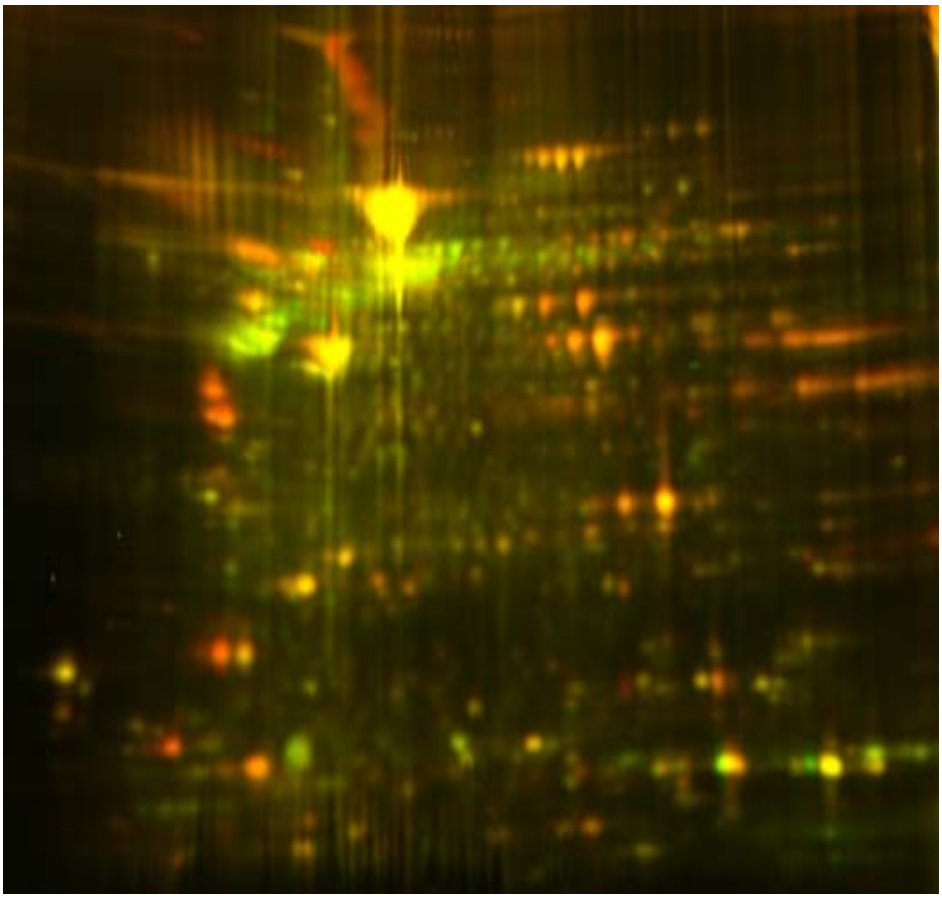


Figure 4-4 Representative overlaid 2-D image of CyDye labeled 25 µg protein lysates of ad libtum feeding mice (cy3) and 25 µg of DCR mice (cy5).



CHAPTER 5 - General Conclusions

During the last twenty years, the number of obese and overweight people increased rapidly (CDC, 2006). Obesity and overweight are positively correlated with increased chronic diseases, including cancer, diabetes, and cardiovascular disease (WCRF/AICR, 2007).Weight control, therefore, has become an important strategy against cancer and/or other chronic diseases. Body weight control is carried out by the balance of energy intake and expenditure. Negative energy balance in adult, which can be achieved by decreased calorie intake or increased expenditure, may help maintain body weight and thus benefit health status. Weight control via dietary caloric restriction and/or physical activity has been demonstrated in animal models for cancer prevention. However, the underlying mechanisms are not fully understood.

Body weight loss has been found to significantly reduce hormones such as IGF-1 and leptin, increase adiponectin and glucocorticoids. Previous study in our lab showed that Ras-MAPK-proliferation and PI3K-Akt-anti-apoptosis pathway were involved in response to weight change by dietary calorie restriction and/or exercise training. We found that the IGF-1-dependent signaling was reduced in exercised mice with limited caloric intake. We hypothesized that IGF-1 reduction in circulation is required in blocking PI3K-Akt signaling by weight control. In order to test our hypothsis, we restored IGF-1 in SENCAR mice that were pair fed with the sedentary control and exercised by treadmill at 13.4 m/min for 90 min/day, 5 days/week, for 10 weeks (PF+Exe). IGF-1 was restored by i.p. injection at 10 μg/kg body weight twice per week in the last two weeks. The body weight and food consumption was recorded weekly, IGF-1 receptor and PI3K protein expression was measured by western blot analysis, and the phospholipid profile was conducted by using ESI-MS-MS in lipidomics center in Kansas State Unvierwsity.

