EFFECT OF EXERCISE-INDUCED WEIGHT CONTROL ON PHOSPHOLIPID PROFILE AND GENE EXPRESSION

by

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B.S., Beijing Agricultural University, P. R. China, 1991

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

Body weight control via increasing exercise and/or decreasing calorie intake has been linked to a reduced cancer risk in animal models. However, the underlying mechanisms are not clear. This study assessed the impact of exercise with or without limited dietary calorie intake on the overview profiling of phospholipids and gene expression in the skin tissues of weight controlled-mice.

Mice were randomly assigned to three groups: *ad libitum*-fed sedentary control, *ad libitum*-fed treadmill exercise at 13.4 m/min for 60 min/d, 5 d/wk (Ex+AL), and exercise but pair-fed with the sedentary control (Ex+PF). After 10 wks, Ex+PF but not Ex+AL mice demonstrated a significant decrease in both body weight and percentage of body fat when compared to the sedentary controls.

Among 338 phospholipids measured in the skin samples by electrospray ionization mass spectrometry, most classes of phosphatidylinositol (PI), phosphatidylcholine-containing lipids with ether linkage (ePC), and some lysophosphatidylcholine (lysoPC) significantly decreased in Ex+PF mice when compared to the controls. Furthermore, some species of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) containing omega-3 18:0-22:6 fatty acyl combinations increased significantly in Ex+PF mice. A total of 25 significantly-changed phospholipids were distinguishable between diet and exercise treatments by discriminant analysis. The reduced PI in Ex+PF mice was observed concomitantly with a significant reduction of PI3K protein expression.

Among the 45,101 probe sets tested in skin tissues, expression of 839 genes was significantly changed by exercise with or without limited dietary calorie intake. The genes with impacted expression were involved in oxidative stress, inflammatory response, lipolysis, protein synthesis, and signaling pathway. Up-regulated expression of genes involved in elongation of long chain fatty acids in Ex+PF but not Ex+AL mice appears to partially account for increased level of 18:0-22:5PC/PE and 18:0-22:6 PC/PE.

Taken together, these data indicate that controlling body weight via exercise with limited dietary calorie intake rather than exercise alone significantly modified phospholipid and gene

expression profiles. The altered profiles may be associated with cancer preventive mechanisms; in particular, reduced PI and PI-related PI3K expression and altered expression of genes involved in protein kinase activity and lipid metabolism may prevent cancer.

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CHAPTER 1 - Review: body weight control, exercise and their influence on cancer prevention

Overweight and obesity

Overweight and obesity are major health problems. In a survey of American adult population in 2004, 66.3% were considered overweight (body mass index or BMI $\geq$ 25.0 kg/m^2), 32.2% were obese (BMI $\geq$ 30.0 kg/m^2), and extremely obese (BMI $\geq$ 40.0 kg/m^2) turned to be 4.8% (1). Overweight and obesity, which may reflect a life style of over-consumption of calorie or less expenditure of energy, has become a major public health problem associated with increased risk of many chronic diseases including cardiovascular disease, diabetes, arthritis, and cancer (2, 3, 4, 5).

Cancer risk

More than one million people have been diagnosed with cancer each year and it was reported that cancer turned to be the second cause of death following heart diseases in the United States in 2003. Based on cancer cases diagnosed during 2000 to 2002, it was estimated that fifty percent of men and one third of women will develop cancer in their lifetime (American Cancer Society. Cancer Facts and Figures 2007. www.cancer.org). The World Health Organization (WHO) reported that non-communicable diseases such as cancer and obesity would become the principal global causes of mobility and mortality.

In addition to the genetic aspect, there are lots of factors that are considered to cause cancer such as a) cigarette smoking, b) constant UV exposure, c) alcohol consumption, d) residues of pesticides, e) asbestos fibers, f) nitrites in preserved food, g) medical X-rays for diagnosis, h) cosmic rays, and i) mistakes occurring in DNA replication and repair (7). In addition, overnutrition has also been considered as a factor in carcinogenesis for more than 80 years (8).

Overnutrition and cancer risk

At the beginning, the role of overnutrition in human tumor formation was the major subject of concern, and experimental studies explored the relationship of cancer risk and a high fat diet. As early as 1913, Hoffman suggested that inappropriate diet may be a factor in the genesis of cancer (9) and later he indicated that overnutrition was the principal factor in

carcinogenesis (10). Watson and Mellanby first reported a role for dietary fat in carcinogenesis in 1930. They found that skin tumors increased from 34% to 57% in coal tar-treated mice by adding 12.5% or 25% butter to the basal diet (3% fat) (11). In 1970, Carroll and Khor reported that mammary tumors in rats were almost doubled by increased fat in the diet from 0.5 to 20%, and the latent period was also reduced. They found that tumor incidence, multiplicity and latency were not changed even when the lower level of dietary corn oil increased about ten times from 0.5 to 5.0%, while when the dietary corn oil was doubled 5 to 10%, the tumor incidence increased 22%, tumor multiplicity increased 74%, and latent period decreased 12%. However, another doubling of corn oil to 20% showed no changes in these tumor parameters. Thus they concluded that there must be a specific calorie level that affects the carcinogenic process (12). Ip and colleagues reported that linoleic acid was a cofactor in the growth of tumors, and both saturated and unsaturated fatty acids became co-carcinogenic when they reached 4% of the intake calories (13). Carroll and Khor showed a positive correlation between total dietary fat intake and death rate from breast cancer (14).

Later the relationship between excess body weight instead of fat intake and cancer mortality was examined in cohort studies over a million people (15, 16). In 1992, a survey of adult body weight and cancer risk was reported by Le Marchand. Colon cancer showed a positive relation with BMI in nine studies out of 12, while six of ten showed a positive correlation between prostate cancer and body weight or BMI (17). Normally, higher body weight major caused by higher calorie intake. In six studies related to breast cancer, four of them showed positive association with energy intake, one inverse and one showed no relation (18). Several studies found energy intake were positively associated with colon cancer (19, 20, 21, 22).

Calorie restriction and cancer risk

The studies related to body weight control included decreasing calorie intake and increasing energy expenditure via exercise. The very early research about decreasing calorie intake, animals were actually underfed instead of calorie restricted. In this situation, the amount of micronutrients may be less than optimum and decreased tumor formation, may be due to the nutrient deprivation rather than to calorie restriction (8).

In 1909, the inhibition of transplanted sarcoma growth in underfed mice was reported by Moreschi (23). Both spontaneous and transplanted tumors were inhibited in underfed mice (24). Furthermore, it was found that spontaneous tumor occurrence was reduced from 88% to 16%, and the life span was enhanced by 35% (25).

In the 1940s, the effects of fat and calories were separated in studies. Lavik and Baumann investigated the skin tumor induced by methylcholanthrene (MCA) in mice, and found that total calorie intake was the major cause of the tumor occurrence instead of fat consumption. Tumor incidence was 66% when both fat and calories were high, it dropped to 54% when diet was high in calorie but low in fat, while the tumor incidence further dropped to 28% when a diet with high fat and low energy (26). Another report which studied the effect of calorie restriction on UV induced tumors in mice also found similar results. Tumor incidence dropped to 7% in low calorie and low fat diet from 87% with high energy and low fat diet (27). These results indicated that energy restriction may affect basic metabolic pathway(s) thus decreasing tumor formation, rather than the effect being on carcinogen metabolism related to calories or fat.

In order to determine the degree of calorie restriction to inhibit mammary tumorigenesis, rats treated with DMBA were energy restricted by 10, 20, 30 and 40%. In 10% calorie restricted rats, tumor multiplicity and tumor burden were reduced by 32% and 47%, respectively, although the tumor incidence was almost the same as the *ad libitum* control. In 20% calorie restricted rats, tumor incidence was reduced slightly, and it was restricted more significantly by 30 and 40% restriction. Tumor multiplicity and tumor burden were reduced dramatically in these more calorie restricted rats. The results also suggested that body weight, body fat, and fasting serum insulin levels were associated with mammary tumor promotion, as well as suggesting that insulin might be a growth factor for DMBA-induced tumors (28). Messenger RNA expression of c-*fos* and c-ki-*ras* was also found to be inhibited in energy restricted mice (29). Expression of 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced AP-1-DNA binding, c-Jun protein and c-*jun* mRNA were significantly decreased by 40% dietary calorie restriction (DCR) (30). A study conducted on *Apc*^{min} mice found that 40% DCR significantly reduced intestinal polyps by 57%, as well as serum insulin-like growth factor I and leptin (31). Another study on the effects of energy balance and cancer using genetic altered mice found that 20 to 40% calorie restriction decreased serum IGF-1 and leptin levels. Furthermore, the tumor burden was decreased and

tumor latency was enhanced (32). These results further illustrated the role of tumorigenesis prevention by DCR.

The mechanisms of the inhibition of carcinogenesis by energy restriction are unclear. It may be due to 1) reduced oxidative DNA damage (33), 2) increase DNA repair (34), 3) increase the anti-oxidant enzymes activity (35), and 4) decreased level of circulating hormones (36).

Physical activity and cancer risk

Besides calorie restriction, increasing physical activity through exercise is another principal method to control body weight and reduce cancer risk. Recently, WHO emphasized, "Physical inactivity…is the result of a progressive shift of lifestyle toward a more sedentary pattern, in developing countries as much as in industrialized ones." Over the past 20 years, abundant evidence from epidemiological, prospective cohort, and intervention studies has shown that physical activity, diet, as well as combined activity and diet interventions could mitigate the progression of chronic diseases, and even reverse existing diseases (37). Data indicated that 400,000 deaths in 2000 were caused by physical inactivity and poor diet with these factors ranking second after tobacco as causes of death. Indeed, it is likely that physical inactivity and inappropriate diet consumption will soon become the leading cause of death in the United States (38).

The benefits of physical activity have been found to be better quality of life through either improving bodily function via increasing sleep quality and sexual function, or preventing chronic diseases including cardiovascular disease, obesity, diabetes, arthritis, and cancer (4, 5). Although the results were controversial in terms of the relationship between physical activity and cancer risk due to the type, frequency, intensity, and duration of exercise, there is accumulated evidence indicating that exercise gives benefits in reducing cancer risk. Evidence from some cohort and case-control studies showed that physical activity reduced cancer risk with dose dependency (39). Physical activity has been found to affect cancer risk at different body sites. Increased death rates for all cancer patients with cancer at multiple sites were also found to be associated with increased body weight (40). The most studies were focused on colon or rectum, breast, prostate, lung, liver, kidney, gallbladder, esophagus, endometrium, tests, and skin cancers.

1). Colon cancer

Colon or rectum cancer is the second leading cause of cancer death in men and the third in women, and is estimated to be the third common cancer for both sexes in the United States for 2006 (American Cancer Society. Cancer Facts and Figures 2007. www.cancer.org). Cancer occurring in the large bowel was investigated most commonly related to physical activity. Friedenreich and Orenstein stated that physical activity reduced colon or colorectal cancer risk by about 50% by combing data from 43 studies out of total of 51. In 29 studies which considered dose response, 25 showed a negative association between physical activity and cancer risk (41). Several potential mechanisms have been discussed. First, physical activity can reduce the exposure of carcinogens in the bowel by increasing the movement of food through the gastrointestinal tract (American Cancer Society. Physical Activity and Cancer www.cancer.org). Second, the growth and proliferation of colonic epithelial cells may be affected by the alteration of insulin level via exercise (42, 43). Third, physical activity may affect the level and metabolism of bile acid (38, 43).

2). Breast cancer

Breast cancer is the second leading cause of cancer death and the number one common cancer in women of the United States (American Cancer Society. Cancer Facts and Figures 2007. www.cancer.org). According to Friedenreich and Orenstein, among 44 studies of the effect of physical activity in women breast cancer, 32 of them were found reduced to an average to 30 to 40%, and most of them also were found dose dependent (41). In a case-control study, Verloop and co-workers assessed the relation of breast cancer risk and lifetime physical activity among 918 case subjects and 918 age-matched subjects. They found women who did recreational exercise and who had a lower BMI (less than 21.8 kg/m^2) had a lower cancer risk than those with a higher BMI (more than 24.5 kg/m^2). The odds ratio was 0.57. Furthermore, there was no difference between women starting recreational activity earlier in their life (before 20 years old) and who began exercise recently (last 5 years) (44). In a cohort study with 90,509 women included people who were at high risk by being overweight, having a family history of breast cancer, being nulliparous, and women on hormone replacement therapy, experienced a linear decrease in breast cancer risk when they increased their recreational activities (45). Physical

activity may alter the excretion and metabolism of sex hormones. Higher steroid hormones (46) and lower sex hormone binding globulin (SHBG) were found in sedentary, postmenopausal women (47). Both insulin and IGF-1 have been purported to be risk factors for breast cancer in sedentary women (48, 49). Reports also showed cross talks exist between estrogen receptor and IGF-1 pathway in breast cancer (50, 51).

3). Prostate cancer

Prostate cancer is the most common cancer and the third leading cause of cancer death in men of the United States (American Cancer Society. Cancer Facts and Figures 2007. www.cancer.org). Thune and Furberg reported that among 28 studies, 14 found that prostate cancer risk was reduced to 10 to 70% through increasing physical activity, and a dose response was identified in 10 out of 19 (39). Similar to breast cancer, prostate cancer reduction through exercise may also be due to the alteration of sex hormones. Fasting insulin and IGF-1 were found to be decreased, while IGF-1 binding protein was increased through physical activity (52). The changes in serum insulin may affect the IGF-1 level, and IGF-1 regulates cell growth and suppresses apoptosis. Furthermore, IGF-1 suppresses p53 function which is involved in cell cycle arrest or apoptosis, as well as DNA repair (53).

4). Lung cancer

Lung cancer is the number one leading cause of cancer death and second common cancer in both sexes in the United States (American Cancer Society. Cancer Facts and Figures 2007. www.cancer.org). Most research about lung cancer risk focuses on cigarette smoking, and there are few studies investigating physical activity and lung cancer prevention. The available data show that lower risk of lung cancer in exercised individuals, and there is also dose response relationship (54, 55). It has been suggested that the concentration of carcinogenes in airways are reduced through the improved pulmonary ventilation and perfusion via exercise (54). IGF-1 was also found to be a risk factor in lung cancer development (51).

5). Skin cancer

It is estimated that more than 1 million new cases of skin cancer will occur in the States in 2007, and which represents over half of all new cancer cases. Among this, 80% are basal cell

carcinoma, 16% are squamous cell carcinoma, and only 4% are melanoma. However, melanoma causes more than 75% of the skin cancer deaths. Form 1950 to 2001, the incidence of melanoma increased almost seven times (American Academy of Dermatology. 2007 Skin Cancer Fact Sheet. www.aad.org). The relationship between physical activity and skin cancer was poorly studied, and some controversial results obtained maybe due to the confounding effect of sun exposure during outdoor exercise. Development of skin cancer in adults even could relate to overexposure to sun and sunburns at an early age. According to Schnohr and colleagues, by investigating 28,000 men and women aged 20 to 93 years old for 14 years, vigorous physical activity was found to significantly increase non-melanoma skin cancer in men with the odds ratio of 1.72 ($p = 0.001$) but not in women. The different results obtained in men and women may due to more outdoor exercise for men than women, and men tend to wear less clothing and probably do not protect themselves as carefully with sunscreen as women do (56). The first report about the relationship between physical activity and melanoma was a case control study conducted in Washington State. It investigated 386 melanoma patients diagnosed in 1997 and 727 controls selected by random-digit dialing. After collecting information by questionnaire and analyzing it by logistic regression adjusted for age, color of hair, vegetable and fruit consumption, and lifetime sun exposure, they found that melanoma occurs more frequently in men who were taller, have heavier body weight, and had a higher body surface area. Furthermore, they found melanoma risk decreased (odds ratio = 0.7) in people who exercised five to seven days a week (57). Animal studies give more accurate results since which are conducted in a well controlled environment. A well known study was conducted by Andrianopoulos and co-workers (58). After intraperitoneally injecting the carcinogen, dimethylhydrazine (DMH) in Sprague Dawley rats for 6 weeks, the incidence of colon cancer was significantly decreased ($p < 0.05$) in wheel running mice and the total number of tumor were much lower ($p = 0.06$) in physically active mice compared to sedentary controls. In 2006, Michna and co-workers reported a study investigating the relationship between exercise and UVB-induced skin cancer in SKH-1 mice. They found that exercise by running on wheels reduced tumor incidence, the number of tumors per mouse, and tumor volume in each mouse. Nonmalignant tumors and total tumors in exercised mice were decreased by 34% and 32%, respectively. Furthermore, the formation of squamous cell carcinomas was decreased by 27% in exercised mice. They also found that in exercised mice parametrial fat pad weight and dermal fat layer thickness were decreased, but dermal muscle

layer thickness was increased compared to the sedentary control. They supposed that running wheels increased epidermis apoptosis and skin tumor apoptosis (59). A study on SANCAR mice in Dr. Wang's lab found that exercise on treadmill combined with moderate calorie restriction could decrease PI3K expression in epidermal cells (60). Recently, a review paper concluded that independent of weight loss, physical activity has been suggested to have a specific effect on cancer prevention (61)

The underlying mechanisms of the possible health benefits of exercise to cancer prevention are not well understood. However, a variety of biological mechanisms have been postulated response to it: 1) lifestyle alteration may change the oxidant/antioxidant status (62); 2) increased physical activity may reduce sex hormone levels, such as estrogen, testosterone, and dihydrotestosterone (63); 3) physical activity might decrease metabolic hormone levels, such as insulin (64), IGF-1 (65), and leptin (66); 4) increased exercise may improve immune function (67).

Fat consumption and cancer risk

Besides all the changes caused by body weight control through calorie restriction and exercise mentioned above which may relate to cancer risk, it is accepted recently that more than one third of human cancers were found to be associated directly with some specific dietary component (68). The alteration of lipid content has been reported also in cancer patients. Previously, nutrients were not assumed to regulate the gene expression directly as hormone and growth factors did. However, accumulated evidence indicates that lipids may affect gene expression (69) including the expression of some oncogenes (70).

1). Omega-3 fatty acids and cancer risk

Over the last 150 years, the consumption of fat type and amount has been changed mostly (71). The relationship between dietary fatty acids consumption and the risk of cancer became a research area attracting more and more scientists. However, there are no definite conclusions obtained which may due to the confounding and the methodological issues in nutritional epidemiology. The effects and mechanisms of omega-3 polyunsaturated fatty acids (PUFAs) in cancer prevention attracted the most attention.

a) Classification of Omega-3 fatty acids:

Omega-3 family include α-linolenic acid (18:3), stearidonic acid (18:4), eicosatrienoic acid (ETA 20:3), eicsoatetraenoic acid (20:4), eicosapentaenoic acid (EPA 20:5), docosapentaenoic acid (DPA 22:5), docosahexaenoic acid (DHA 22:6), and tetracosahexaenoic acid (24:6) (www.lipomics.com/resources/fatty_acids/index.htm)

b) effects of Omega-3 fatty acids on cancer risk

About 30 years ago, some epidemiological studies on Eskimo people in Inuit, Greenland triggered interest in ω-3 PUFAs. Those studies found that although this population has a diet rich in fat, there is a low incidence of myocardial infarction. The Inuit's diet is rich in ω-3 PUFAs compared to a western diet, due to their high consumption of fish and some meat of fish-fed mammals such as seals and walrus (72, 73). After that many remarkable studies focused on the effects of EPA and DHA which came from fish oil. In 1999, Chajès and co-workers reported that no significant association was found between ω-3 polyunsaturated fatty acids and breast cancer risk in their cohort studies, but it showed protective effect of stearic acid in breast cancer (74). According to Leitzmann and colleagues, the results of their 14 years cohort study on 47,866 US men found that EPA and DHA intake was associated with decreased prostate cancer (75). In some animal models, high ω-6 polyunsaturated fatty acids in diet showed strong tumor-enhancing effect at levels under 4% of total calories and that saturated fats have a weaker tumor-enhancing effect. While high ω-3 PUFAs in diet appeared to have a protective effect on tumor growth and metastasis, it was not statistically significant (76). Others reported that dietary supplements rich in ω-3 PUFAs indicated the inhibition of the growth of human breast cancer cell lines *in vitro* and in nude mice (77). The growth of tumors was slowed due to EPA and DHA intake have been found in other types of cancers such as lung (78) and colon (79) although some people do not agree to that (80). Nevertheless, in a review paper published in 2006 reported that "most of the studies on the relationship between ω-3 intake and onset of neoplastic pathologies lead to conclude that such substances have a protective effect towards some kind of cancer, independently from the place and the kind of cancer" (81).

