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Anti-aging activity of Korean pine (*Pinus koraiensis*) leaf extracts demonstrated through the induction of mitochondrial biogenesis in mice

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Age-related increases in cellular damage and death have been associated with oxidative stress. Mitochondrial biogenesis is likely to be involved in the regulation of cell metabolism and signal transduction. In addition, there is a growing body of evidence suggesting that mitochondrial biogenesis is a key regulator of mitochondrial reactive oxygen species (ROS). Our results demonstrate that *Pinus koraiensis* leaf (PKL) extract reduces oxidative stress and, at the same time, stimulates the proliferation of mitochondria through a peroxisome proliferation-activated receptor coactivator 1α (PGC-1α) signaling pathway. The effects of PKLs were associated with the induction of genes associated with oxidative phosphorylation and mitochondrial biogenesis pathways, and were largely explained by PKL-mediated decrease in PGC-1α acetylation and an increase in PGC-1α activity. PKL extracts induced a PGC-1α dependent pathway in mitochondria capable of efficient and balanced bioenergetics to reduce oxidative stress and attenuate endogenous oxidative damage.

Key words: *Pinus koraiensis*, mitochondrial biogenesis, reactive oxygen species (ROS), peroxisome proliferation-activated receptor coactivator 1α (PGC-1α).

INTRODUCTION

Reactive oxygen species (ROS) have been implicated as one of the major factors affecting the lifespan of organisms (Sohal and Weindruch, 1996) as it is the products of respiration which is believed to contribute substantially to aging (Ho et al., 2010; Bonda et al., 2011). Oxidative stress and mitochondrial dysfunction are important factors that contribute to aging (Cui et al., 2012). A series of protein complexes (I-IV) is embedded in the inner mitochondrial membrane and generates a proton gradient known as the mitochondrial membrane potential (MMP) (Finley et al., 2009). The mitochondrial respiratory chain is a major site of ROS production in the cell. Generations dysfunction and represent putative targets of anti-aging of ROS play an important role in mitochondrial strategies (Mammucari and Rizzuto, 2010). There is evidence that mitochondrial biogenesis is dysregulated in these organs, and it is believed that the resulting decline in cellular mitochondrial mass may contribute to the increased mitochondrial generation of ROS. Previous studies have showed that smooth muscle cells exhibit increased mitochondrial ROS production (Wallace, 2005).

Natural antioxidants are safer and healthier than synthetic antioxidants used in food (Velioglu et al., 1998; Elmastas et al., 2007). Pine trees native to different soil environments appear to contain different biological active substances, and may contain compounds that have not

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yet been described. Korean pine (*Pinus koraiensis*) is one of the most important sources of timber and is a traditional medicinal plant (Yang et al., 2010). *Pinus* plants have reportedly been used in traditional medicine for many years and have many effects, being known particularly for their fatigue relieving, anti-ageing, and anti-inflammatory properties (Watanabe et al., 1995). The antioxidant ability of phenolic compounds has been widely accepted by the scientific community and is predicted to play a beneficial role in disease prevention (Chewa et al., 2008).

The antioxidant capacity of *P. koraiensis* seed (PKS) extract could be exploited in the production of health foods to prevent ageing and degeneration. Therefore, detailed studies of the activity of antioxidants from the Korean pine leaf to determine if they are useful as herbal medications and functional foodstuffs are warranted. The mechanisms responsible for the antioxidant effects of *P. koraiensis* leaf (PKL) extract remain uncertain, but its ability to reduce oxidative stress within mitochondria remains a major focus of research. In this study, we investigated the antioxidant activity of PKL extract in mice by measuring the induction of mitochondrial biogenesis.

MATERIALS AND METHODS

Sample extraction

Korean pine (*Pinus koraiensis*) leaves were collected from Jecheon City (Chungcheongbuk-do, Korea). The dried PKL (500 g) was ground using a pulveriser and extracted with ethanol (70%) by ultrasonification at 200 W for 2 h at room temperature. The extract was centrifuged at 3,000 rpm for 5 min and the residue was extracted once again with new solvent under the same conditions. The supernatant was evaporated and the extract powder was obtained by freeze drying. The PKL extract powder was stored at 4°C until required. Prior to the assays, the extract was dissolved to the required concentration.