Compared to the controls, body weights and plasma IGF-1 levels were significantly reduced in PF+Exe mice. However, the circulation level of IGF-1 was not significantly changed

by IGF-1 restoration. A followed pharmacokinetic study showed that plasma IGF-1 may reach to peack within one hour and almost back to basal level at 24 hours after a single dose injection. In the IGF-1 restoration study, we collected plasma sample about 26 hours after the last injection, therefore it is no surprising that we did not observe plasma IGF-1 increasing. Although the degradation rate of IGF-1 in plasma after injection seems to be very fast, acute elevation of plasma IGF-1 after mutilple injections still partially reversed the reduced PI3K protein expression and phosphatidylinositol 38:4 level.in PF+Exe mice. IGF-1 injection also significantly enhanced some lysophosphatidylcholine species such as 20:3, 20:4, and 20:5 in PF+Exe group. It is interesting that IGF-1 restoration had no significant effect on IGF-1 receptor protein expression, and also on ether phospholipid profile. Overall, the results indicated that the PI3K pathway and the phospholipids that are related to signaling pathway, cell proliferation, and/or cell death were selectively reduced by PF+Exe treatment, with IGF-1 restoration apparently able to reverse, at least in part, the impact of weight control by physical exercise and moderately caloric restriction.

Many studies have showed that exercise, especially the negative balance conducted by more expenditure via exercise, is able to reduce plamas triacylglycerols. However, all the previous studies are only focused on total triacylglyerols. In the second, we hypothesized that weight control will change TAG and DAG profile in the plama and TPA-induced mice skin. We tested the hypothesis by devide our animals into four group and they are: ad libitum sedentary control (control), 20% calorie restriction (CR), ad libitum fed treadmill exercise at 13.4 meter/min for 60 minutes per day, five day a week (AL+Exe) and exercise but pair-fed at the amount as the sedentary control (PF+Exe). DAG and TAG profile was analyzed by using ESI-MS-MS in lipidomics center.

Comparing to control, AL+Exe was found to have no effects on body weight, and both DAG and TAGs. The treadmill exercise, 13.4 meters /min for 60 minutes a day, is considered moderate intensity. If the mice are fed ad libitum, their calorie intake might be slightly increased. In this situation, exercise without consideration of dietary intake maybe can not produce sufficient negative energy balance for significant body weight loss as well as TAG and DAG species. When the food consumption of the exercised mice was adjusted to that of sedentary control, the body weight, plasma TAG subtales, total DAG in the plasma was significantly decreased. The data suggested that to maintain body weight through exercise, dietary intake should be considered as an important factor. Both PF+Exe and DCR changed the DAG profile skin tissue, where DAG may be important in TPA induced signaling induction. The association between lipids, DAG and signal protein, PKC are quite complicated. Evidence showed that individual DAG species may have different binding activity with regard to different PKC isomers (cPKC or nPKC). Our results showed some of the DAGs such as DAGs 16:0/20:2, 18:0/20:3, 18:0/20:4, 18:1/20:2, 18:1/20:4, or 16:0/16:0 were significantly increased by weight control (PF+Exe and DCR) and some DAGs such as 16:0/18:2, 16:1/18:2, 18:1/18:2, 18:2/18:2 were significantly decreased in both PF+Exe and DCR mice. The results indicate a significant impact of weight loss upon TAG and DAG profiles. It suggested that DAG-related signal transduction system, which is important in skin carcinogenesis, are mediated by weight control. Our study provides insight into the mechanism of weight control on cancer prevention.

Although the cancer prective mechanism of weight control is not well known, it is clear that this process is quite complicated including multiple factors and multiple signaling pathways. Hundreds of biological molecules may cooperate in this network complex. It seems traditional molecular biology techniques are not efficient to meet the requirements for a broader and deeper

overview of the mechanisms. Fortunately, recently developed technologies named "-omics" may provide us a chance to take a global view of these biological processes. The transcriptome is a study of the entire range of transcripts produced by a given organism. The development of microarray technology provides a useful tool in the assessment of gene expression profile in a large scale or even the whole genome. The proteome is the final outcome of genome expression compring all the proteins present in a cell at a particular time. Proteomics, the global overview of proteome, is to investigate the expressions, modifications, interactions and regulation of proteins in organisms comprehensively. We identified genes that are significantly changed by DCR, PF+Exe, and AL+Exe, respectively. PF+Exe and DCR showed similar impact on signaling pathways-related gene expression as analyzed by GenMAPP. Braf and Akt1 appeared to be the key modulator in MAPK pathway and insulin signaling pathway respectively in response to weight control via DCR or PF+Exe. Using proteomcis approach, we identified total 86 proteins. There are about 20 proteins were significantly changed by DCR. Although this is our initial step and we could not clarify all the relationship between significant impact to cancer related mechanisms in the scope of this study, overview of all the gene and protein alteration profiles as well as lipid profile may provide intrinsic mechanisms of cancer prevention by weight control in the future studies.

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