Furthermore, supplying ω-3 fatty acids in the diet for tumor-bearing rodents or cultured medium of cells has been shown increase the sensitivity to radiation therapy and chemotherapy drugs. For example, Cha and co-workers found that low dose of DHA supplementation not only enhanced the effect of chemotherapy of arabinosylcytosine in colon cancer, but also can protected normal tissues (82). Similar results of enhancing the chemotherapy effect were also

found with other chemotherapy drugs such as 5-fluorouracil (83), doxorubicin (84), mitomycin C (85), tamoxifen (86), epirubicin (87), and CPT-11 (83). In radiotherapy, enhanced antitumor effect was also found in mice after injection of ω-3 fatty acids (88).

c) Mechanisms of ω-3 PUFAs inhibition of cancer growth and enhanced cancer therapy

The mechanisms of ω-3 PUFAs protecting from cancer formation are not clear, but it has been proposed that many mechanisms maybe involve and that cancer prevention possibly due to a combination of them.

First, dietary ω-3 PUFAs are incorporated into cell membrane phospholipids in second position replacing the arachidonic acid (AA). Membrane-bound phopholipase-A_2 (PLA_2) releases EPA and other ω-3 PUFAs and AA which does not compete as successfully as EPA for cyclooxyenase (COX) and lipoxygenase (LOX) activity (89, 90). Eicosanoid synthesis from AA was inhibited by DHA (91). EPA is converted to prostaglandins. Then less of inflammation-producing and more prostaglandin E2 are produced in both tumor and normal cells (92).

Second, in contrast to AA, ω-3 PUFAs do not promote growth through stimulated mitosis by activating protein kinase C (93). Ras and AP-1 are transcription factors for many oncogenes which promote growth, and ω-3 PUFAs also decreased the expression of these transcription factors (94, 95).

Third, programmed cell death or apoptosis was found to be blocked by Bcl-2 family and COX 2. ω-3 PUFAs induce apoptosis and increase differentiation of cancer cells through down-regulating Bcl-2 family genes (96) and COX 2 expression (97). Nuclear factor κB (NFκB) can also block apoptosis when it is activated. The up-regulation of NFκB in cancer cells will resistance to chemotherapy drugs or radiation. There is a report that ω-3 PUFAs can down-regulate NFκB and restore functional apoptosis (98).

Furthermore, the enhanced effect in cancer therapy by ω-3 PUFAs may also be due to the altered membrane composition and function. Membrane fluidity was increased which might enhance transport capabilities and accumulation of selective anti-cancer agents. Thus, the activity of drug selected enzymes was increased, and the signaling pathways associated with cancer progression were alterated (99).

Finally, inhibiting angiogenesis is also important in controlling the growth of tumor because new blood vessels are required for nutrients supply and wastes remove for the fast

divided cells. ω-3 PUFAs were found to inhibit angiogenesis through down-regulating of protein kinase C and alteration eicosanoid production (100, 101).

2). Olive oil and cancer risk

Some confounding exists in the epidemiological studies related the level of ω-3 fatty acids consumption and the risk of neoplasia, and that may cause the results of the studies disagree with each other. Consumption of olive oil, which is rich in monounsaturated fatty acids-primarily oleic acid (18:1 n-9), is one of those factors due to its higher cancer protective effect than ω-3 fatty acids (81).

a) Effects of olive oil on cancer risk

European nations in Mediterranean region (Spain, Italy, Greece, and Portugal) were found tend to have lower risk of cardivascular and cancer diseases although the mean fat consumption is higher compared to people in other western countries. In spite of the different food habit between the countries around Mediterranean Sea due to the culture and religion, the Mediterranean diet containing high olive oil, wine, fish, fruits and vegetable which is rich in phytosterols, phenolic substances, antioxidants, and polyunsaturated acids (101).

b) Mechanisms of olive oil inhibit cancer growth

Olive oil is a principal ingredient of the Mediterranean diet and accumulating evidence showed that consumption of olive oil may potentially reduce risk of neoplasms such as breast, colon, stomach, ovary, and endometrium cancer, while the mechanisms are not well understood (102). Salvini and co-workers reported that in a randomized cross-over trial the significant reduction of DNA damage in healthy postmenopausal women was identified after eight weeks in each period of extra-virgin olive oils consumption which was high in phenolic compounds that have antioxidant properties (103). Another paper published in 2006 reported that virgin olive oil phenols could inhibit proliferation by inducing apoptosis (104). Oleic acid was found to enhance apoptosis (105) and reduce the gene expression of *cox-2* and *c-fos* in human prostate cancer cells (106), as well as PGE in rats (107). In 2005, Wahle and colleagues reported that oleic acid down-regulated the *Her2* expression and increased the anticancer effect of anti-Her2. Overexpression of *Her2* played an important role in tumorigenesis and metastasis, and it was suppressed specifically by olive acid (102).

Lipid alteration in cancer patients

The lipid components of biological membranes are the structural building blocks for the permeability barrier of cells and organelles, as well as precursors of signaling components. Some lipids regulate the activities of enzymes, channels and transport proteins, as well as cell surface receptors (such as, G-protein coupled receptors, growth factors, and integrins) that generate first and second messengers (www.lipidmaps.org).

Recently, lipid alteration has been reported in cancer patients in some studies, suggesting that membrane phospholipids could be involved in modulating of tumor growth. In 1990s, Bougnoux and colleagues found that breast cancer patients had a higher risk of early occurrence of visceral metastasis if their polyunsaturated fatty acids were at low levels. For cancer patients who remained metastasis-free, the stearic acid of membrane phosphatidylcholine (PC) in their carcinoma tissues was significant higher than that in the patients who developed metastasis (108). Furthermore, lower essential fatty acid levels were found in breast tumor (109), while lower levels of linoleic acid as well as a combination of high monounsaturated fatty acids and a low ω6/ω3 fatty acid ratio were associated with increased the risk of breast cancer (110). Li and co-workers found that compared to primary foci, hepatic metastasis has higher phosphatidylethanolamine (PE) level. There was also a positive association between colorectal cancer genesis and phosphatidylinosital (PI) as well as PC (111).

Lipid alteration due to exercise

Lipid compositional changes happen in response to both internal and external changes. Exercise training was reported to affect lipid content. A meta-analysis of 95 studies conducted between 1955 and 1983 which investigated human serum lipid and lipoprotein levels in response to exercise training found that exercise could significantly reduce total cholesterol, low density lipoprotein cholesterol (LDL-C), cholesterol/high-density lipoprotein cholesterol (HDL-C), and triglyceride in a group with constant body weight. Body weight loss during exercise further decreased the levels of above lipid and lipoprotein (112). However, a lipidomic profile study on athletes and non-athletes found no significant difference in total cholesterol, serum triacylglycerol, LDL, and HDL between the two groups; thus might be due to the limited sample number of 28 men between 18 to 26 years old (113). In another human study, regular exercise training significantly increased levels of oleic acid 18:1 (ω-9) and docosahexaenoic acid (DHA) 22:6 (ω-3) in muscle membrane (114).

The content of sphinganine and sphingosine were increased significantly in rat skeletal muscle after prolonged exercise (115). According to Dobrzyń and co-workers, total content of ceramide-fatty acids were reduced by 64% in rat heart muscle by prolonged exercise via treadmill running three hours per day for 6 weeks, but not the total content of sphingomyelin-fatty acids. For ceramide-fatty acids, the percentage of palmitic and oleic acid was decreased significantly, that of stearic, linolenic, and eicosapentaenoic acids was stable, whereas that of other acids including palmitoleic, arachidonic, and docosahexaenoic was elevated significantly compared to the control. For sphingomyelin-fatty acids, the percentage of palmitic, eicosapentaenoic, and nervonic acid were reduced significantly, that of linoleic and arachidonic acid was stable, and those of palmitoleic, stearic, linolenic, and docosahexaenoic acid were increased. The authors also found reduced enzyme activities of sphingomyelin hydrolysis in exercised rats suggesting that the decreased in the content of ceramide was due to the lower sphingomyelin formation after exercise. However, the biological function of the changes in ceramide content caused by exercise is still unknown (116).

In almost all the lipid studies mentioned above, lipid analysis was conducted by gas chromatography or high-performance liquid chromatography. While "lipidomics" is a recently developed method of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and electrospray ionization tandem mass spectrometry (ESI/MS-MS). "The 'soft' ionization does not cause extensive fragmentation, is highly sensitive, accurate, and reproducible" (117). This new technique has been applied to study the important roles of lipids in both normal and stress responses or diseased cells (118, 119). However, almost no research of membrane phospholipids profiling related to exercise training has been reported. Dr. Wang's lab investigated phospholipid alteration via ESI/MS-MS in mice skin tissues caused by exercise and modulate calorie intake, and they found that most PI molecular species decreased while some docosapentaenoic acid (DPA, 22:5) and DHA (ω-3 fatty acid) increased in exercise and pair-fed mice (data will be submitted for publication).

Gene expression changes induced by skin tumor promoter and exercise

In the past ten years, DNA microarray has become a powerful and essential tool for gene expression analysis, and "so far, DNA microarray technologies are perhaps the most successful

and mature methodologies for high-throughput and large-scale genomic analyses" (120). According to the type of spotted probes, DNA array can be classified into two main types. Affymetrix (Santa Clara, CA, USA), which is considered as a leading provider, uses small single-stranded oligonucleotides (around 22 nucleotides) synthesized in situ. The other one uses cDNA or open reading frames (ORF) as the bound probes (120).

In order to better understand human neoplasia, one of the best-defined models is mice and *in vivo* epithelial (skin) carcinogenesis induced by chemical (121). In 2003, Schlingemann and colleagues reported gene expression changes induced by tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) in mouse epithelia (122). Tumorigenesis is a very complicated process involving alterations in genome integrity, DNA damage and repair, cell proliferation, differentiation, and program death. In order to offer dependable information about biomarkers for cancer prevention and diagnostic, as well as novel targets for cancer therapy, it is essential to understand the molecular mechanism of tumorigenesis. The variation of RNA expression is associated with the amount of protein expression and will further alter the biochemical activities (122). Phorbol esters including TPA are powerful and prevalent tumor promoters of carcinogenesis in mouse models. They mimic the results of diacylglycerol (DAG), a second messenger, and induce a series of PKC isoforms, then further affect gene regulation and cellular signaling pathways (123). Therefore, genes associated with cell cycle, epithelial differentiation, cell metabolism, transcriptional regulation and signal transduction could be affected by TPA. Overall, the microarray hybridizations found 54 genes were up-regulated and another 35 were down-regulated in dorsal skin tissues six hours after phorbol ester TPA treatment. Metallothionein, s100 calcium binding protein family, omithine decarboxylase, cathepsin, Jun-B oncogene, secretory leukocyte protease inhibitor, and small proline-rich protein family were confirmed to be increased, which were demonstrated to be associated with cancer development. Among them, secretory leukocyte protease inhibitor, s100 calcium binding protein family, as well as serum amyloid A3, interleukin 4 receptor, alpha, and tumor necrosis factor receptor family number which have shown to relate with the processes of epidermal hyperproliferation and skin inflammation were also up-regulated by TPA application (122).

Exercise training has been demonstrated to alter the gene expression related to metabolism, transport, stress and defense on hepatic tissue in high-fat diet (HFD) induced obese mouse (124). Totally 73 gene expression were changed by HFD-fed with or without exercise

compared to normal diet and no exercise control. Gene expression of elongation of very long chain fatty acids-like 2 which related to fatty acid biosynthesis, metallothionein 1 and 2 which are antioxidant factors, P450 (cytochrome) oxidoreductase which is a gene-encoding enzymes of electron transport were down-regulated by HFD-feeding, but were brought back to normal by exercise training. However, heat shock 70-kDa protein 5 families which is involved in immune responses were up-regulated by HDF-fed moderate and moderated by exercise training. Exercise combined with HFD-feeding, but not HFD-feeding alone affected eleven gene transcripts including Lipin 1, involved in regulation of adipogenesis, while serum amyloid P-component (Apcs) and malic enzyme which participated in acute phase responses and lipogenesis were up- and down- regulated by exercise, respectively. Interestingly, the expression of metallothionein and heat shock protein (HSP), which were all related to stress responses were found contrary to each other, and they suggested this may due to the interaction of antioxidant and repair systems.

Recently Dr. Wang's lab reported some cancer related gene expression alteration in exercise and 20% dietary calorie restriction (DCR) mice (125, 60). DCR has shown an overwhelming impact on expression of the oncogenes, and exercise with pair-feeding was similar as DCR in down-regulating the cancer-related gene expression. DCR tended to affect both PI3K and MARK pathways, while exercise showed more inhibition in the PI3K pathway.

In summary, the incidence of cancer is kind high nowadays. It may happen to anybody no matter how strong and how rich you are. Although the mechanisms for cancer prevention through dietary calorie restriction and exercise need further study, and just some primary data related with lipid alteration and cancer risk are available, accumulated evidence shows that combining a nutritious diet and enough physical activity may be the best way to keep healthy. The American Cancer Society recommends that adults should have at least 30 minutes of moderate exercise five days a week. For children and adolescents, at least 60 minutes of moderate to vigorous exercise at least five days a week are necessary (American Cancer Society. Physical Activity and Cancer. www.cancer.org).

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CHAPTER 2 - Alteration of phospholipid composition by exercise with or without moderate caloric restriction in mouse skin tissues

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The abbreviations used are: Ex+AL, exercise with ad libitum-feeding; Ex+PF, exercise and pair-feeding; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; ePC, ether phosphatidylcholine; ePE, ether phosphatidylethanolamine; ePS, ether phosphatidylserine; lysoPC, lysophosphatidylcholine; lysoPE, lysophosphatidylethanolamine; SM, sphingomyelin; PI3K, phosphatidylinositol 3-kinase; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; ESI/MS-MS, electrospray ionization tandem mass spectrometry; DXA, dual-energy X-ray absorptiometer; DMBA, Dimethylbenz(a)anthracene; TPA, 12-O-tetradecanoyl phorbol-13-acetate; BHT, butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HRP**,** horseradish peroxidase; PBS, phosphate buffered saline; IGF-1, insulin-like growth factor 1; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid**;** DHA, docosahexaenoic acid; LDL, low density lipoprotein; DFA, discriminant function analysis; PI-3,4,5-P(3), phosphatidylinositol-3,4,5-trisphosphate.

Abstract

Body weight maintenance via increasing physical activity and decreasing calorie intake has been linked to a reduced cancer risk in animal models. However, the underlying mechanisms are not well known. This study assessed the impact of exercise with or without limited dietary calorie intake on the phospholipid profiles and phosphatidylinositol-related PI3K expression in mouse skin tissues. CD-1 mice were randomly assigned to one of three treatment groups: *ad libitum*-fed sedentary control (sedentary), *ad libitum*-fed treadmill exercise at 13.4 m/min for 60 min/d, 5 d/wk (Ex+AL), and exercise but pair-fed with the sedentary control (Ex+PF). After 10 weeks, Ex+PF but not Ex+AL mice demonstrated a significant decrease in both body weight and percentage of body fat when compared to the sedentary controls. Among 338 phospholipids measured in skin samples by electrospray ionization mass spectrometry, some phosphatidylinositol (PI), phosphatidylcholine-containing lipids with ether linkage (ePC), and lysophosphatidylcholine (lysoPC) species significantly decreased in Ex+PF mice when compared to the controls. In addition, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) species containing 18:0-22:5 and 18:0-22:6 fatty acyl combinations increased significantly in Ex+PF mice. Stepwise discriminant analysis based upon 25 significantly changed phospholipid contents illustrated a clear diverse of discriminating variables among the three treatments. Furthermore, the expression of PI3K as measured by immunohistochemistry was remarkably reduced in phorbol ester-induced mouse skin tissues by Ex+PF treatment. These data indicate that controlling body weight via exercise with limited calorie intake but not exercise alone significantly modified phospholipid profiles by increasing the levels of certain molecular species containing ω-3 fatty acids, decreasing some cancer promoter signaling-related species such as PIs, and suppressing anti-apoptotic PI3K expression. Considering the well-known role of the PI3K signaling pathways in TPA-induced skin cancer promotion, the results observed in this study suggest a novel mechanism by which weight control via exercise with limited dietary calorie intake may protect against cancer.

Introduction

The World Health Organization (WHO) reported that non-communicable diseases such as cancer and obesity would become the principal global causes of mobility and mortality. Recently, WHO emphasized, "Physical inactivity…is the result of a progressive shift of lifestyle toward a more sedentary pattern, in developing countries as much as in industrialized ones". Approximately 60% of the U.S. population is overweight (1). Over the past 20 years, abundant evidence from epidemiological, prospective cohort, and intervention studies has shown that physical activity, diet, as well as combined activity and diet interventions could mitigate the progression of chronic diseases, and even reverse existing diseases (2). Recent data indicated that about 400,000 deaths in 2000 were caused by physical inactivity and poor diet with these factors ranking second after tobacco as causes of death. Indeed, it is likely that physical inactivity and inappropriate diet will soon become the leading cause of death in the United States (3).

The benefits of physical activity have been found to be associated with better quality of life through either improving bodily function via increasing sleep quality and sexual function, or preventing some chronic diseases including cardiovascular disease, obesity, diabetes, arthritis, and cancer (4, 5). Although the relationship between cancer risk and the type, frequency, intensity, and duration of exercise are controversial, there is accumulated evidence that exercise is beneficial in reducing cancer risk. Evidence from some cohort and case-control studies showed that physical activity reduced cancer risk in a dose dependent manner (6). Physical activity has been found to affect risk of various cancers. Increased death rates for cancer patients were also found associated with increased body weight (7). Recently, a review paper concluded that, independent of weight loss, physical activity has been suggested to have specific effect on cancer prevention (8).

A genetically modified mouse model has often been employed to study nutrition and cancer chemoprevention (9), while the relationship between physical activity and cancer prevention were commonly studied using chemical or physical carcinogenesis model on rats and mice (10, 11). A study reported that UVB-induced skin tumorigenesis could be prevented through voluntary running wheel exercise and which may also inhibit the growth of tumor (11).

The underlying mechanisms for physical activity might prevent cancer are not clear. It has been postulated that: 1) exercise could increase antioxidant status of the body (12); 2)

physical activity combined with weight reduction may reduce sex hormone levels, such as estrogen, testosterone, and dihydrotestosterone (13, 3); 3) exercise combined with diet were found to decrease some metabolic hormone levels, such as insulin (11), IGF-1 (14, 15), and leptin (16); 4) increased exercise may improve immune function (17).