Experimental animals

Eight (8-week-old) male C57BL/6 mice were obtained from Koatech (Gyeonggi-do, Korea). The mice were initially adapted for a week in the SPF animal facility, on a diet of rodent chow. The mice were then divided into 3 groups (6 animals per group) and fed the following diets for 8 weeks: 1) high-calorie diet with vehicle treatment (HC); 2) supplemented high-calorie diet with PKL (100 mg kg^{-1} day^{-1}) treatment (HC-P100); 3) high-calorie diet with PKL (300 mg kg^{-1} day^{-1}) treatment (HC-P300). Their bedding was changed once a week, and the temperature and humidity were controlled. Mice were housed under 12 h light/12 h dark photoperiod conditions. A pellet rodent diet with 60% Kcal fat (Central Lab. Animal Inc., Seoul, Korea) was provided to the HC group. In the supplemented groups, each mouse was administered 100 or 300 mg of PKL orally, the dosages being based on the results of previous studies. For the vehicle treatment group, the same volume of distilled water was administered orally every day by the same method used for PKL treatment for the duration of the study. All mice were allowed free access to the described diet and water during experimental periods. Body weights and food intake were measured weekly, maintaining the same times. The plans and protocols for the animal experiments were approved by the Institutional Animal Care and Use Committee of the Korea Institute of Oriental Medicine, Daejeon, Korea.

Protein extraction and western blotting

Skeletal muscle and liver tissues were lysed using a tissue lyser with an ice-cold homogenization buffer containing 50 mM Tris-HCl, (pH 8.0), 150 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecylsulphate (SDS), and a protease inhibitor cocktail (Roche, Indianapolis, IN, USA). The total cell lysates were centrifuged at 14,000 rpm for 20 min at 4°C to remove insoluble materials. The protein concentrations were determined using a DC Protein Assay Kit (Bio-Rad, Rockford, IL, USA). Protein extracts (50 µg) were resolved by 10% SDS-polyacrylamide gel electrophoresis at 150 mA for 1 h and then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked for 2 h at room temperature with phosphate buffer saline (PBS) containing 5% skimmed milk and 0.1% Tween 20, incubated with 1:2000-fold dilutions of primary antibodies overnight at 4°C, and then with horseradish peroxidase-conjugated anti-rabbit secondary antibody for 1 h at room temperature. Peroxidase activity was visualized using an ECL kit (Las-3000, Fuji Photo, Tokyo, Japan).

RNA isolation and quantitative RT-PCR analysis

Total RNA was isolated from frozen tissues using a guanidinium thiocyanate-phenol-chloroform extraction method. Prepared total RNA was reverse-transcribed using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) at 37°C for 1 h. Polymerase chain reactions (PCRs) for the amplification of mRNAs encoding nuclear respiratory factor-1 (NRF-1), nuclear respiratory factor-2 (NRF-2), estrogen-related receptor α (ERRα), and smooth muscle actin (SMA) were performed using the following primer pairs: NRF-1 forward, 5''-TTGATGGACACTTGGGTAGCTT-3'', NRF-1 reverse, 5''-CCCACAGGACCTAGTATATGCG-3''; NRF-2 forward, 5''-TGGAGCGAGACCCTCCTAAT-3'', NRF-2 reverse, 5''-CACTGGTTCACACTGGCCTC-3''; ERRα forward, 5''-CCATGATGGACTGAGGCAAG-3'', ERRα reverse, 5''-ATCCCTTCCCTCCCTGAGAT-3''; SMA forward, 5''-GGGAGCCAAAACAGGGGTTG-3'', SMA reverse, 5''-GCCTCTGCAGACCCAACGTG-3''; mtDNA reverse, 5''-TGTCAGATATGTCCTTCA-3''. RT-PCR products were visualized by 1% agarose gel electrophoresis, and the intensity of the bands was measured using a DNR Bio-Imaging system (Kiryat Anavim, Jerusalem, Israel).

Blood assays

Blood glucose concentrations were determined using a glucose analyzer (Model 2300; Yellow Springs Instruments, OH, USA). Plasma insulin was assayed with a radioimmunoassay kit (Phadesepa Insulin RIA, Phamacia AB, Sweden). Plasma free fatty acids (FFA) were determined with a commercial kit (Wako Pure Chemical Industries, Osaka, Japan).

Measurement of ROS generation in liver tissue

Two independent assays were performed to measure ROS production in muscle tissue. Lucigenin-enhanced chemiluminescence was used to detect superoxide (lucigenin (bis-N-methylacridinium nitrate luminesces specifically in the presence of superoxide) (Li et al., 2007). After exposing the liver, the portal vein was cannulated with 24G catheter and ligated with 4 to 0 silk. The liver was perfused