Besides all the alteration caused by exercise mentioned above, some studies reported that fat mass and lipid content could be affected via exercise training. Increasing physical activity could lower fat mass via reducing adipocyte volume, and the cell number in each epididymal fat pad was also reduced (18, 19). A meta-analysis of 95 studies conducted between 1955 and 1983 which investigated human serum lipid and lipoprotein levels in response to exercise training found that exercise could significantly reduce total cholesterol, low density lipoprotein cholesterol (LDL-C), cholesterol/high-density lipoprotein cholesterol (HDL-C), and triglyceride in the group without a change in body weight. Body weight loss during exercise further decreased the levels of above lipid and lipoprotein (20). Increased levels of sphinganine and sphingosine were found in rat skeletal muscle (21), and reduced total content of ceramide-fatty acids were obtained in rat heart muscle (22). In a human study, regular exercise training significantly increased levels of oleic acid 18:1 (ω-9) and docosahexaenoic acid (DHA) 22:6 (ω-3) in muscle membrane (23).

It is well known that eukaryotic cellular membranes consist of as many as 1000 molecular species of lipids. The lipid components of biological membranes are the structural building blocks for the permeability barrier of cells and organelles, as well as precursors of signaling components. Some lipids regulate the activities of enzymes, channels and transport proteins, as well as cell surface receptors (such as, G-protein coupled receptors, growth factors, and integrins) that generate first and second messengers (www.lipidmaps.org). Phospholipid alteration in tumor tissues has been reported in some previous studies and that suggested membrane phospholipids could involve in modulating of tumor growth. For example, lower essential fatty acids level were found in breast tumor (24), while lower level of linoleic acid as well as combining high mono-unsaturated fatty acids and low ω6/ω3 fatty acid ratio was associated with against the risk of breast cancer (25). Breast cancer patients were found to have higher risk of early occurrence of visceral metastasis when they have lower level of polyunsaturated fatty acids of PE in their primary tumor, and lower level of stearic acid of PC were found in breast cancer patients who developed metastasis compared to those remained

metastasis-free (26). However, most lipidomic profiles related with cancer were focused on total cholesterol, lipoproteins, triglyceride and so on (27, 28). Phospholipids which are important structure of membrane and some of them have important biological functions were measured as one group when they were detected (28).

Lipid compositional changes happen in response to both internal and external changes, which were monitored by "lipidomics" via recently developed methods of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and electrospray ionization tandem mass spectrometry (ESI/MS-MS). "The 'soft' ionization does not cause extensive fragmentation, which is highly sensitive, accurate, and reproducible" (29). This new technique has been applied to study the important roles of lipids in both normal and stress responses or diseased cells (30, 31). However, almost no research of membrane phospholipid profiling related to exercise training has been reported.

In order to overcome the confounding variable involved in human exercise studies such as body mass, dietary intake, lifestyle, smoking and alcohol use, exercised mice were used in this study because 1) they are more uniform, 2) mouse and human have similar genetic and physiological characteristics, and 3) some common naturally developed diseases shared in the two species, such as cancer, atherosclerosis, diabetes, heart diseases and so on (www.genome.gov). Furthermore, mouse skin tumor model is a well established model in studies related to cancer prevention and therapy due to the visible of tumor formation, high survival rate even by large dose of chemical application, agents reach target tissue directly before significant diluted and metabolism, and the molecular signaling pathway of tumor formation caused by dimethylbenz(a)anthracene (DMBA) inducer and 12-O-tetradecanoylphorbol-13-acetate (TPA) promoter is quite clear (32).

In this study, for the first time we measured 12 membrane phospholipid classes including PI, a substrate of PI3K in mouse skin tissues by phospholipid profiling that corresponded to exercise with or without combined with limited calorie intake. We found that the difference of lipid profiling existed between exercise alone and exercise combined with limited calorie intake mice. More decrease or normalization of ether PC and lysoPC were found in exercise with limited calorie intake mice while some were increased by exercise alone. However, phospholipid species containing long chain polyunsaturated fatty acids including 22:5 and 22:6, an omega-3 fatty acid, were increased significantly in exercise and pair-fed mice. The results suggest that

changes in phospholipids composition and an alteration in PI3K expression may contribute reducing cancer risk through exercise with limited calorie intake.

Experimental procedures

Animals and animal treatments — Female CD-1 mice (NIH, Frederick, MD) obtained at 8 weeks old were randomly divided into three groups: sedentary and *ad libitum* (sedentary) (n=10), exercise and *ad libitum* feeding (Ex+AL, n=12), and exercise and pair feeding (Ex+PF, n=15). *Ad libitum* groups 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. Water was provided *ad libitum*. A speed adjustable rodent treadmill (Boston Gears, Boston, MA, USA) was used for mice exercise. Exercise training started from 6.7 m/min for 5 min and increased gradually in intensity and duration in the first two weeks training until the 13.4 m/min and one hour per day was reached. Then exercise groups ran on the treadmill 5 days a week for 10 weeks during the entire experiment. All mice were housed individually at 24 ± 0.6ºC and 80% relative humidity with 12 hr light/12 hr dark cycle. Body weight and food consumption were recorded weekly.

Mice dorsal skin was shaved, followed by treatment with acetone on three to five mice chosen randomly from each group, and other mice in each group were applied 3.2 nmol 12-O-tetradecanoylphorbol-13-acetate (TPA) which was dissolved in acetone. Dorsal mouse skin was collected two hours after acetone or TPA treatment, snap-frozen in liquid nitrogen immediately, and then stored at -80ºC for following analysis.

Body fat analysis — In the last week, percentage of body fat of the mice was determined by a dual-energy X-ray absorptiometer (DXA, GE lunar-General Electric, Milwankee, WI, USA).

Lipid extraction and profiling — Each skin sample was ground with liquid nitrogen. After 2 ml of solvent [chloroform: methanol 1: 2 + 0.01% butylated hydroxytoluene (BHT)] were added to 1 g tissue and shaken well, 1 ml of chloroform and 1 ml of water were added and centrifuged at 1,000 rpm, and the lower layer was collected. Then, twice, 1 ml of chloroform was added to the tissue, the samples were centrifuged and the lower layer was collected. The combined lower layers were washed once with 300 μl 1 M KCl and once with 300 μl water. The lower layer was removed to a new tube and the samples were sent to Kansas Lipidomics Research Center for lipid profiling. An automated electrospray ionization-tandem mass spectrometry approach was

used and data acquisition and analysis as well as acyl group identification were carried out as described previously (30, 33) with minor modifications. Internal standards were obtained and quantified as previously described (30). Twelve phospholipid classes or subclasses, including phosphatidic acid (PA), PI, PC, lysoPC, ePC, PE, lysoPE, ePE, phosphatidylserine (PS), phosphatidylserine-containing species with an ether linkage (ePS), sphingomyelin, and PE-ceramide were determined. For 40:5-PC, 40:6-PC, 40:5-PE, and 40:6-PE acyl composition analysis, four samples chosen randomly from each group were analyzed. Acyl ions of PE species were identified after collisionally induced dissociation of the $[M-H]^-$ ions, and acyl ions of PC species were identified following collisionally induced dissociation of the $[M+OAc]^-$ ions.

PI3K expression detected by immunohistochemistry — The frozen dorsal skin tissues of mice applied by acetone or TPA were fixed in -70ºC absolute ethanol overnight in the freezer, followed by 70% ethanol in refrigeration, 50% ethanol at room temperature, and rinsed with PBS before adding 10% formaldehyde. Then the skin tissues were set on edge in paraffin blocks and were sectioned. Extra paraffin sections were de-paraffinized by incubating 3 times with xylene, twice with absolute ethanol, twice with 70% ethanol, and once with PBS. Then slides were exposed to steam in the target retrieval solution (Dakocytomation, Carpinteria, CA, USA) at 95ºC for 30 minutes for antigen retrieval. PI3Kinase p85 (mouse monoclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a primary antibody, and the secondary antibody was BioGenex QP900 SS multilink HRP kit (BioGenex, San Ramon, CA, USA). Slides were counterstained with Gills hematoxylin for 60 seconds followed by dehydration in alcohol and xylene and coverslipping with permanent adhesive. Staining was developed with diaminobenzidins chromogen (BioGenex, San Ramon, CA, USA) and the density of the stain for each section was scored by a pathologist in a blind study. Ten to fifteen sections for each group were scored.

Statistical analysis — Lipid data outliers were detected using a Q test. Statistical analyses were performed using a commercial statistical package from SAS (SAS Institute Inc., Cary, NC, U.S.A.). Results of phospholipid profiling of acetone applied mice and TPA applied mice were found no significant different, then data were analyzed according to treatment. Difference in phospholipid levels among the three treatment groups were compared using a one-way analysis of variance (ANOVA) and an F-test for significance followed by pair comparisons by the LSD method. Data are expressed as mean ± S.D. The level of significance of all statistical tests was

set at the 5% level. An automatic backward stepwise discriminant analysis was performed on 57 lipid species which showed statistical significant changes among the three groups in order to test the grouping of the samples according to the treatments (Sedentary, Ex+AL, and Ex+PF) as well as to determine the lipid variables contributing the most to this discrimination observed.

Results

Body weight change — Body weight changes of sedentary, Ex+AL, as well as Ex+PF mice during the 14 weeks of experimentation are shown in Fig. 2-1A. Adult CD-1 mice in sedentary control group gained weight slowly throughout their life span. Exercise alone did not prevent weight gain. However, starting from the 5^{th} week of the study, the body weight of Ex+PF mice was statistically significant lower compared with either sedentary or Ex+AL mice.

Body fat analysis —As shown in Fig. 2-1B, the percentage of body fat of Ex+AL mice was similar to that of control. While the percentage of body fat of the Ex+PF mice showed significantly lower than that of control and Ex+AL mice.

Changes in lipid molecular species through exercise — Crude lipid extracts from CD-1 mice skin tissues were analyzed by ESI-MS/MS. A total 338 kinds of phospholipids were measured and 57 of them were found significantly different among the three treatment groups. Lipid profile data of acetone applied and TPA applied were combined for each group due to no significant difference found between them.

PC, PE, PS and PI lipid profiles are shown in Fig. 2-2. Some PCs containing short chain fatty acids, including 30:0-PC, 32:0-PC, and 32:1-PC, were significantly lower in Ex+PF mice than in either group of *ad libitum*-fed mice. However, 40:4-PC, 40:5-PC and 40:6-PC were significantly higher in Ex+PF mice compared to sedentary as well as Ex+AL mice. Most species of PE and PS were not changed statistically significant among the three treatment groups. The level of 40:5-PE, 40:6-PE, and 40:7-PE were similar between sedentary control and Ex+AL. That of Ex+PF mice were 56%, 36%, and 31% higher compared to sedentary mice, respectively, although they were not statistically significant different. 40:6-PS was significant higher in Ex+PF mice compared with Ex+AL mice, but not significant difference with sedentary control. Not many PI species could be detected. The level of the lipids of Ex+AL mice were similar as

that of sedentary control, but five of them were significantly lower in the Ex+PF mice compared to sedentary and Ex+AL mice.

Ether PC is showed in Fig. 2-3A. Exercise alone increased some ePC level such as 38:2-ePC and 40:2-ePC, but that were normalized by exercise and pair-fed to the similar amount as sedentary control. Most of the ePC in the Ex+PF mice were significantly lower compared to the other two *ad libitume* groups. Two ePE (36:2-ePE and 38:2-ePE Fig. 2-3B) were increased significantly in Ex+AL mice, but were normalized in the Ex+PF mice as similar as control. 38:5-ePE was higher in Ex+AL mice compared to sedentary control although not statistically significant, while it was lower significantly in Ex+PF mice compared to that in Ex+AL mice. The results of SM are shown in Fig. 2-4. Some SM species were lower in Ex+PF mice compared to sedentary control as well as Ex+AL mice. Fig. 2-5 shows the lysoPC (A) and lysoPE (B) profile. LysoPC of Ex+AL were not different significantly compared to sedentary control. Some of the them including 16:0-lysoPC, 18:1-lysoPC, 18:2-lysoPC, and 20:4-lysoPC were significantly lower in Ex+PF mice compared to sedentary and Ex+AL. 22:6-lysoPE was significantly higher in Ex+PF mice compared to both sedentary control and exercise alone mice, while 22:5-lysoPE was higher in Ex+PF mice compared to exercise alone, but not sedentary control.

As in most mammalian tissues, phosphocholine (PC) (Fig. 2-2A) was the most common head group class in mouse skin; PC (diacyl) species represented 45.5% of the phospholipids analyzed, PC species with masses indicating the presence of one ether linkage (Fig. 2-3A) represented 3.0%, sphingomyelins (Fig. 2-4) represented 6.8%, and lysoPCs (Fig. 2-5A) represented 1.2%. Phosphoethanolamine (PE) (Fig. 2-2B) was the second most common head group with PE (diacyl) species as 31.5% of the phospholipids analyzed, PE species with masses indicating the presence of an ether linkage (Fig. 2-3B) as 2.3%, and lysoPEs (Fig. 2-5B) as 0.4%. PSs (Fig. 2-2C) diacyl species made up 4.6% of the total phospholipids, while PIs (Fig. 2-2D) up 4.6%.

Phosphatidylcholines had a broad range of chain lengths, from a total of 28 carbons in the two fatty acyls group to 44 carbons (Fig. 2-2A). Compared to other classes, PC has the largest amount of shorter chain species with 28, 30 and 32 total acyl carbons making up 9.1% of diacyl PCs, while species of 38 carbons or more made up 26.7% of diacyl PC. PE has less amount of short chain species with 28, 30 and 32 total acyl carbons making up 0.5% of diacyl PEs, and

species with 38 or more carbons made up 68.4% of the diacyl PE. Likewise lysoPC was predominantly 16- and 18- carbon species, while about half (51%) of lysoPE was relatively enriched in species with 20 or 22 carbons. PI has the simplest fatty acyl composition of the classes with 38:4 PI, representing 91.4% of the total PI. Species 16:0 and 24:1 sphingomyelin were found decreased in Ex+PF mice. They represent about 70% of total sphingomyelin.

In order to identify the lipid species in 40:5-PC/PE and 40:6-PC/PE, acyl composition analysis was conducted, and product ion analysis of 40:5-PC chain showed that the acyl composition included 18:1-22:4, 18:0-22:5 and 20:1-20:4 (Fig. 2-6A), and 40:6-PC included 18:1-22:5 and 18:0-22:6 species (Fig. 2-6B). For 40:5-PE, the acyl compositions were 18:1-22:4 and 18:0-22:5 (Fig. 2-6C), while for 40:6-PE, the acyl composition were 18:2-22:4, 18:1-22:5 and 18:0-22:6 (Fig. 2-6D). The 18:0-22:5 and 18:0-22:6 pair species of PC and PE were similar between Ex+AL and control groups, but showed significantly higher in Ex+PF mice compared to sedentary as well as Ex+AL ($p < 0.001$) (Fig.2-7).

Discriminant function analysis —The automatic backward stepwise discriminant analysis generated two discriminant functions. Based upon selected 25 lipid variables including 16:1-LysoPC, 18:1-LysoPC, 18:2-LysoPC, 20:4-LysoPC, 28:1-PC, 32:0-PC, 34:1-PC, 40:4-PC, 40:5-PC, 40:6-PC, 42:5-PC, 44:2-PC, 44:5-PC, 32:0-ePC, 32:2-ePC, 32:3-ePC, 34:0-ePC, 34:1-ePC, 36:1-ePC, 36:2-ePC, 36:4-ePC, 36:5-ePC, 38:5-ePC, 38:0-PS and 36:1-ePS as listed in the table in Fig. 2-8. The three treatments (sedentary, Ex+AL, Ex+PF) were significantly separated (Wilks' lambda = 0.001 at $p < 0.00001$). Using the two discriminant functions, 92% of the mice were classified correctly according to their respective treatment-group.

Class I PI3K expression detected by immunohistochemistry — The expression and localization of class I PI3K in skin tissue is shown in Fig. 2-9. The brown color illustrates the stain of DAB (3,3-diaminobenzidine) which shows the PI3K protein location in the skin tissue. The picture shown for each group in figure 2-9 depicts the sample median expression score in each group. In sedentary control mice, PI3K expression in epidermal cells (arrows) was higher in TPA applied mice (Fig. 2-9B, scored as 10) than that in acetone applied mice (Fig. 2-9A, scored as 5). For exercise groups, all samples were TPA applied, and PI3K expression was higher in Ex+AL mice (Fig. 2-9C, scored as 15) compared to sedentary control which is not statistically significant, while the PI3K expression was normalized to acetone applied control in Ex+PF mice (Fig. 2-9D, scored as 5).

Discussion

This study shows that exercise with or without limited dietary calorie intake showed difference effects on body weight, percentage of body fat, and phospholipids profiling. Exercise combined with limited calorie intake was successfully prevented the body weight gain and reduced body fat efficiently in mice, and modified the phospholipid profile in mice skin tissues more than exercise alone, especially decreased some lysoPC, most PI and ePC, while increased some long chain polyunsaturated fatty acids level including ω-3 fatty acid in PC, PE and lysoPE, as well as decreased PI3K protein expression in skin epidermis.

Our data showed that exercise alone did not affect body weight and percentage of body fat significantly, while combined exercise with limited calorie intake could significantly decreased body weight and body fat of CD-1 mice. These data demonstrated that total calorie intake contribute to the effect of body weight control through exercise training.

The phospholipid profiling results found in PC and PE were very interesting. Compared to sedentary control, exercise alone did not alter PC and PE level significantly, but some short chain fatty acids in PC decreased in Ex+PF mice. The biological meaning of these changes is not very clear. Some long chain polyunsaturated fatty acids such as 40:4-PC, 40:5-PC and 40:6-PC showed a significant increase in Ex+PF mice compared to control or exercise alone. The results of increased 40:5-PE and 40:6-PE were not significant when compared to sedentary control and Ex+AL group may due to the large variation. However, product ion analysis showed that 18:0-22:5 and 18:0-22:6 were major fatty acid pairs in 40:5-PC/PE as well as 40:6-PC/PE, respectively, and they were increased significantly in exercise combined with limited calorie intake mice. There are two isomers of docosapentaenoic acid (DPA, 22:5) fatty acids including all-cis-4,7,10,13,16-docosapentaenoic acid (ω-6) which is also called adrenic acid and all-cis-7,10,13,16,19-docosapentaenoic acid (ω-3) which is also called clupanodonic acid that is one of the three principal ω-3 long chain polyunsaturated fatty acids was found mostly in seal oil, and is an intermediary between eicosapentaenoic acid (EPA, 20:5 ω-3) and docosahexaenoic acid (DHA, 22:6 ω-3) (http://en.wikipedia.org/wiki/Docosapentaenoic_acid). Burdge and co-workers found that liver was the major site for DPA and EPA production through desaturation and elongation of alpha-linolenic acid (18:3 ω-3) (34). DHA was also significantly increased in

lysoPE in exercise combined with limited calorie intake mice. Because some mammalian tissues can form 22:6 (ω-3) from 22:5 (ω-3) chain elongation and ω-6 desaturation followed by 2-carbon chain shortening (35), the fatty acid elongation enzymes may be enhanced during exercise with food control. This result confirmed the increased levels of DHA found in human study through exercise, and it was suggested an enhanced ω-9-desaturase activities induced by exercise itself, or by catecholamine stress via exercise (23, 36). Omega-3 fatty acids were reported to be benefit for preventing heart disease (37, 38) and cancer (39, 40), and help for anti-inflammation (41) via inhibit the synthesis and release of tumor necrosis factor alpha and interleukin-1-β which are proinflammatory cytokines produced during inflammation (42).

Unlike PC and PE which are most common head group classes in mouse skin tissue, few PI species could be detected and it has the simplest fatty acyl composition. Classes with 38:4 PI represented absolutely the most of total PI. This is consistent with the typical pattern of PIs found in mammals, and in particular, in mice tissues, where PI has been demonstrated to consist mainly of sn-1-18:0 2-20:4 species (43). Higher percentage of 16:0 and 24:1 sphingomyelin found in mice skin tissues is similar to sphingomyelin found in human lens membranes (44).

In addition to that, we also found that two ether PE increased significantly in exercise alone mice compared to control, but that were normalized by exercise combined with limited calorie intake. Most of the ether PC in exercise combined with limited calorie intake mice were significantly reduced compared to other two *ad libitum* groups. A lower level of ether-linked PCs in skin tissue may reduce the formation of platelet-activating-factor (PAF).

Some lysoPC were reduced in exercise combined with limited calorie intake mice compared to other two *ad libitum* groups, but 22:6 ω-3 fatty acid in lysoPE was increased significantly in this group in our study. Lysophospholipids regulate both membrane phospholipids synthesis and signaling molecules bioactive. LysoPC is formed during low density lipoprotein (LDL) oxidation and then locates within atherosclerotic plaques, which has proinflammatory and proatherogenic properties (45, 46). LysoPC regulates different cellular functions, such as promotion of atherosclerotic lesion development, enhancement of cell proliferation (47, 48), attraction of monocytes (45), retardation of the migration of endothelial cell (49), permeability of plasma membranes (50, 51), and regulation of stem cells (52) and their progenitors (53, 54). LysoPC-specific oxidant stress enhances injures of variety cells due to its "detergent-like" properties, such as relates to lysis of red blood cells (55, 56). Furthermore,

lysoPC has been illustrated to increase the expression of CXCR4 dose- and time-dependently in human CD4 T cells (57). Reduced level of lysoPC in exercise combined with limited calorie intake mice may decrease the cell damage and also be beneficial with respect to cancer prevention.

Because the results of phospholipid profiling for the two exercise groups showed different trends for different individual phospholipids, discriminant function analysis (DFA) was applied to all the 57 significant changed phospholipids to test the difference among three treatment groups. About 25 phospholipids represent most of the variation of the total phospholipid profiling change. There are some factors that affect the phospholipid profiling among the three groups. In particular we were able to distinguish the sedentary control, exercise alone mice, as well as exercise combined with limited calorie intake mice from the DFA results which confirmed that exercise combined with limited calorie intake mice are significantly different from the sedentary mice we observed from the results of body weight and percentage of body fat. Furthermore, the exercise alone mice as well as exercise combined with limited calorie intake mice can also be distinguished by some discriminant functions. As shown in the inserted table in Fig. 2-7, lysoPC, PC and ether PC contributed to the group distinguish mostly. 40:5-PC, 40:6-PC, and 32:2-ePC contribute more for discriminant function 2 which distinguishes the sedentary group from two exercise groups, while 16:1-lysoPC, 18:2-lysoPC, 20:4-lysoPC, 28:1-PC, 40:4-PC, 44:2-PC, 44:5-PC, 36:1-ePC, 36:4-ePC, 38:5-ePC, 38:0-PS, and 36:1-ePS contribute more to discriminant function 1 which distinguishes the two exercise groups. Although the discriminant functions are not identified, we could use the 25 phospholipids as biomarkers to distinguish the mice from the treatments. The discriminant analysis gave 92% classification efficiency.

The observation that most of phosphoinositides lipids decreased in exercise combined with limited calorie intake mice determined through ESI-MS/MS prompted us to further investigate PI3K expression in those mice, because PI is a substrate of PI3K. Decreased PI in exercise combined with limited calorie intake mice may cause a down-regulation of the PI3K pathway.

Interestingly, the expression of *ras* and PI3K has been found significantly increased by swimming (58) and running (59). However, the study in our lab found that PI3K expression was decreased in skin tissue of exercise combined with limited calorie intake mice by western blot

analysis. Considering that skin cancer development is associated with uncontrolled proliferation of epidermal cells (60), the protein expression of class I PI3K in epidermal cells was detected by immunohistochemistry and the results confirmed the discovery obtained by western blot. Furthermore, the different expression level of class I PI3K in the sedentary group showed that TPA triggered PI3K expression in epidermal and dermal cells. PI3K expression in epidermal and dermal cells was enhanced by exercise alone compared to TPA treated sedentary control, but it was reduced in exercise and pair-fed mice. Decreased PI3K level in exercise combined with limited calorie intake mice suggests the second messenger phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P(3)) might be reduced. It is widely recognized that PI-3,4,5-P(3) recruits some signaling proteins with pleckstrin homology (PH) domains to the membrane such as protein serine-threonine kinases (Akt and PDk_1), exchange factors for GTP-binding proteins, and adaptor proteins. Finally, these proteins initiate some complex reactions which control protein synthesis, cell cycle entry, cell survival, and actin polymerization (61). As the same effect of PTEN, wortmannin or LY294002, the results of down-regulated of PI3K expression in our study suggest that body weight control through exercise with limited calorie intake may contribute to cancer prevention. PI is also a substrate of class II PI3Ks which is a monomeric (C2α/β/γ) (62). Detection of class II PI3K expression will make the results more interesting. The fact that PI3Ks activated by growth factors, and the PI3K plays an important role in insulin signaling suggest that the down regulation of both class I PI3K and PI in our exercised and pair-fed mice may be caused by the decrease of some growth factors such as IGF-1 (data not show).

This study for the first time detected the phospholipid profiling of weight control mice via exercise with *ad libitum* feeding or pair feeding, and showed down-regulated of class I PI3K expression in exercise and pair-fed mice. It is interesting to find that the combination of exercise with limited calorie intake contribute to the mice lipid profile. The significant decrease of some lysoPC, most PI and ePC, as well as significant increase of some long chain polyunsaturated fatty acids including ω-3 fatty acid in exercise combined with limited calorie intake mice showed some effects which may be benefit for cancer prevention. PI3K pathway is targeted more frequently than other pathways in cancer formation due to the high genomic abnormality such as rearrangement, amplification and mutation. Recently, several studies demonstrated that inhibition of PI3K-AKT pathway enhanced sensitivity to chemotherapy in different tumor types such as ovarian cancer cell lines (63, 64), pancreatic cell lines (65), myeloid leukemia cell lines

(66), and small cell lung cancer cells (67). Ultimately, future studies of phosphoinositide signaling pathways are likely to contribute to the cancer prevention, cancer therapy, and may also reveal new drug targets for some chronic diseases including cancer and diabetes since this is an ideal target because an activated pathway makes the inhibition easier than the replacement of lost tumor suppressor function in p53 or other tumor-suppressor pathways (68).

In summary, our data demonstrate that body weight control through exercise should combine with limited calorie intake, which can alter phospholipid composition such as decrease PI, significantly reduce class I PI3K protein levels, and significantly increase some long chain fatty acids including ω-3 fatty acid those may be the potential cancer preventive targets for cancer prevention.

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Figures

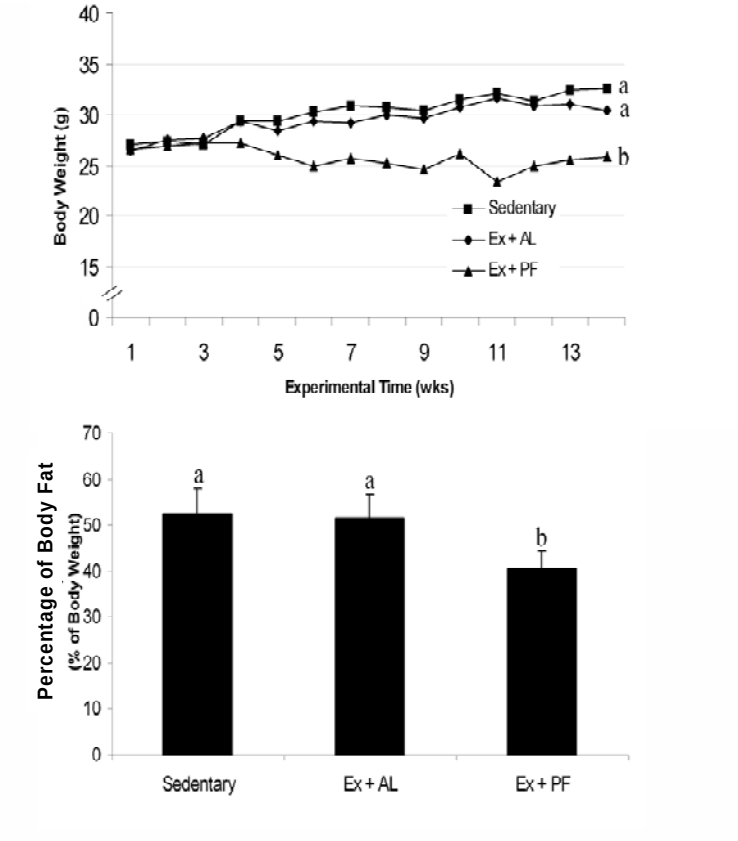

Figure 2-1 Mouse body weight and body fat composition changes. A: Adult mice lost weight significantly in Ex+PF group compared with sedentary and Ex+AL groups during the 14 weeks of experiment. Different letters shows statistically significant differences ($P < 0.05$, $n = 10 \sim 15$). B: Mouse body fat determined by dual-energy X-ray absorptiometer (DXA). The percentage body fat of Ex+PF mice was reduced significantly compared to sedentary and Ex+AL mice. The bars with different letters differ significantly ($P < 0.05$, $n = 10 \sim 15$).

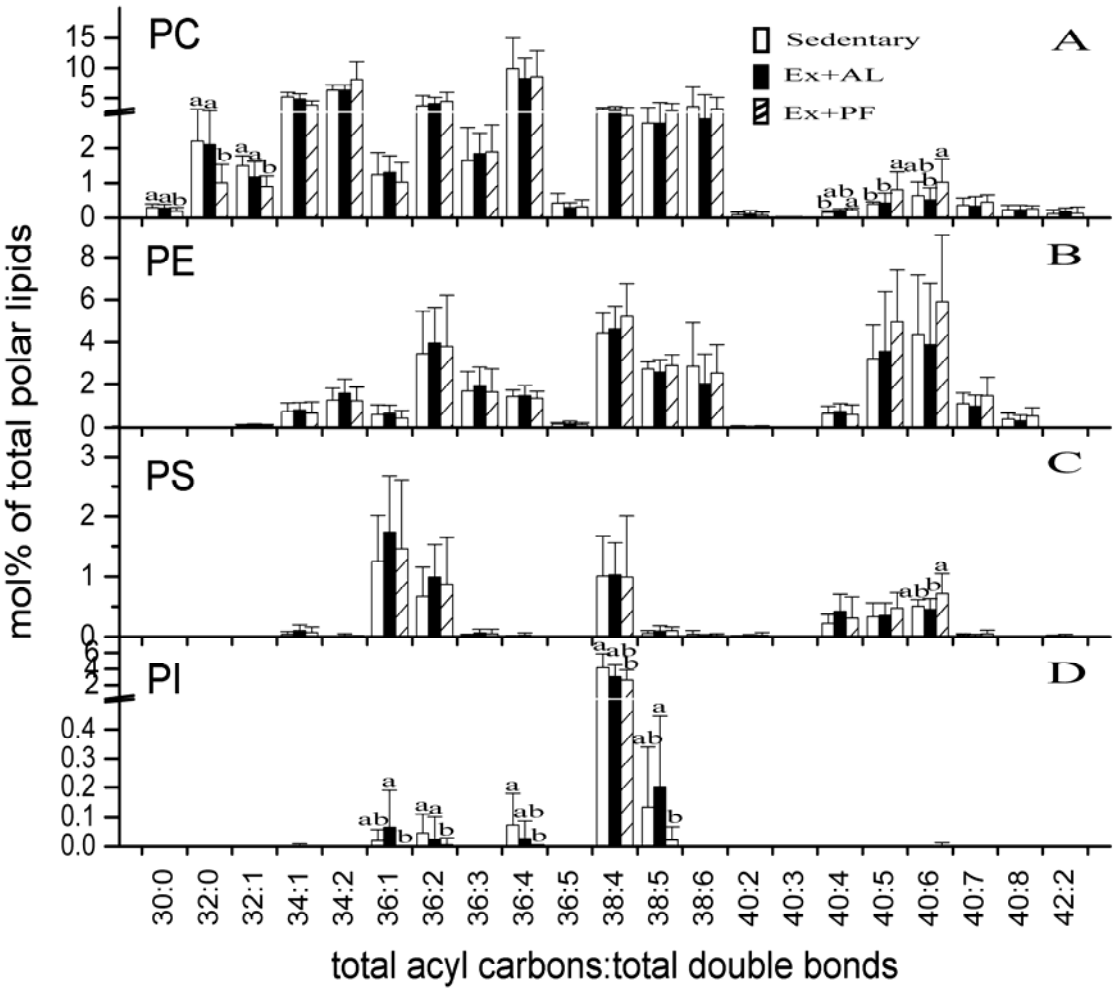

Figure 2-2 Exercise induced changes in phospholipid profiles of mice skin tissue. phosphatidylcholine (PC) (A), phosphatidylethanolamine (PE) (B), phosphatidylinositol (PS) (C), and phosphatidylethanolamine (PI) (D), in mouse skin samples were detected by ESI-MS/MS. The blank bars represent sedentary mice, the black bars represent Ex+AL mice, and the hatched bars represent Ex+PF mice. The bars with different letters differ significantly ($P < 0.05$, means ± S.D., n = 8 ~ 15).

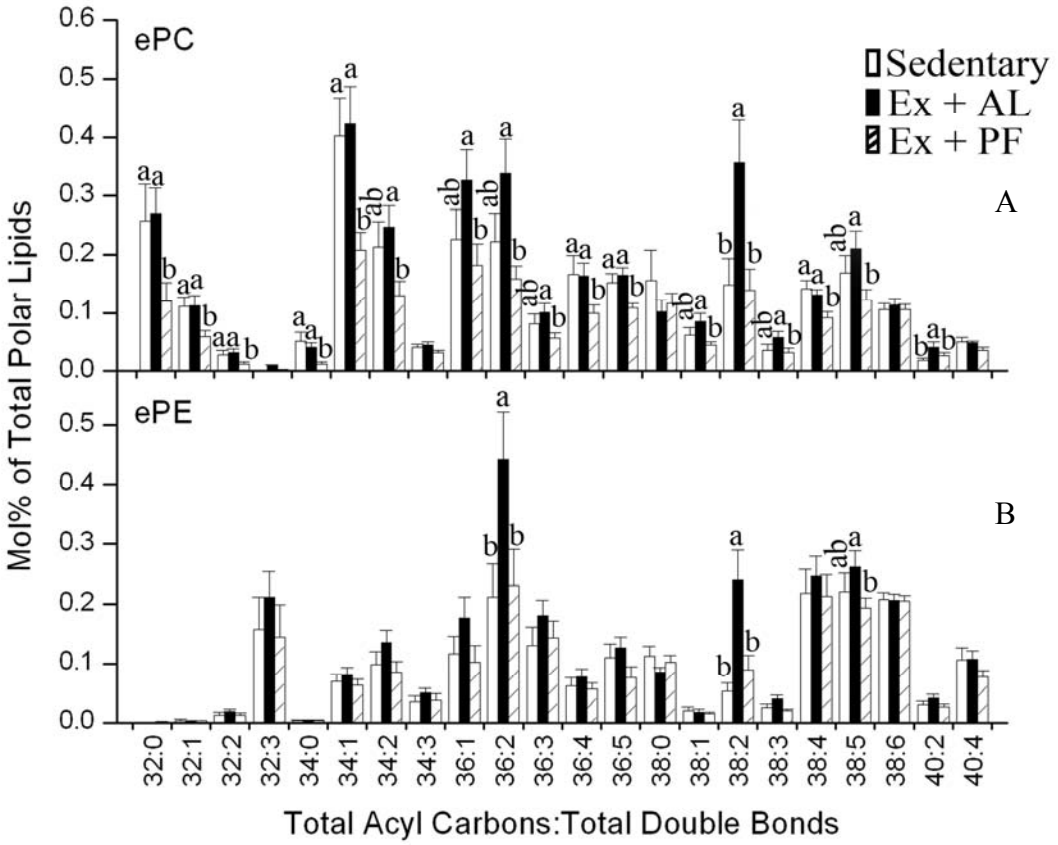

Figure 2-3 Exercise induced changes in ether phospholipid profiles of mice skin tissue.
Ether phosphatidylcholine (ePC) (A) and ether phosphatidylethanolamine (ePE) (B) in mice skin samples were detected by ESI-MS/MS. The blank bars represent sedentary mice, the black bars represent Ex+AL mice, and the hatched bars represent Ex+PF mice. The bar with different letters differ significantly ($P < 0.05$, means ± S.D., $n = 8 \sim 15$).

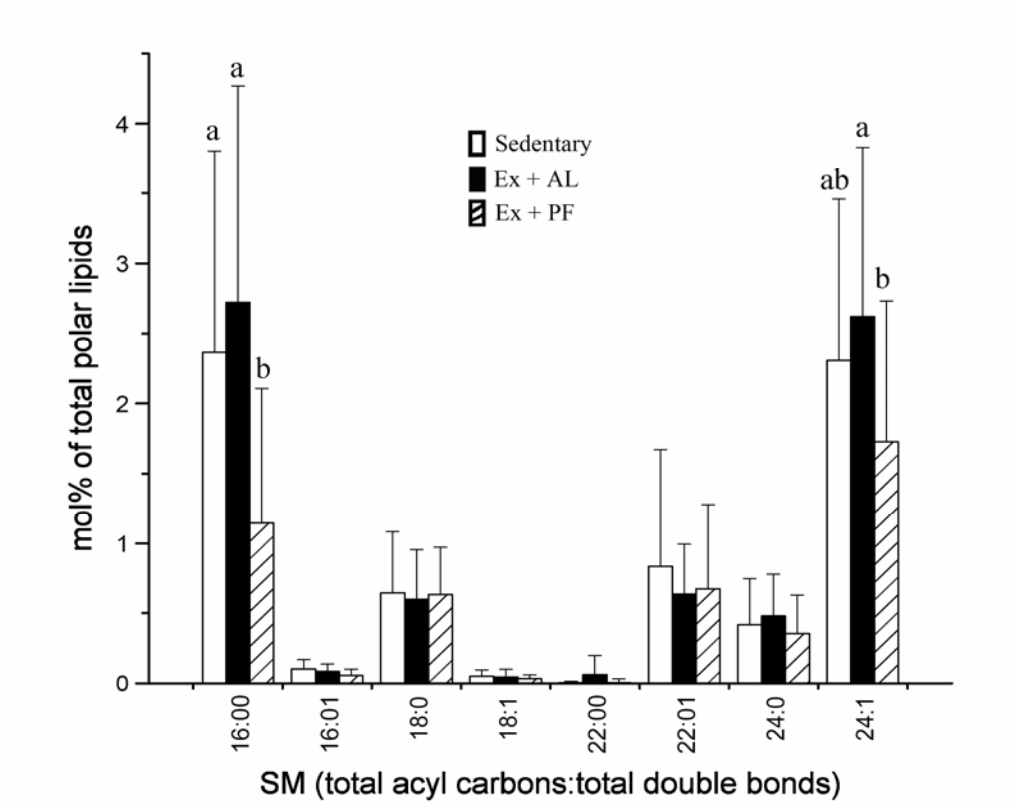

Figure 2-4 Exercise induced sphingomyelin (SM) changes in mice skin tissue.