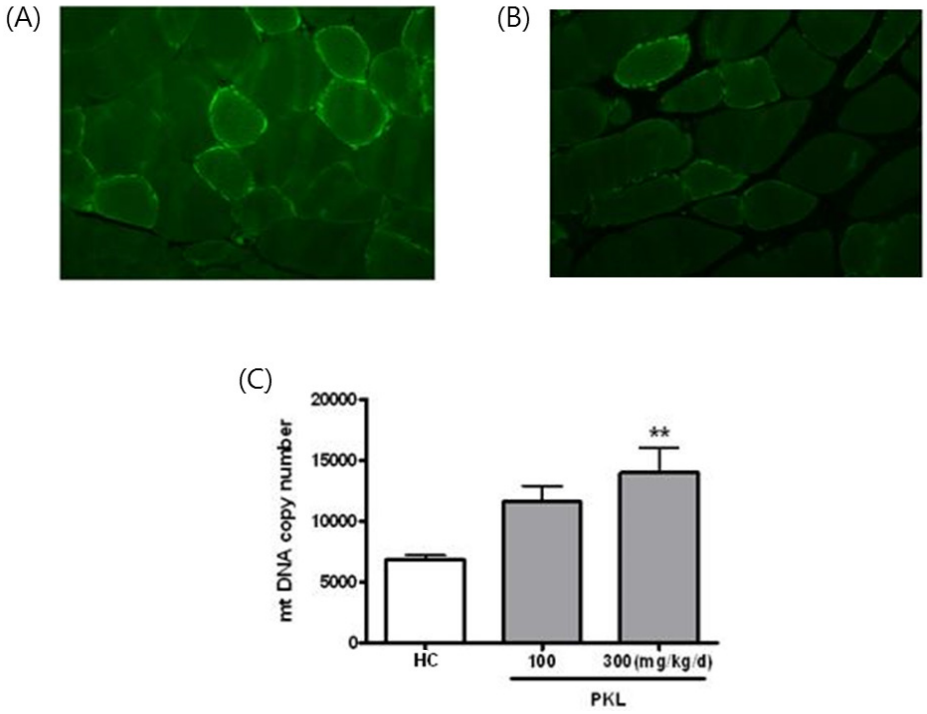

Figure 1. Fluorescence microscopy characteriazation of mitochondria in PKL- treated mice skeletal muscle. (A) High- calorie diet with vehicle treatment (HC). (B) Supplemented high- calorie diet with PKL (300 mg kg^{-1} day^{-1}) treatment (HC-P300). (C) mtDNA copy number of muscle from PKL-treated and non-treated mice. Asterisks indicate significant differences from the corresponding control group (**$P < 0.01$).

slowly with 1 to 2 ml of solution 1 (Hank's balanced salt solution, HBSS [Ca, Mg-free], 1 mM EGRA, 20 mM HEPES [pH 7.4], preoxygenated and prewarmed to 37°C) and then with solution 2 (HBSS, 5 mM $CaCl_2$, 0.05% collagenese, 20 mM HEPES [pH 7.4], preoxygenated and prewarmed to 37°C) for 5 to 10 min until it softened. The left hepatic lobe was excised and cut into 1 to 2 mm pieces. After gently pipetting the tissue in solution 2 for 5 min, the homogenate was filtered through gauze and the cell suspension was centrifuged at 100 × *g* for 3 min. The chemiluminescent signal was measured using a luminometer, and monitored every 5 min for a total of 30 min. The emitted light units after subtracting a blank and integrating over 15 min were used as a measure of superoxide production.

Immunohistochemistry

Paraffin sections (5 µm thickness) of skeletal muscle tissue was deparaffinized and incubated in sodium citrate (pH 6.0) for antigen retrieval. The sections were incubated with 1:250 anti-MITO1 antibodies, washed, mounted in Vecta-shield H-1200 (Vector Labs, Burlingame, CA, USA) and fluorescence with standard methods. Immunofluorescence images (Figure 1A and B) were acquired with an INFINITY3 fluorescence microscope, fitted with a 60× objective and appropriate filters, and analyzed with the Image-J software package.

Statistical analysis

Statistical evaluation of the results was performed using the 2-tailed Student's *t*-test performed on GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA).

RESULTS AND DISCUSSION

Body weight

Body weights were monitored throughout the feeding period. After 8 weeks, the body weight of the mice in all HC groups was significantly higher than that of the mice in the NOR group, with a 23% greater weight gain for the HC (data not shown). No significant differences in body weight or weight gain occurred between the different HC groups.