Sphingomyelins in mice skin samples were detected by ESI-MS/MS. The blank bars represent sedentary mice, the black bars represent Ex+AL mice, and the hatched bars represent Ex+PF mice. The bars with different letters differ significantly ($P < 0.05$, means ± S.D., $n = 8 \sim 15$).

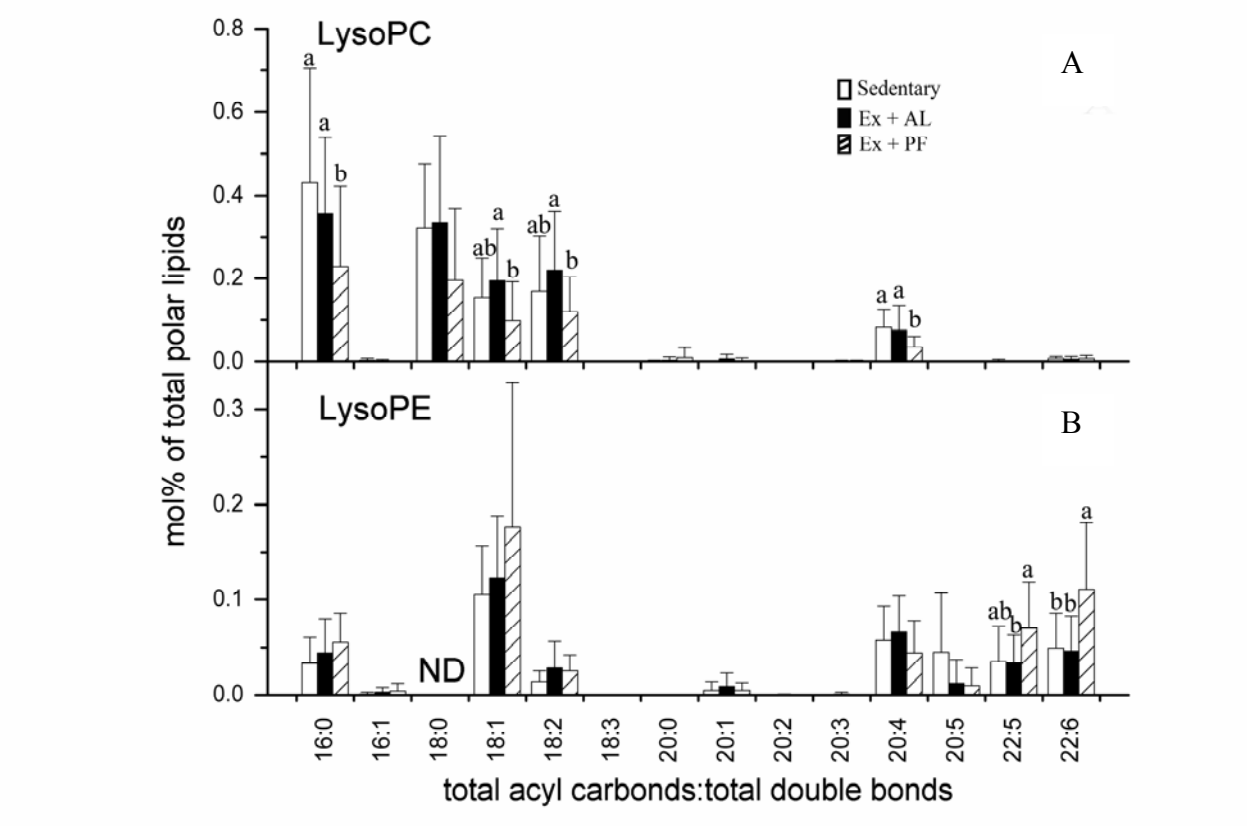

Figure 2-5 Exercise induced changes in lysophospholipids of mice skin tissue.
Lysophosphatidylcholine (LysoPC) (A) and lysophosphatidylethanolamine (LysoPE) (B) in mice skin samples were detected by ESI-MS/MS. The blank bars represent sedentary mice, the black bars represent Ex+AL mice, and the hatched bars represent Ex+PF mice. The bars with different letters differ significantly ($P < 0.05$, means ± S.D., $n = 8 \sim 15$). "ND" represents not detected.

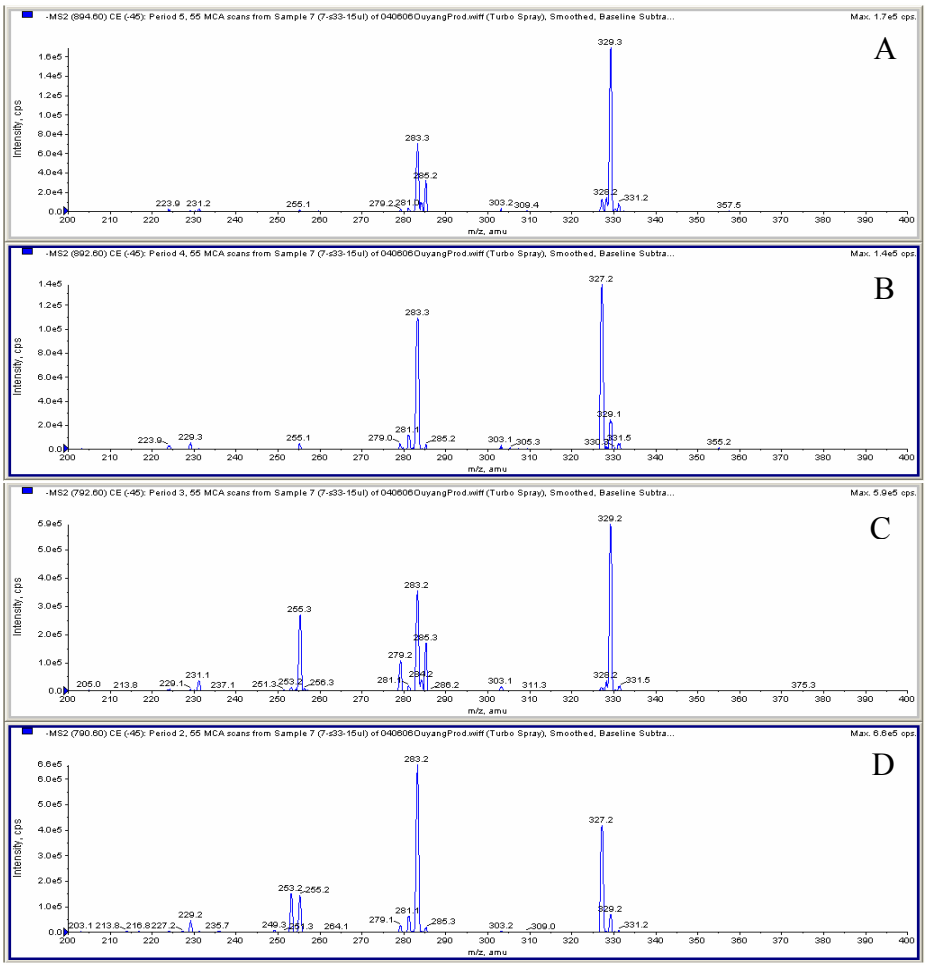

Figure 2-6 Spectral images of acyl group identification. 40:5-PC (A), 40:6-PC (B), 40:5-PE (C) and 40:6-PE (D) from a sample of an Ex+PF mouse were identified for the acyl group composition. The lines show the pair for acyl composition. These product ion analyses were performed on selected molecular ions in the total lipid extract.

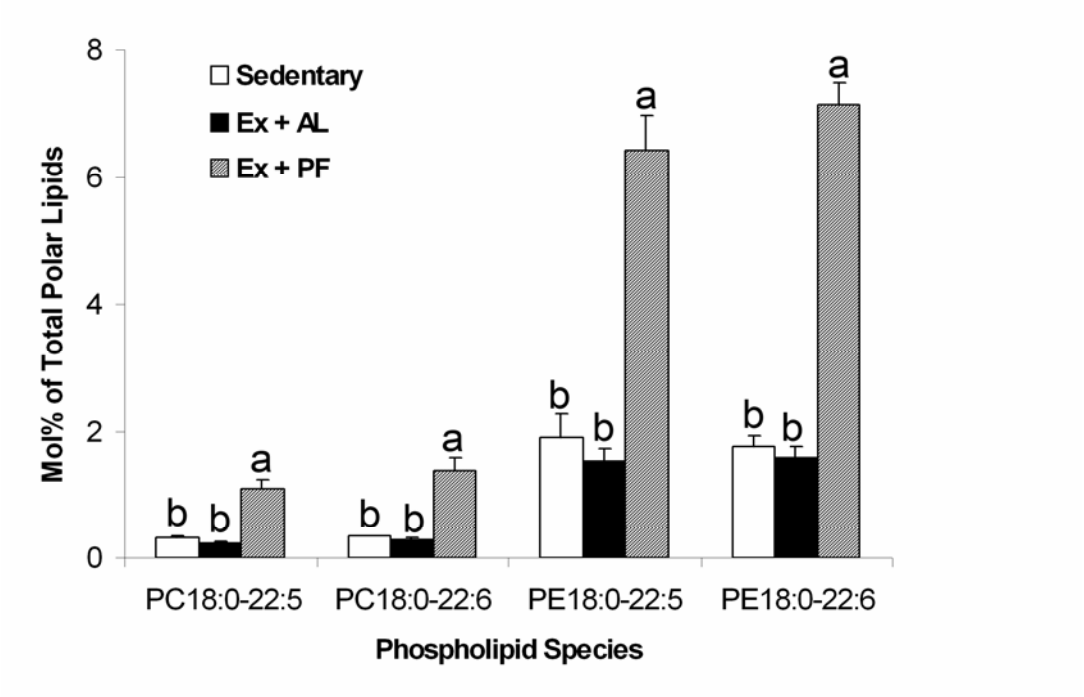

Figure 2-7 Quantification of individual acyl species of some 40:5 and 40:6 phospholipids. The 18:0-22:5 PC/PE and 18:0-22:6 PC/PE were significantly increased in Ex+PF mice compared to the sedentary control as well as Ex+AL mice. The bars with different letters differ very significantly ($P < 0.001$, $n = 4$ for each group).

Phospholipids	Discriminant Functions	
	DF1	DF2
PC28:1	-15.071	3.851
PC32:0	5.465	7.06
PC34:1	-1.171	1.962
PC40:4	-7.352	-0.202
PC40:5	-0.185	-10.099
PC40:6	1.943	8.766
PC42:5	-4.596	0.256
PC44:2	7.085	1.416
PC44:5	-6.527	0.745
EPC32:0	8.266	5.085
EPC32:2	0.706	-3.997
EPC32:3	5.454	-1.055
EPC34:0	-3.755	-4.932
EPC34:1	-10.969	-3.117
EPC36:1	24.831	-2.36
EPC36:2	-6.271	4.669
EPC36:4	-18.957	-0.097
EPC36:5	-6.092	-2.403
EPC38:5	13.323	2.575
LYSOPC16:1	8.051	1.723
LYSOPC18:1	9.275	-5.011
LYSOPC18:2	-28.868	-7.611
LYSOPC20:4	13.928	5.195
PS38:0	-1.268	0.021
EPS36:1	1.99	0.035

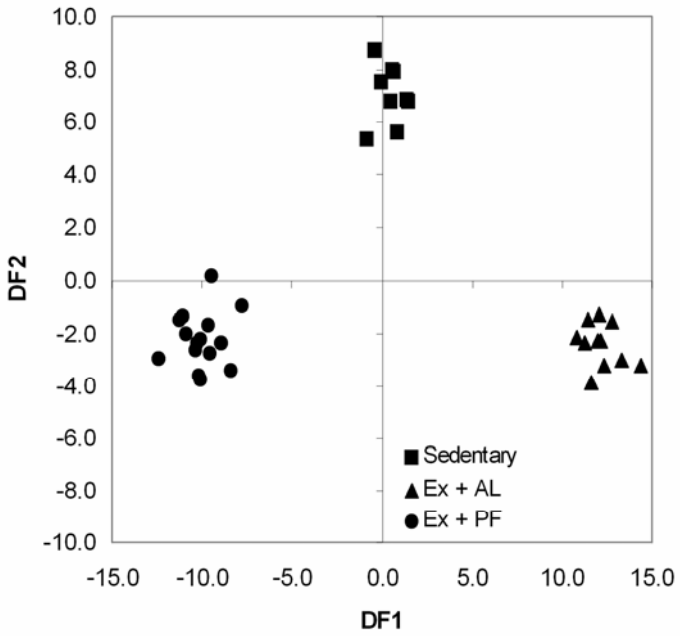

Figure 2-8 Discriminant function analysis of phospholipid profiling data and the phospholipids that distinguish the three treatment groups. X axis represents discriminant function (DF) 1 and Y axis represents DF2. DF1 separated Ex+AL group from the Ex+PF group, and DF2 separated sedentary control group from the two exercise groups. Inserted table shows two discriminant functions generated by automatic backward stepwise discriminant analysis. This process selected 25 lipid variables (Wilks' lambda=0.001 at $p<0.00001$). DF means canonical discriminant functions.

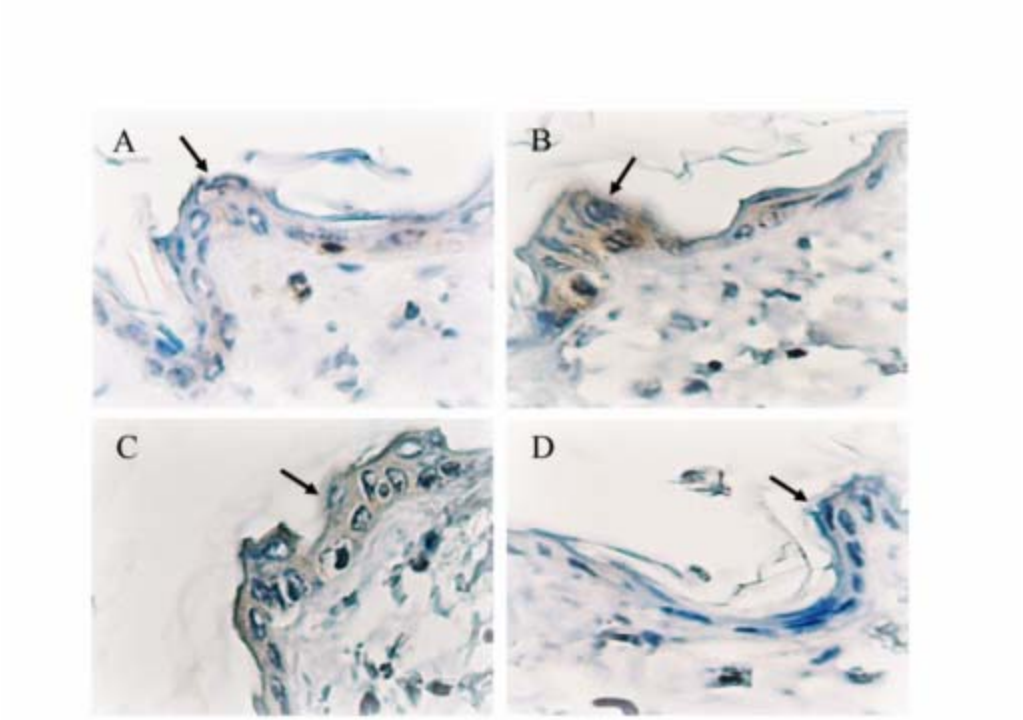

Figure 2-9 Detection of class I PI3K expression in epidermis by immunohistochemistry staining. Staining of PI3K expression by immunohistochemistry of skin tissues obtained from sedentary *ad libitum* control mice applied by acetone (A), sedentary *ad libitum* control mice applied by TPA (B), Ex+AL mice applied by TPA (C), and Ex+PF mice applied by TPA (D). Arrows point the PI3K expression in brown color stained by DAB (3,3-diaminobenzidine) in epidermal cells.

CHAPTER 3 - Profiling of gene expression in skin tissues of exercised or weight-controlled via exercise mice

The abbreviations used are: Ex+AL, exercise with ad libitum-feeding; Ex+PF, exercise and pair-feeding; PA, phosphatidic acid; PC, phosphatidylcholine; PE, lysoPC, lysophosphatidylcholine; lysoPE, lysophosphatidylethanolamine; PI3K, phosphatidylinositol 3-kinase; TPA, 12-O-tetradecanoyl phorbol-13-acetate; IGF-1, insulin-like growth factor 1; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; TNFR, tumor necrosis factor receptor; PPAR-α, peroxisome proliferator activated receptor alpha; Hsp, heat shock protein; Elovl1, elongation of very long chain fatty acids-like 1; Elovl6, elongation of long chain fatty acids, member 6; DMH, dimethylhydrazine; IL-1β, interleukin-1β; SOD, superoxide dismutase; GPx, glutathione peroxidase; GLUT4, glucose transporter-4; ORF, open reading frames; SNPs, single nucleotide polymorphisms; DAG, diacylglycerol; HFD, high-fat diet; MT, metallothionein; PCR, polymerase chain reaction; P, present; A, absent; M, marginal; ANOVA, analysis of variance; FDR, false discovery rate; PCA, principle component analysis; GOI, gene of interested; HK, house keeping gene; PPIase, peptidyl-prolyl isomerase; SPRR, small proline-rich protein; TNFR, tumor necrosis factor receptor; ZNF 185, zinc finger protein 185; IGFBP-3, insulin-like growth factor binding protein 3; GTPase, guanosine triphosphatase; Figf, c-fos-induced growth factor; Vegf-D, vascular endothelial growth factor D; L-PGDS, lipocalin-type PGD synthase; FAD2, fatty acid desaturase 2; and PLA_2, phospholipase A_2.

Abstract

Increasing physical activity and body weight control has been linked to a reduced cancer risk. However, the underlying mechanisms are not well understood. This study assessed the impact of exercise with or without limited dietary calorie intake on the gene expression in mouse skin tissues. SANCAR mice were randomly assigned into one of the three treatment groups: *ad libitum*-fed sedentary control (sedentary), *ad libitum*-fed treadmill exercise at 13.4 m/min for 60 min/d, 5 d/wk (Ex+AL), and exercise but pair-fed at the amount as the sedentary control (Ex+PF). After 10 wks, Ex+PF but not Ex+AL mice demonstrated a significant decrease in both body weight and percentage of body fat when compared to the sedentary controls. The results of gene expression showed that among the 45,101 probe sets tested in skin tissues of four mice randomly chosen from each treatment group, 960 probe sets related with 839 genes were significantly changed by exercise training with or without limited calorie intake. The expression of some genes products related with oxidative stress, inflammatory response, lipolysis, protein synthesis, and signaling pathway have been found modified by exercise training. For example, the expression of s100 calcium binding protein family, tumor necrosis factor receptor (TNFR) superfamily, peroxisome proliferator activated receptor alpha (PPAR-α), heat shock protein (Hsp) family, protein kinase inhibitor, phospholipase A2, and elongation of long chain fatty acids-like member 6 (*Elovl6*) were significantly altered in exercise alone or body weight loss via exercise and pair feeding mice. Down-regulation of phospholipase A2 and up- regulation of *Elovl6* in exercise and pair fed mice mostly likely lead the decreased level of lysophoplipids and increased level of 18:0-22:5PC/PE and 18:0-22:6 PC/PE in exercise and pair fed mice demonstrated in our previous phospholipid profiling study. These data indicate that comparing with exercise alone, controlling body weight via exercise with limited dietary calorie intake modified higher number of gene expression, and might contribute even more for anti-inflammatory response and reducing cancer risk.

Introduction

Overweight and obesity might reflect a life style of over-consumption of calorie or less expenditure of energy which associated with increased risk of various kinds of chronic diseases. Numerous evidence from epidemiological, prospective cohort, and intervention studies has shown that physical activity and diet as well as the combined exercise and diet interventions could mitigate the progression of chronic diseases (1). The benefits of physical activity have been found to control body weight, lower body fat, improve human health and then lead a better quality of life through either improving bodily function or preventing some chronic diseases including cardiovascular disease, obesity, diabetes, arthritis, and cancer (2, 3). Recently, a review paper concluded that independent of weight loss, physical activity has been suggested to have specific effect on cancer prevention (4)

Animal models are commonly used on cancer prevention (5, 6). The well known study was published in 1987 by Andrianopoulos and co-workers (7). After intraperitoneally injected carcinogen dimethylhydrazine (DMH) to Sprague Dawley rats for 6 weeks, the incidence and total number of tumor were much lower in the wheel running mice than that of control. In 2006, Michna and co-workers reported that studies on voluntary wheel running SKH-1 mice showed exercise inhibited UVB-induced skin carcinogenesis significantly in delaying the appearance of tumors, decreasing the number of tumors per mouse, and reducing tumor volume per mouse even without losing body weight due to the increased consumption of food (6).

The biological mechanisms of physical activity benefits for cancer prevention were not well understood. Some postulated explanations have been suggested to: 1) induce antioxidant defense mechanism; 2) change growth factors level; 3) reduce metabolic hormone and sexual hormone levels; and/or 4) improve immune function (4).

The expression of some genes products related with oxidative stress, inflammatory response, lipolysis, protein synthesis, and signaling pathway have been found modified by exercise training. The influence of physical activity on tumor necrosis factor-α (TNF-α) signaling has been detected in rat epididymal adipocytes by Sakurai and co-workers (8), and up-regulated expression of survival signals and the ability of protect against oxidative stress in adipocytes were induced by enhanced TNF-α via exercise training. Strenuous exercise appears to elevate interleukin-1β (IL-1β), IL-6, and TNF (9-10), which further enhanced IL-8 (9) and serum C-reactive protein concentration (11) as well as activated and accumulated neutrophils in skeletal

muscle (12). In addition, superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) were also found increased in exercised animals (13). Thus physical activity showed the modification of inflammatory response and appeared to restrict inflammation stimuli (3). Exercise training also elevated the amount of transporters of glucose transporter-4 (GLUT4) which altered the cell membrane sensitivity to insulin and increased glucose and amino acids transportation (14).

In the past ten years, DNA microarray has become a powerful and essential tool for gene expression analysis, and "so far, DNA microarray technologies are perhaps the most successful and mature methodologies for high-throughput and large-scale genomic analyses" (15). DNA microarray technologies were designed initially for measuring the variation of RNA transcription of enormous genes within a single experiment, and allow the researchers to investigate the gene expression related to physiological cell states for responding of stimuli and pressure, disease progression, and drug target identification (15).

Tens of thousands of gene-specific single-stranded DNA sequences, mostly derived from the 3'end of RNA transcripts, are hosted on glass or silica platforms which are called "chips" in DNA microarrays. Messenger RNA extracted from tissues or cell cultures are labeled with biotin or fluorescent dye and hybridized to DNA microarrays. After washing off the unbounded or not strongly bounded material in salt buffers, the platform goes to the scanner for signal detection. The sample gene expression can be estimated through the signal intensity which represents the relative proportion of each labeled sequence (16).

According to the type of spotted probes, DNA array can be classified into two mainly types. Affymetrix (Santa Clara, CA, USA) which is considered as the leading provider of one type uses small single-stranded oligonucleotides (around 22 nucleotides) synthesized in situ. The other one uses cDNA or open reading frames (ORF) as the bound probes (15). The advantages of Affymetrix GeneChips are: 1) very high uniformity and reproducibility; 2) extremely high density of genes per chip (~20,000 genes/chip); 3) direct comparability among experiments. The disadvantages include: 1) relatively expensive (~$700/chip); 2) hardly justified for theory of "average difference"; 3) hybridization is affected by DNA polymorphism (Kansas State Microarray workshop given by Dr. Greg Gibson). DNA microarray technologies have been applied in more extensive areas for biomedical studies recently such as detecting single

nucleotide polymorphisms (SNPs) of human genome, pathogen detection, gene copy-number changes, RNA splicing alternation, and methylation pattern aberrations (15).

Several studies have been published about using microarray technique for investigating the alteration of gene expression in skin tissues associated with the effects of skin carcinogenesis. In 2003, Schlingemann and co-workers published the results of gene expression changes induced by tumor promoter TPA in mice epithelial cells (17). Tumorigenesis is a very complicated process involved in genome integrity, DNA damage and repair, cell proliferation, differentiation, and cell program death. In order to offer dependable information of biomarkers for cancer prevention and diagnostic, as well as novel targets for cancer therapy, it is essential to understand the molecular mechanism of tumorigenesis. The variation of RNA expression is associated with the amount of protein expression and will further alter the biochemical activities (17). Phorbol esters including TPA are powerful and prevalent tumor promoters of carcinogenesis in mouse models. They mimic the results of diacylglycerol (DAG), a second messenger, and induce a series of PKC isoforms, then further effect the gene regulation and cellular signaling pathways (18). Therefore, genes associated with cell cycle, epithelial differentiation, cell metabolism, transcriptional regulation and signal transduction could be affected by TPA. Schlingemann and co-worker using microarray hybridizations found 54 cDNA were up-regulated and another 35 were down-regulated in dorsal skin tissues six hours after phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment. Some genes which were demonstrated to be associated with cancer development were triggered by TPA such as metallothionein, s100 calcium binding protein family, omithine decarboxylase, cathepsin, Jun-B oncogene, secretory leukocyte protease inhibitor, and small proline-rich protein family and so on were conformed to up-regulated. Among them, secretory leukocyte protease inhibitor, s100 calcium binding protein family, as well as serum amyloid A3, interleukin 4 receptor, alpha, and tumor necrosis factor receptor family number which were also up-regulated by TPA application have been shown to relate with the processes of epidermal hyperproliferation and skin inflammation (17).

In 2006, Lee and co-workers reported that exercise training altered the expression of genes related to metabolism, transport, stress and defense on hepatic tissue in high-fat diet (HFD) induced obese mouse (19). Totally 73 gene expression were changed by HFD-fed with or without exercise compared to normal diet and no exercise control. Exercise training was found to

normalize some gene expression that were down-regulated by HFD-fed such as elongation of very long chain fatty acids-like 2 which related to fatty acid biosynthesis, metallothionein 1 and 2 which are antioxidant factors, P450 (cytochrome) oxidoreductase which is a gene-encoding enzyme of electron transport, as well as moderate some gene expression that were up-regulated by HDF-fed such as the heat shock 70-kDa protein 5 families which involved in immune responses. Eleven gene transcripts showed alteration by exercise combined with HFD-fed mice, but not HFD-fed alone. For example, Lipin 1, involved in regulation of adipogenesis was up-regulated by exercise, while serum amyloid P-component (Apcs) and malic enzyme were down-regulated which participated in acute phase responses and lipogenesis, respectively. Interestingly, they found that the expression of metallothionein and heat shock protein (HSP) which related to stress response were on the contrary, and they suggested this may due to the interaction of antioxidant and repair systems.

Considering the limitation of microarray analysis such as huge data obtained from very few sample size, normally quantitative real-time RT-PCR (qPCR) has been widely used for confirming the microarray data and becomes the most powerful technique for analysis the quantitative of nucleic acids in scientific, diagnostic and medical communities. Polymerase Chain Reaction (PCR) was developed by Kary Mullis and colleagues in 1980s, and the 1993 year's Nobel Prize in chemistry was awarded to Kary Mullis for this achievement (20). In 1992, Higuchi and co-workers (21) developed real-time PCR based on the original PCR technique which provides the possibility for calculating the initial amount of DNA molecules in the sample more sensitively and accurately through monitoring the amount of product formed during the reaction using fluorescent reporter which binds to the product. Currently, real-time PCR has been widely applied in gene expression analysis, pathogen detection, chromosome aberrations analysis, analysis of single nucleotide polymorphism (SNP), and protein detection via real-time immuno PCR (20).

The previous works in Dr. Wang's lab reported that physical activity combined with limited calorie intake down-regulated PI3K pathway. Some cancer related gene expression alteration in exercise and 20% dietary calorie restriction mice, and the results further showed different influence on signaling pathway between exercise and 20% dietary calorie restriction (22, 23). My study is focused upon the different effect of exercise alone and exercise combined with limited calorie intake on the phospholipids profiling and gene expression alteration.

In this study, we focused on the modification of gene expression related with cellular process and molecular function of lipid metabolism. We hypothesized that exercise alone and exercise with pair-fed had different effects on mice gene expression related with cell cycle, cell death, cell proliferation and differentiation, apoptosis, and lipid metabolism. Especially, decreased PI-related PI3K pathway might restore apoptosis induction. Furthermore, down-regulated phospholipase A_2 in exercise and pair fed mice may likely lead to the reduced level of lysoPC, and up regulated elongation of very long chain fatty acid, member 6 might be partially explained the increased of 18:0-22:5PC/PE and 18:0-22:6PC/PE level. The results would suggest exercise combined with limited calorie intake should contribute cancer prevention at least via apoptosis enhancement and anti-inflammatory response mechanisms.

Experimental procedures

Animals and animal treatments — Female SANCAR mice purchased from National Institute of Health (Frederick, MD, USA) obtained at 8 weeks old were randomly divided into three treatment groups: sedentary and *ad libitum* (sedentary), exercise and *ad libitum* feeding (Ex+AL), and exercise and pair feeding (Ex+PF). *Ad libitum* groups 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. Water was provided *ad libitum*. A speed adjustable rodent treadmill (Boston Gears, Boston, MA, USA) was used for mice exercise. Exercise training started from 6.7 m/min for 5 min and increased gradually in intensity and duration in the first two weeks training until the 13.4 m/min and one hour per day was reached. Then exercise groups ran on the treadmill 5 days a week for 10 weeks during the entire experiment. All mice were housed individually at 24 ± 0.6ºC and 80% relative humidity with 12 hr light/12 hr dark cycle. The light cycle was adjusted to run nighttime exercise at 0400-0500 hrs.

Mice dorsal skin was shaved, followed by treatment with 3.2 nmol 12-O-tetradecanoylphorbol-13-acetate (TPA) which was dissolved in acetone. Dorsal mouse skin was collected two hours after acetone or TPA treatment, snap-frozen in liquid nitrogen immediately, and then stored at -80ºC for following analysis.

Microarray analysis—Skin tissues from four mice were chosen randomly from each treatment group for the microarray analysis. Each skin tissue was homogenized in TRIZOL® Reagent (Invitrogen, Carlsbad, CA, USA) at ratio 0.4 g tissue: 1 ml reagent. Total RNA was purified by using the RNeasy cleanup kit (Qiagen Inc., Valencia, CA, USA), and the RNA quality and quantity were measured by Agilent 2100 Bioanalyzer (Agilent Technologies, Inc. Santa Clara, CA, USA). After the annealing of total RNA (10 μg) with 100 pmol T7(dT)24 at 70.0ºC for 10 mins, the annealed mRNA was reverse transcribed into cDNA using Superscript Choice System Kit (Invitrogen Crop., Carlsbad, CA, USA). Enzo BioArray High-Yield RNA Labeling Kit (Enzo Diagnostics, Farmingdale, NY, USA) was used for synthesizing biotinylated antisense RNA followed by purification with the RNeasy RNA Purification Mini Kit (Qiagen Inc., Valencia, CA, USA). Then, 20 μg biotin-labeled cRNA was incubated in fragmentation buffer (40 mM Tris-acetate, 100 mM postassium acetate, 30 mM magnesium acetate, pH 8.1) at 94ºC for 35 mins. Finally, the labeled cRNA was applied to GeneChip Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA, USA) which contained 39,000 transcripts with 45,101 probe sets, and after hybridization and wash, the intensity was scanned by Affymetrix equipment. Microarray analysis was conducted by the Microarray Core of the Mental Retardation Research Center at the University of Kansas Medical Center (Kansas City, KS, USA).

Analysis of Microarray Data—The intensity of probe sets were quantified by GeneChip operating software 1.0 (GCOS 1.0; Affymetrix). First, normalizations were applied via filter by flag. The intensity of probe sets considered present (P) represents that the p value was lower than 0.04, absent (A) represents p value greater than 0.06, and marginal (M) represents p value between 0.04 and 0.06. Data labeled by M and A were excluded, and screen the data by 4 out of 12 present (P) for each probe set to increase the accuracy and reliability. The data were normalized again by taking log 2. One–way ANOVA and False Discovery Rate (FDR, Benjamini, $p < 0.1$) were applied to identify the gene expression difference between the treatment and control group. Then the data were filtered by using 1.5 fold changes as a cutoff. Volcano plots and scatter plots were analyzed by GeneSpring Software (Agilent Technologies, Inc. Santa Clara, CA, USA). Principal component analysis (PCA) was applied to analyze the similarity of the three treatment groups. Finally, Gene Ontology Slims were applied to classify

the categories based on cellular process or molecular functions that significantly modified genes involved.

Gene expression for Elovl family—Gene expression of elongation of (very) long chain fatty acids family was further analyzed due to their function involved in (very) long chain fatty acid synthesis, since 18:0-22:5 PC/PE and 18:0-22:6 PC/PE have shown statistically significant increased in exercise with pair feeding mice obtained in the former study.

Real-Time PCR—Mouse skin tissues were collected 2 hrs after TPA application. Total RNA was isolated using TRIZOL® Reagent (Invitrogen Crop., Carlsbad, CA, USA), and was purified by TRIZOL® Plus RNA Purification Kit (Invitrogen Crop., Carlsbad, CA, USA). After measuring the concentration of the RNA, RNA quality was tested by the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc. Santa Clara, CA, USA). The total RNA was reverse-transcribed into cDNA using SuperScript ™ III First-Strand Synthesis System (Invitrogen Crop., Carlsbad, CA, USA). The PCR was conducted using cDNA as template, and primers of gene elongation of very long chain fatty acid-like 1 (*Elovl1*) and elongation of very long chain fatty acid-like 6 (*Elovl6*) which shown significantly increased in microarray analysis in Ex+PF mice were designed by IDT software (Integrated DNA Technologies, Inc., Coralville IA, USA). The primers for (*Elovl1*): forward: 5'-CCCAAGTGTCCTCTCTACCTAC-3', reverse: 5'-GAAGTAAGTGTCCA GCCCTTTC-3'; the primers for gene *Elovl6*: forward: 5'-CGGCAAAGTGAAGAAAGCCACGAA-3', and reverse: 5'-GGCAACTGAACAACGGATGCTTGA-3'. The products were analyzed on 1.0% agarose gels to check the molecular weight and specificity. After the PCR products were purified using Qiagen PCR Product Purification kit (Qiagen Inc., Valencia, CA, USA), they were sent to DNA Sequencing and Genotyping Facility of Kansas State University using Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) for DNA sequencing. Then the double-stranded DNA fragments from PCR reactions was purified from primers, nucleotides, polymerases, and salts using MinElute spin columns (Qiagen Inc., Valencia, CA, USA), and were used as template for real-time PCR analysis.

Real-time PCR was performed for the genes in *Elovl* family which showed 1.5 fold changed and FDR < 0.1 in microarray analysis in Ex+PF mice. Primers were designed using

Beacon Designer (v2.0) software (Biosoft International, Palo Alto, CA, USA) by avoiding cross homology and template structure such as cross dimmer, self dimmer, hairpins, runs and repeats. The product length was set from 75 bp to 200 bp for accuracy control. Then, the primers quality evaluated with best or good were selected for real time PCR reaction. The primers for *Elovl1* forward: 5'-TGATGTCTGGTTGGCTGAG-3', and reverse: 5'-GTCACTTGCCCGTCCTTC-3'; the primers for *Elovl6* forward: 5'- GTTGTAAGTTTATGATCCTTTC-3', reverse: CTAATCTCTGCCCAATCTC-3'; the primers for peptidyl-prolyl isomerase (*PPIase*) as a house keeping gene: forward: 5'-CTCCAGCGTGAGTTCTTC-3', reverse: 5'-TTTGCGTGGGATGTGTAG-3'. Real time PCR reactions were conducted by a Bio-Rad iCycler real-time detection system (Bio-Rad Hercules, CA, USA) using iQ SYBR Green Supermix. The efficiency of all primers was tested first by serial dilution of the cDNA to generate a calibration standard curve, then the measurement of the gene expression was set as following: 95ºC for 5 min first and followed by 45 cycles of denaturation at 95ºC for 20 s and annealing at 53ºC for 45 s. Then after all the cycles, the amplification specificity of PCR was verified by obtaining the dissociation curve. Samples were set at 55ºC, then totally run 80 cycles with 0.5ºC increase for every cycle and 10 s treatment for each cycle. For every sample measurement, results obtained by calculating the mean of values got from at least two independent PCR reactions and from more than two independent biological replications.

Fold change was calculated using the following formula:

$(1+\text{Efficiency of GOI})^{-\Delta CtGOI} / (1+\text{Efficiency of HK})^{-\Delta CtHK}$

GOI represents for gene of interested, and HK represents for house keeping gene.

Results

Microarray analysis— By using Affymetrix microarray analysis for messenger RNA expression in the mice skin tissues collected two hours after TPA application, totally 960 probe sets were filtered out by 1.5 fold change combined with FDR less than 10% analysis through the expression of 39,000 transcripts with 45,101 probe sets. Because some genes were tested by more than one probe set, there were 839 gene expressions statistically significant altered by treatment after deleting the redundant. Among them, 417 genes significantly changed by exercise

with *ad libitum* feeding (Ex+AL, Fig. 3-1 left), and 626 genes significantly altered by exercise with pair feeding (Ex+ PF, Fig. 3-1 right) compared to sedentary control shown in the volcano plots. Gene expression modified in both exercise groups with the same direction or reverse directions existed. The x-axis represents the fold change, and the y-axis represents the minus log $_{10}$ p-value. The spots above the horizontal green line represent the gene expression change with p-value less than 0.1 for FDR.

The results of PCA analysis showed in figure 3-2. PC1 represents 77.9% of the variance, and PC2 represents 4.9% of the variance. Three treatment groups were separated by PC1 and PC2, and with no overlapping.

When comparing the gene expression change of Ex+AL to that of sedentary control mice, 300 gene expression was increased including keratin associated protein family; breast carcinoma amplified sequence 1; and caspase 14, while 117 gene expression was decreased such as caspase 4, apoptosis-related cysteine protease; and c-fos induced growth factor. For Ex+PF mice, the expression of 349 genes was enhanced, and the expression of 277 genes was reduced compared to that of control. For example, Ex+PF increased the expression of interleukin 7 receptor, caspase recruitment domain family, member 10; elongation of very long chain fatty acids-like 1 and member 6. While Ex+PF decreased the gene expression for ras homolog gene family; prostaglandin D2 synthase; neuroblastoma ras oncogene; neuroblastoma myc-related oncogene 1; myeloblastosis oncogene; and myeloblastosis oncogene-like 2. Exercise with or without limited dietary calorie intake altered the expression of genes participated in different cellular processing and molecular functions such as cell cycle, cell death, cell proliferation, cell differentiation, apoptosis, response to stress, and lipid metabolism (Table 3-1).

Gene or gene family expression change was also found in both exercise groups with the same direction or with reverse directions. The expression of 16 genes or gene families was elevated in both exercise groups including cytochrome P450, family1, 2, 4 & 11, zinc finger protein 185 and subfamily 1A, solute carrier family, procollagen, and kinesin family, member 7 & 21A. The expression of 15 genes or gene families was lowered in both exercise groups such as B-cell receptor-associated protein 37; transglutaminase 2 & 3; s100 calcium binding protein A8 in Ex+AL group and s100 calcium binding protein A3 in Ex+PF; and heat shock protein family, member 7. There were 23 genes and gene families which expression was decreased in Ex+AL, but increased in Ex+PF mice. For example, human immunodeficiency virus type I enhancer

binding protein 2 was enhanced in Ex+AL mice, but human immunodeficiency virus type I enhancer binding protein 3 was reduced in Ex+PF mice; and ATP-binding cassette, sub-family A, member 5 & 8a and sub-family B, member 1B were increased by Ex+AL mice, while ATP-binding cassette, sub-family C, member 9 was decreased in Ex+PF mice. More gene expression (about 84) changed in reverse direction were found in Ex+AL up and Ex+PF down group. For example, prostaglandin D2 synthase, arachidonate 15-lipoxygenase, cathepin 3; lipin 3; ATPase, H+/K+ transporting, nongastric, alpha polypeptide; fatty acid desaturase 2; G protein-coupled receptor, family C, group 5, member D; lymphoid enhancer binding factor 1; myeloblastosis oncogene; myeloblastosis oncogene-like 2; neuroblastoma myc-related oncogene 1; s100 calcium binding protein A3; transglutaminase 3, E polupeptide; uridine phosphorylase 1; vitamin D receptor; phospholipase A2, group IIE increased in Ex+AL group, but phospholipase A2, group IIE and group IVB decreased in Ex+PF group. Some more complicated expression obtained in the treatment groups that Ex+AL enhanced small prolline-rich protein 2A, while Ex+PF increased small prolline-rich protein like2, 5, 7, & 10, but decreased small proline-rich protein 2F. Tumor necrosis factor receptor (TNFR) superfamily, member 12a was reduced in both exercise groups, but Ex+PF enhanced the expression of tumor necrosis factor receptor (TNFR) superfamily, member 18, 21 and tumor necrosis factor, alpha-induced protein 3.

Gene expression for Elovl family—All members of Elovl family could be detected in mice skin tissues by microarray analysis included 11 probe sets for 5 *Elovl* genes. The gene expression for Ex+AL verse control (Fig. 3-3, left), and Ex+PF verse control (Fig. 3-3 right) was shown in scatter plots. *Elovl2* could not be detected in this study due to the specific expression found in liver only. The expression of *Elovl* family had not been altered much in Ex+AL mice compared to control, but *Elovl1* and *Elovl6* were increased in Ex+PF mice compared to control. Four measurements of different probe sets for *Elovl1* were all increased with the highest of 2.2 fold. The expression for one of *Elovl6* probe set showed 1.68 fold enhanced. No large decreased expression was found for this family in both exercise groups compared with control.

Real-Time PCR—Real-time PCR was performed to measure the expression alteration for *Elovl1* and *Elovl6* using the peptidyl-prolyl isomerase (*PPIase*) as the housekeeping gene. The efficiency of primers for *Elovl1*, *Elovl6*, and *PPIase* were 0.728, 0.974, and 0.964, respectively.

Results showed that the gene expression of *Elovl1* was 0.58 fold compared to that of control, and gene expression of *Elovl6* was 3.4 fold compared to that of control (Fig. 3-4).

Discussion

The microarray technique is a powerful tool for analyzing the alteration of global gene expression affected by treatment. Totally, the expressions of 839 genes were statistically significant modified by exercise alone and exercise with pair feeding compared to that of sedentary control. Generally, exercise combined with limited calorie intake which leads to body weight loss affected more gene expression than exercise alone did.

Several genes trigged in skin tissues by TPA application illustrated in Schlingemann's study were affected by exercise with or without limited calorie intake in the response for TPA application in our study. Metallothionein, omithine decarboxylase, Jun-B oncogene, and secretory leukocyte protease inhibitor were not significantly changed in the two exercise groups which mean exercise normalized the expression of these genes to the same level of control. s100 calcium binding protein family, A3 was also enhanced by TPA application in exercise alone mice, but it was down-regulated in exercise and pair fed mice, and s100 calcium binding protein family, A8 was also down-regulated in exercise alone mice. This protein family expresses specifically in skin tissue and has affinities for Ca2+ (Zn2+ or Cu 2+ in some cases) and interacts with 40 target proteins. They regulate protein phosphorylation, cell proliferation, differentiation, and inflammation. Their altered expression has been found associated with several human disorders including cancer, inflammation, diabetes, neurodegenerative diseases, cardiomyopathies, and allergies (24, 25). The high level of s100 A8 has been identified in tumor associated stroma (26) and tumors such as pancreatic, breast, colorectal, and lung cancer (27). Down-regulation of s100 calcium binding protein family in the exercised mice might be benefit for reducing cancer risk. Cathepsin E showed up-regulation in exercise alone mice, but was down-regulated in exercise with pair fed mice. Cathepsin E locates and expresses mostly in endosomal structures of antigen-presenting cells and involves in immune responses (28). The expression of some small proline-rich protein (SPRR) family (e.g. SPRR 2A in exercise alone mice, SPRR like 2, 5, 7 & 10 in exercise with pair fed mice) was up-regulated also in this study, but exercise with pair feeding down-regulated the SPRR 2F expression. Controlling the dosage

of SPRR protein has shown functional importance, and was regulated coordinately in high expressed epidermal differentiation complex in papillomas (29). Interleukin 7 (IL-7) receptor has been up-regulated in exercise and pair fed mice in this study. The homeostasis, development, and proliferation status of T and B cells in cell-mediated immunity was critically regulated by the IL-7 signaling pathway. IL-7 binding to its alpha-receptor can stimulate the IL-7 pathway. Over- or under-regulation of the IL-7 signaling pathway resulted in serious autoimmune reactions, immunodeficiency, heart problems and cancers (30). Similar as the results found by Schlingemann's study that the expression of tumor necrosis factor receptor (TNFR) superfamily, member 18, 21 and tumor necrosis factor, alpha-induced protein 3 was enhanced by TPA application in exercise with pair feeding mice, however, TNFR superfamily, member 12a was down-regulated in both exercise groups in this study. TNF α is a cytokine with multifunction produced by activated macrophages, T lymphocytes, and some epithelial tumor cells. TNFRs on the cell surface from wide range of cell types mediate the activities triggered by THF α. TNFR I contains a high conserved "death domain" and induce cell death, and TNFR II mostly mediate cell proliferation (31, 32).

In addition to these genes, numerous genes related with cell proliferation, differentiation, apoptosis, participated in PI3K/Akt signaling pathway, and affected disease processes have been affected by exercise with or without moderate calorie intake. For example, zinc finger protein 185 (*ZNF 185*) was enhanced in both exercise groups. *ZNF 185* is a novel LIM domain gene and regulates cellular differentiation and proliferation. In human tissues, the expression of it is limited, but most found in prostate, and its expression has been shown negatively associated with prostate cancer progression (33). The increased level of *ZNF185* might due to the exercise training alone. Insulin-like growth factor binding protein 3 (IGFBP-3) and peroxisome proliferator activated receptor alpha (PPAR-α) were up-regulated by exercise with pair feeding. IGFBP-3 is the most abundant binding protein exists in circulation, and can inhibit cell growth by competitively binding with IGF-1. In addition to this, IGFBP-3 can also induce apoptosis (34). PPAR-α is intracellular transcription factor which activated by fatty acids and plays an important role in anti-inflammation through inhibition of NF-kB activity and suppressing inflammatory response (35, 36). The expression of heat shock protein (*Hsp*) family, member 7 was down-regulated in both exercise groups, and the moderate expression has been also found in Lee's study in HFD-fed exercised mice. In our study, the mice were fed normal diet. Thus,

exercise showed down-regulation of *Hsp* which expression was demonstrated positively associated with cell proliferation, antiapoptosis, tumor development, metastases of lymph node, resistant to chemotherapy, and lower survival (37). With no questions, decreased *Hsp* expression by exercise would benefit for cancer prevention. Interestingly, protein kinase inhibitor alpha and beta were up-regulated also in exercise with pair feeding mice. Activated protein kinase leads to expression of inflammation genes through enhancing NF-kB and Ap-1 transcriptional activity (38). Cell signaling pathway mediated by protein kinase has been proved related to many diseases such as inflammation, cancer, and diabetes via regulating cell metabolism, cell cycle, proliferation, DNA damage repair, and apoptosis. Increased protein kinase inhibitor might lead to down-regulate a number of signaling pathway including PI3K/Akt and MARK signaling pathway, and promote cell apoptosis (39). The results also showed the gene expression of ras homolog gene family, member f and G were reduced in exercise with pair fed mice. Ras which is a small guanosine triphosphatase (GTPase) bind the guanine nucleotides GTP and becomes active form that affects down stream proteins and associates with cancer. High activity of unregulated Ras has been well accepted in forming cancer (40). Gene expression of c-fos-induced growth factor was down-regulated in exercise alone mice. The expression of c-fos-induced growth factor/vascular endothelial growth factor D (Figf/Vegf-D) is controlled by the nuclear oncogene *c-fos*, and might play an important role in tumor cell growth, invasion, and angiogenesis (41).

Many gene expression has been found up-regulated by exercise alone but down-regulated by exercise with pair feeding in our study such as p55 regulatory subunit of phosphatidylinositol 3 kinase, Vitamin D receptor, prostaglandin D2 synthase, phospholipase A2, and fatty acid desaturase 2. PI3K is a critical component in growth factor regulated pathway and induce inhibition of apoptosis, and which was illustrated reduced in exercise with pair feeding mice by immunohistonchemistry in the former study discussed in chapter 2. The results of immunohistonchemistry study also showed protein expression increased in TPA treated exercise alone mice, while reduced in TPA treated exercise with pair feeding mice. However, the PI3K we tested in immunohistonchemistry study was p85 PI3K, but not p55, which gene expression was not found significantly changed. The effect that 1,25 $(OH)_2D$ induces differentiation and apoptosis, and could inhibit tumor cell invasion and metastasis in breast and other cells has been illustrated by numerous studies. In breast and other tissues, the vitamin D receptor regulates the

1,25 $(OH)_2D$ effects and reduce cancer risk (42). Prostaglandins (PGs) have been found with large amount in inflammatory tissues which play important role in lipid regulation, and induction of lipocalin-type PGD synthase (L-PGDS) has been regulated negatively by p53, but positively by Ap-1. PGD2 involves in many essential processes including pain response, inflammation and allergy (38).

In addition to PGDS, there were more altered expression for genes involved in lipid metabolism such as cytochrome 450, fatty acid desaturase 2, phospholipase A2, and elongation of (very) long chain fatty acids-like 1 (*Elovl1*) and member 6 (*Elovl6*). In Lee's study, cytochromes P450 was down-regulated by HFD and has been normalized by exercise. In our study cytochromes P450, family 1,2 and 4 were up-regulated in exercise with pair feeding mice, but not changed in exercise alone mice. Cytochromes P450 was named by the wavelength of light (450 nm) absorbed by it, and it is a very critical element in oxidative metabolism and interacts with phospholipids which mechanism is not well understood (43). Among them, fatty acid desaturase 2 (*FAD2*) and phospholipase A_2 (*PLA_2*) expression has been enhanced by exercise alone, but decreased by exercise with pair feeding. The function of *FADS1* and *FADS2* is they catalyze endogenous synthesis of fatty acids that are highly unsaturated such as eicosanoids and arachidonic (44). In contradict, some long chain fatty acids including 18:0-22:5 PC/PE and 18:0-22:6 PC/PE were increased in exercise with pair fed mice, but not exercise alone mice in our previous study. Interesting results were found in *PLA_2* expression. PLA_2 specifically hydrolyzes the sn-2-acyl bond of phospholipids of cell membrane and lipoproteins and produces free fatty acids and lysophospholipids. Both free fatty acids and lysophospholipids are precursors of various lipid mediator for proinflammation such as eicosanoids, leukotrienes, platelet-activationg factors, and prostaglandins. Coincidentally, the prostaglandin D2 synthase expression was also up-regulated by exercise alone, but down-regulated by exercise with pair feeding. The results of phospholipid profiling showed 18:0-, 18:1-, 18:2-, 20:1-lysoPC and 16:0-, 16:1-, 18:1-, 18:2-, 20:1- and 20:4 lysoPE were increased in exercise alone mice compared to sedentary control, however, the increase was not statistically significant. While 16:0-, 18:1-, 18:2-, and 20:4- lysoPC in exercise and pair fed mice were all significantly decreased than that in exercise alone mice, and 16:0- as well as 20:4- lysoPC in exercise and pair fed mice were significantly decreased than sedentary control. The down-regulated expression of *PLA_2* in exercise with pair feeding mice may lead to lower lysoPC and lysoPE. Lysophospholipids involve in both

metabolism of membrane phopsholipid synthesis and regulation of signaling molecular bioactivity (45). LysoPC regulates different cellular functions, such as promotion of atherosclerotic lesion development, enhancement of cell proliferation (46), attraction of monocytes (47), retardation of the migration of endothelial cell (48), and permeability of plasma membranes (49). LysoPC-specific oxidant stress enhances injures of variety cells due to its "detergent-like" properties, such as relates to lysis of red blood cells (50). Reduced level of lysoPC in exercise combined with limited calorie intake mice may decrease the cell damage and also be beneficial with respect to cancer prevention.

Elovl1 and *Elovl6* were up-regulated in exercise with pair feeding mice, but only *Elovl6* has been conformed by real-time PCR which may because of the low efficiency of the *Elvol1* primers or due to the probe sets tested in microarray technique. There are few probe sets labeled as "_x_at" which may represent less specific, and the probe set used for testing the *Elovl1* gene was labeled with "_x_at". Totally there are 6 major *Elovl* genes demonstrated in this family (51). *Elovl1* specifically express in lung, brain, kidney, and some in heart and skin, and it regulates the synthesis of saturated FAs and sphingolipid synthesis. *Elovl2* was illustrated down-regulated in liver tissue by high fat diet feeding, but was normalized by exercise training in Lee's study (19). *Elovl2* specifically expresses in liver which is the major location for lipid synthesis, and *Elovl2* regulates the polyunsaturated fatty acid synthesis. *Elovl3* which is a skin specific gene participates in saturated and monounsaturated FAs and triglyceride formation. *Elovl4* might involve in elongation of DHA (22:6, n=3), but it is specifically expressed in retina, brain and testis tissues. *Elovl5* expresses in liver, lung, kidney, heart, and skin, and it regulates the elongation of polyunsaturated long-chain FAs (C18-C20). *Elovl6* significantly increased in exercise and pair fed mice in our study, and it regulates the elongation of saturated fatty acids form C12 to C18 which may partially explain the increase of 18:0-22:5PC/PE and 18:0-22:6PC/PE fatty acids enhanced in exercise with pair feeding mice found in our former study. In addition, increased omega-3 fatty acid (DHA) was demonstrated in human study through exercise training (52. 53). Thus, it would be interesting for investigating the relationship between the expression of *Elovl2, Elovl4* and *Elovl5* and the level of polyunsaturated long-chain fatty acids in exercise with or without limited calorie intake mice in the future research.

The challenge for microarray analysis is that the huge capacity dimension of the sample compare to the very limited sample size. Every probe sequence on a chip represents a single

experiment from the viewpoint of statisticians. For a multiple comparison, false discovery rates should be considered, but it has been always ignored. Since tens or hundreds of thousands experiments are conducted simultaneously in DNA microarray, an obvious number of results could pass the p-value limit randomly. Both probe number on the array and p-value cutoff would affect the number of false positives obtained (16).

Due to the high cost for the experiments, the lack of samples from different time series becomes the limitation for gene expression measurement. The level of mRNAs doesn't necessarily predict the levels of the corresponding protein in a cell. New protein generation could be affected by different stability of mRNAs and different efficiencies in translation. Furthermore, mRNA does not able to reflect the regulatory status of the corresponding proteins. However, microarray analysis provides an overview for gene expression alteration under the treatment and screens out the significantly changed genes which we might be interested in. Thus, proteomics, the study of proteomes, has been defined as the characterization of patterns of gene expression at the protein level or defined more widely as the link between proteins and genomes. The further study would focus on the analysis of protein expression to conform the alteration found in this microarray analysis.

Due to the lack of antibodies for most of *Elovl* family except *Elovl4*, the proteomics was conducted and has been tried to measure the protein expression of *Elovl* family and others. However, although more than 96 spots on the 2-D gel were detected and more than 50 peptides were identified, we still could not detect the proteins we are interested in (data did not shown).

In summary, we illustrated numerous gene expression alteration occurred in TPA applied exercise alone and exercise combined with limited calorie intake mice. Many significantly modified genes were involved in cell proliferation, differentiation, apoptosis, and affect signaling pathway which related with disease process. More changed were found in exercise combined with limited calorie intake mice which showed that moderate limitation of calorie intake and body weight loss combined with exercise affected the gene expression. Among them, most changes showed benefit for cell apoptosis, lower the risk of inflammation, and may contribute for cancer prevention.

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Figures and Tables

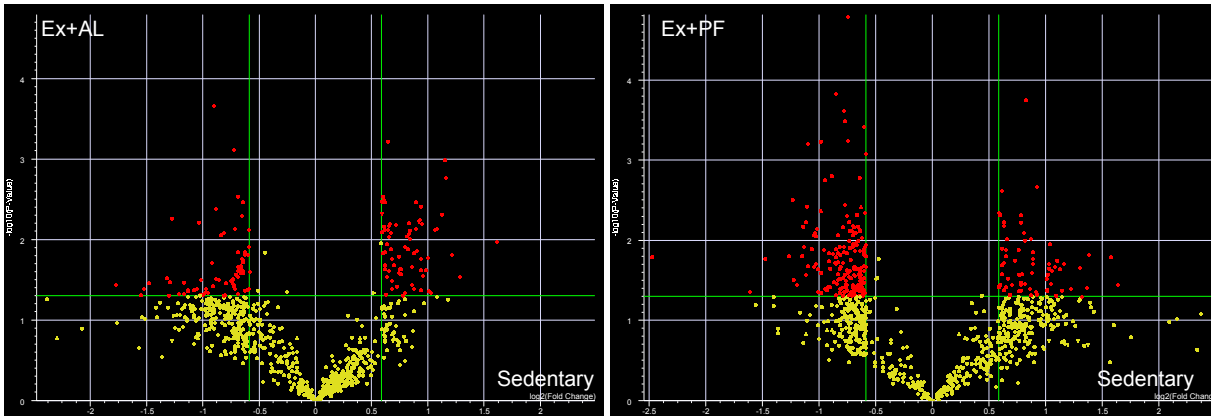

Figure 3-1 Volcano plots for all gene expression in TPA treated skin tissues of exercised mice compared to sedentary control. Totally 45,101 probe sets were measured in the skin tissues. Fold change at 1.5 was set for cutoff, and ANOVA as well as FDR ($p \leq 0.1$) were applied to assess gene expression difference between Ex+Al and sedentary (top, 417 gene expression was up/down-regulated), as well as Ex+PF and sedentary (bottom, 626 gene expression was up-/down-regulated) (n = 4).

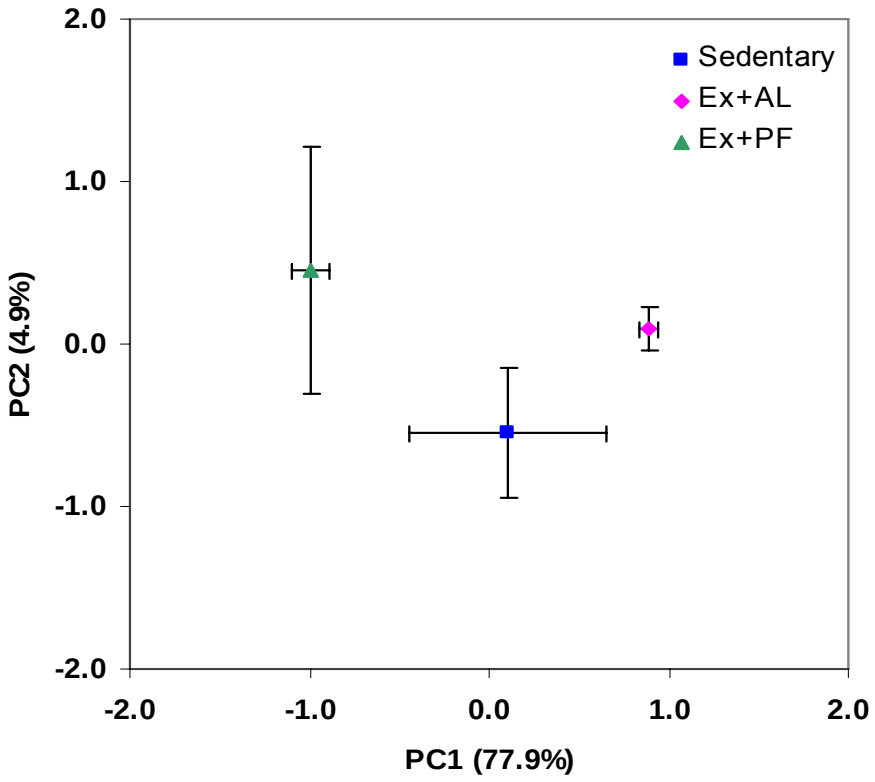

Figure 3-2 Principal Component Analysis (PCA) of the first two principal components in three treatment groups. It demonstrates difference for gene expression in the 839 variable data set which were significantly different by 1.5 fold change and FDR less than 0.1. Means ± S.E. in both directions (n=4).

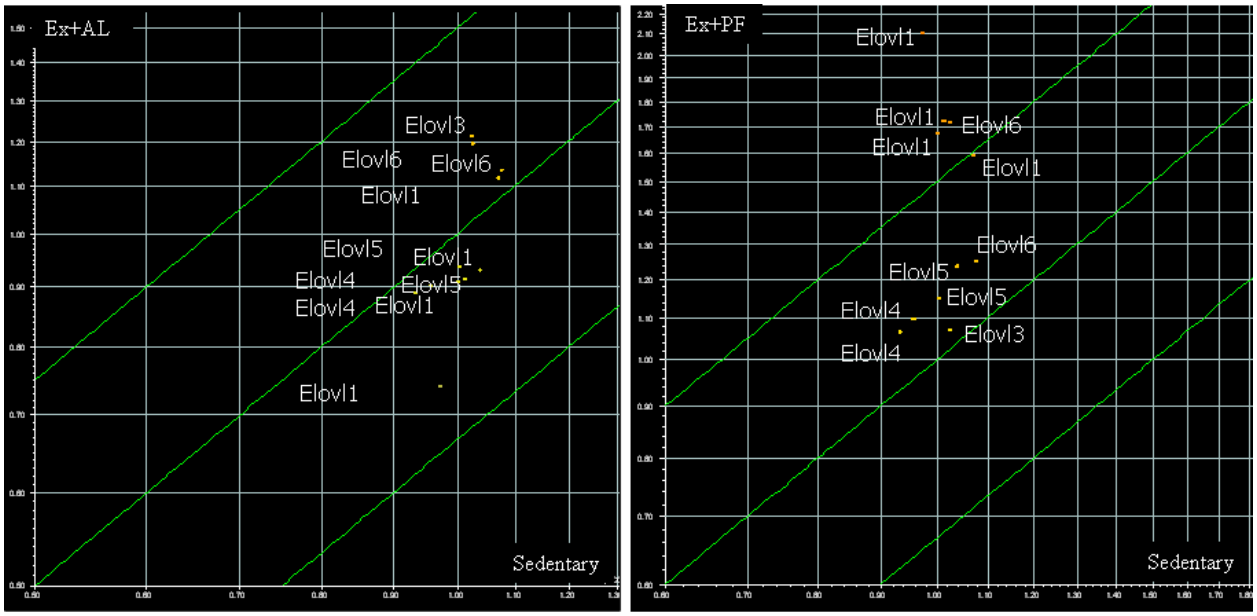

Figure 3-3 Distribution of expression of eleven probe sets for five *Elovl* genes in skin tissues.
Ex+AL vs. Sedentary (left) and Ex+PF vs. Sedentary (right) (n = 4).

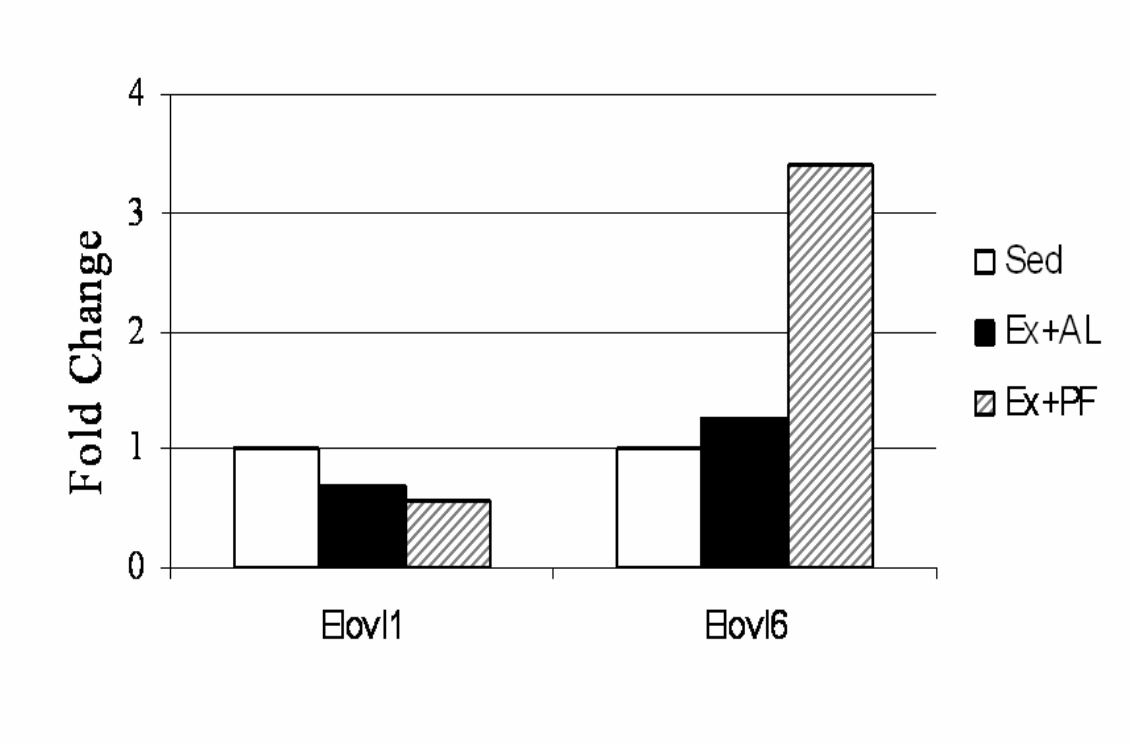

Figure 3-4 Gene expression changed for *Elovl1* and *Elovl6* in TPA applied mice skin tissues from exercised mice compared to sedentary control using real-time PCR. The gene expression in TPA applied skin samples for *Elvol1* and *Elovl6* was compared among three treatment groups using RT-PCR (n = 2~5).

Table 3-1 Gene expression changed in two exercise groups classified by gene function.

The up-/down-regulated directions were showed by arrows.

Function	Probe set ID	GeneBank	Gene Title	Symbol	Ex +AL	Ex+PF
cell cycle	1416076_at	NM_007629	cyclin B1	Ccnb1	↑	
	1416309_at	BC009096	nucleolar and spindle associated protein 1	Nusap1	↑	
	1417155_at	BC005453	neuroblastoma myc-related oncogene 1	Nmyc1	↑	↓
	1417656_at	NM_008652	myeloblastosis oncogene-like 2	Mybl2	↑	↓
	1417856_at	NM_009046	avian reticuloendotheliosis viral (v-rel) oncogene related B	Relb		↑
	1417911_at	X75483	cyclin A2	Ccna2	↑	
	1422687_at	BB018528	neuroblastoma ras oncogene	Nras		↓
	1424144_at	AF477481	retroviral integration site 2	Ris2		↑
	1425761_a_at	AF239169	nuclear factor of activated T-cells, cytoplasmic 1	Nfatc1	↓	
	1434079_s_at	BB699415	minichromosome maintenance deficient 2 mitotin (S. cerevisiae)	Mcm2		↓
	1435541_at	AV231340	betacellulin, epidermal growth factor family member	Btc		↑
	1437580_s_at	C77054	NIMA (never in mitosis gene a)-related expressed kinase 2	Nek2	↑	
	1438953_at	BB359521	c-fos induced growth factor	Figf	↓	
	1441855_x_at	BB554288	chemokine (C-X-C motif) ligand 1	Cxcl1		↑
	1449519_at	NM_007836	growth arrest and DNA-damage-inducible 45 alpha	Gadd45a		↓
	1450119_at	NM_010081	dystonin	Dst		↑
	1450194_a_at	NM_033597	myeloblastosis oncogene	Myb	↑	↓
	1451246_s_at	BC003261	aurora kinase B	Aurkb	↑	
	1453313_at	AK017464	sestrin 3	Sesn3		↓
cell death	1416505_at	NM_010444	nuclear receptor subfamily 4, group A, member 1	Nr4a1		↑
	1417956_at	NM_007702	cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	Cidea		↑
	1418571_at	NM_013749	tumor necrosis factor receptor superfamily, member 12a	Tnfrsf12a	↓	↓
	1418626_a_at	NM_013492	clusterin	Clu	↑	↓
	1418748_at	NM_009809	caspase 14	Casp14	↑	
	1419463_at	AF108501	chloride channel calcium activated 2	Clca2		↑
	1420639_at	BF227962	junction-mediating and regulatory protein	Jmy	↑	↓
	1424278_a_at	BC004702	baculoviral IAP repeat-containing 5	Birc5	↑	
	1430135_at	AK018651	deoxyribonuclease II alpha	Dnase2a	↑	

	1433699_at	BM241351	tumor necrosis factor, alpha-induced protein 3	Tnfaip3		↑
	1433775_at	BB163333	tyrosyl-tRNA synthetase	Yars		↑
	1437578_at	AV231113	chloride channel calcium activated 2	Clca2		↑
	1440085_at	AV246296	RIKEN cDNA 9430060M22 gene	9430060M22Rik	↓	
	1449317_at	NM_009805	CASP8 and FADD-like apoptosis regulator	Cflar		↓
	1449491_at	NM_130859	caspase recruitment domain family, member 10	Card10		↑
	1449591_at	NM_007609	caspase 4, apoptosis-related cysteine protease	Casp4	↓	
	1450731_s_at	BG972377	tumor necrosis factor receptor superfamily, member 21	Tnfrsf21		↑
	1460259_s_at	AF108501	chloride channel calcium activated 2	Clca2		↑
cell proliferation	1427691_a_at	Y09864	interferon (alpha and beta) receptor 2	Ifnar2		↑
	1430533_a_at	BI134907	catenin beta	Catnb		↓
	1435227_at	BM117007	B-cell leukemia/lymphoma 11B	Bcl11b		↑
	1435541_at	AV231340	betacellulin, epidermal growth factor family member	Btc		↑
	1436869_at	AV304616	sonic hedgehog	Shh	↑	
	1438953_at	BB359521	c-fos induced growth factor	Figf	↓	
cell growth	1416576_at	NM_007707	suppressor of cytokine signaling 3	Socs3		↑
	1423062_at	AV175389	insulin-like growth factor binding protein 3	Igfbp3		↑
physiological death	1416505_at	NM_010444	nuclear receptor subfamily 4, group A, member 1	Nr4a1		↑
	1417956_at	NM_007702	cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	Cidea		↑
	1418571_at	NM_013749	tumor necrosis factor receptor superfamily, member 12a	Tnfrsf12a	↓	↓
	1418626_a_at	NM_013492	clusterin	Clu	↑	↓
	1418748_at	NM_009809	caspase 14	Casp14	↑	
	1420640_at	BF227962	junction-mediating and regulatory protein	Jmy		↓
	1424278_a_at	BC004702	baculoviral IAP repeat-containing 5	Birc5	↑	
	1430135_at	AK018651	deoxyribonuclease II alpha	Dnase2a	↑	
	1433699_at	BM241351	tumor necrosis factor, alpha-induced protein 3	Tnfaip3		↑
	1433775_at	BB163333	tyrosyl-tRNA synthetase	Yars		↑
	1437578_at	AV231113	chloride channel calcium activated 2	Clca2		↑
	1440085_at	AV246296	RIKEN cDNA 9430060M22 gene	9430060M22Rik	↓	
	1449317_at	NM_009805	CASP8 and FADD-like apoptosis regulator	Cflar		↓
	1449491_at	NM_130859	caspase recruitment domain family, member 10	Card10		↑
	1449591_at	NM_007609	caspase 4, apoptosis-related cysteine protease	Casp4	↓	
	1450731_s_at	BG972377	tumor necrosis factor receptor superfamily, member 21	Tnfrsf21		↑

	1416505_at	NM_010444	nuclear receptor subfamily 4, group A, member 1	Nr4a1		↑
regulation of apoptosis	1418748_at	NM_009809	caspase 14	Casp14	↑	
	1420639_at	BF227962	junction-mediating and regulatory protein	Jmy	↑	↓
	1424278_a_at	BC004702	baculoviral IAP repeat-containing 5	Birc5	↑	
	1449317_at	NM_009805	CASP8 and FADD-like apoptosis regulator	Cflar		↓
	1449491_at	NM_130859	caspase recruitment domain family, member 10	Card10		↑
	1449591_at	NM_007609	caspase 4, apoptosis-related cysteine protease	Casp4	↓	
development cell differentiation	1417856_at	NM_009046	avian reticuloendotheliosis viral (v-rel) oncogene related B	Relb		↑
	1418571_at	NM_013749	tumor necrosis factor receptor superfamily, member 12a	Tnfrsf12a	↓	↓
	1419150_at	NM_008657	myogenic factor 6	Myf6	↓	
	1420715_a_at	NM_011146	peroxisome proliferator activated receptor gamma	Pparg		↑
	1422222_at	NM_008412	involucrin	Ivl	↑	
	1425611_a_at	AA023906	cut-like 1 (Drosophila)	Cutl1		↓
	1425792_a_at	AJ132394	RAR-related orphan receptor gamma	Rorc		↑
	1426314_at	BB770914	endothelin receptor type B	Ednrb		↓
	1430135_at	AK018651	deoxyribonuclease II alpha	Dnase2a	↑	
	1430533_a_at	BI134907	catenin beta	Catnb		↓
	1432410_a_at	AK020411	bone morphogenetic protein 7	Bmp7	↑	
	1435190_at	BB378591	close homolog of L1	Chl1		↑
	1436869_at	AV304616	sonic hedgehog	Shh	↑	
	1437231_at	AV246497	SLIT and NTRK-like family, member 6	Slitrk6		↑
	1447655_x_at	BB257593	SRY-box containing gene 6	Sox6	↓	
	1448877_at	NM_010054	distal-less homeobox 2	Dlx2		↓
	1449077_at	NM_133245	erythroid associated factor	Eraf	↑	↓
	1449465_at	NM_011261	reelin	Reln		↑
	1449863_a_at	NM_010056	distal-less homeobox 5	Dlx5		↑
	1454078_a_at	AK002510	galactosylceramide sulfotransferase	Gcst		↑
	1455188_at	BQ176283	Eph receptor B1	Ephb1		↓
	1456258_at	BG072869	empty spiracles homolog 2 (Drosophila)	Emx2		↑
response to stress	1434927_at	BM124741	heat shock protein family, member 7 (cardiovascular)	Hspb7	↓	↓
response to DNA damage stimulus	1429835_at	AK009778	X-ray repair complementing defective repair in Chinese hamster cells 1	Xrcc1		↓
	1449519_at	NM_007836	growth arrest and DNA-damage-inducible 45 alpha	Gadd45a		↓

response to oxidative stress	1423414_at	BB520073	prostaglandin-endoperoxide synthase 1	Ptgs1		↑
	1449106_at	NM_008161	glutathione peroxidase 3	Gpx3	↓	
	1449279_at	NM_030677	glutathione peroxidase 2	Gpx2	↑	
lipid metabolism	1415823_at	BG060909	stearoyl-Coenzyme A desaturase 2	Scd2		↓
	1415977_at	NM_023627	RIKEN cDNA 1300017C10 gene	1300017C10Rik	↑	
	1417404_at	NM_130450	ELOVL family member 6, elongation of long chain fatty acids (yeast)	Elovl6		↑
	1418266_at	NM_009659	arachidonate 12-lipoxygenase, 12R type	Alox12b		↑
	1420674_at	NM_012044	phospholipase A2, group IIE	Pla2g2e	↑	↓
	1418601_at	NM_011921	aldehyde dehydrogenase family 1, subfamily A7	Aldh1a7		↑
	1420994_at	BM214359	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5	B3gnt5		↓
	1423414_at	BB520073	prostaglandin-endoperoxide synthase 1	Ptgs1		↑
	1423860_at	AB006361	prostaglandin D2 synthase (brain)	Ptgds	↑	↓
	1425376_at	BC015253	arachidonate 15-lipoxygenase, second type	Alox15b	↑	↓
	1426818_at	BC025091	RIKEN cDNA 2410003C09 gene	2410003C09Rik	↑	
	1428484_at	AK004768	oxysterol binding protein-like 3	Osbpl3		↑
	1434465_x_at	AV333363	very low density lipoprotein receptor	Vldlr	↓	
	1435039_a_at	BB822856	phosphatidylinositol-4-phosphate 5-kinase, type 1 beta	Pip5k1b		↑
	1436640_x_at	BE994529	1-acylglycerol-3-phosphate O-acyltransferase 1 (lysophosphatidic acid acyltransferase, delta)	Agpat4		↑
	1438152_at	BB550093	GPI anchor attachment protein 1	Gpaa1	↑	
	1438579_at	BM213836	fatty acid Coenzyme A ligase, long chain 3	Facl3	↑	
	1439183_at	AI662009	N-acylsphingosine amidohydrolase (alkaline ceramidase) 3	Asah3		↑
	1439947_at	C87524	cytochrome P450, family 11, subfamily a, polypeptide 1	Cyp11a1	↑	↓
	1440852_at	AV248340	RIKEN cDNA 4833405L16 gene	4833405L16Rik	↓	
	1449237_at	NM_011786	arachidonate lipoxygenase 3	Aloxe3		↑
	1449450_at	NM_022415	prostaglandin E synthase	Ptges		↑
	1450966_at	BB283187	carnitine O-octanoyltransferase	Crot		↓
	1454078_a_at	AK002510	galactosylceramide sulfotransferase	Gcst		↑
	1455994_x_at	BB145065	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 1	Elovl1		↑
	1456047_at	NM_145378	phospholipase A2, group IVB (cytosolic)	Pla2g4b		↓
	1456147_at	BB486599	sialyltransferase 8 (alpha-2, 8-sialyltransferase) F	Siat8f		↓