PKL stimulates mitochondrial biogenesis

Exercise and reduced caloric intake increase the number of hepatic mitochondria (Nisoli et al., 2005; Lopez-Lluch et al., 2006), and we wondered whether PKL might induce the same effect (Figure 1C). Morphologically, the livers of the PKL-treated mice had considerable difference compared with those of the HC group. Mitochondrial biogenesis in the liver and muscle is controlled predominantly by the transcriptional coactivator

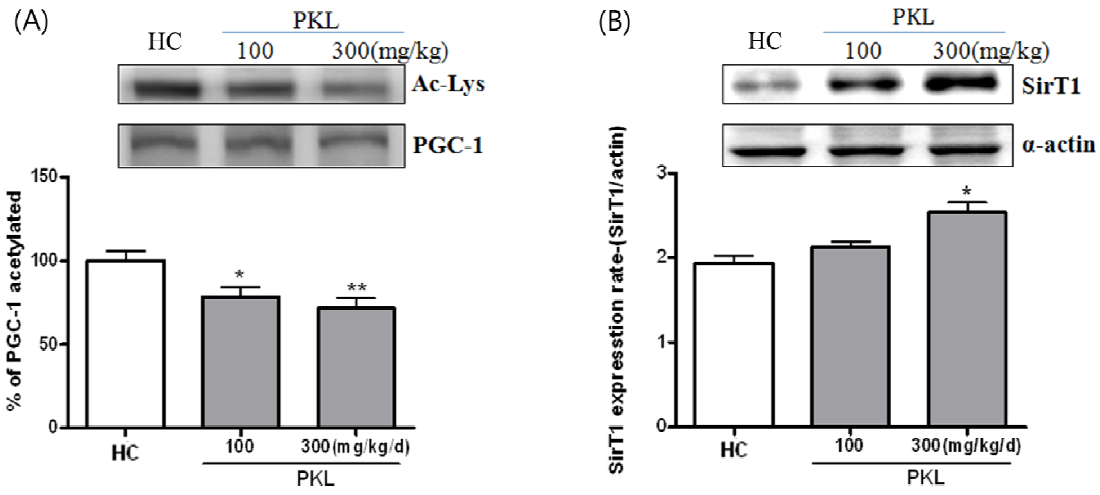

Figure 2. PKL extract induces PGC-1α activity through SirT1. (A) Representative western blots and quantification exhibiting the relative amount of acetylated versus total PGC-1 protein for skeletal muscle. PGC-1 was immunoprecipitated from nuclear extracts and then immunoblotted with either an anti-acetylated lysine antibody to determine the extent of PGC-1 acetylation(Ac-Lys) or a PGC-1 antibody, to determine the total amount of PGC-1. (B) Representative western blots exhibiting the expression levels of SirT-1. Asterisks indicate significant differences from the corresponding control group (*$P < 0.05$, **$P < 0.01$).

PGC-1α (Wu et al., 1999; Zhu et al., 2009), the activity of which in turn is positively regulated by SirT-1 mediated deacetylation (Rodgers et al., 2005; Lerin et al., 2006). Hence, the acetylation status of PGC-1α is considered a marker of SirT-1 activity *in vivo* (Rodgers et al., 2005). The acetylation status of PGC-1α in the PKL-treated mice was 30% lower than in the diet-matched HC group (Figure 2A). Increases in SitT-1 protein levels were detected in a dose-dependent manner in the PKL- treated mice, indicating that SirT-1 enzymatic activity was enhanced by PKL (Figure 2B).

PKL modulates regulators of mitochondrial transcription

Functional NRF-1 and NRF-2 binding sites have been identified in the promoter of the nuclear gene that encodes mtTFA, a transcription factor that regulates mitochondrial DNA transcription and replication. There was a significant increase in mt-TFA shown in Figure 3A, B and C. ROS are byproducts of metabolism that are physiologically and continuously generated in the mitochondria, and are also one of the main targets in factors implicated in the causality of detrimental effects (Turrens, 2003; Kowaltowskia et al., 2009). Oxidative alterations to biomolecules increase with age, and are an obvious outcome of redox-imbalance (Fukagawa, 1999; Rebrin and Sohal, 2008). It is hence expected in decreasing ROS production or increasing antioxidant effects. Thus, we determined ROS levels in the mice after administering PKL (Figure 4B).

In humans, high-calorie diets cause numerous pathological conditions, including increased insulin levels leading to diabetes, cardiovascular disease, and non-alcoholic fatty liver disease, a condition for which there is no effective treatment (Siebler and Galle, 2006). The HC-fed mice exhibited alterations in their plasma insulin levels of markers that predict mitochondrial dysfunction by ROS (Bonnard et al., 2008). Plasma insulin levels were lowered in a dose-dependent manner in PKL group, paralleling the HC group (Figure 4A).

Our data demonstrated that PKL is an antioxidant that protects cells both from mitochondrial dysfunction and insulin resistance by scavenging free radicals of mitochondrial biogenesis. PGC-1α has been shown previously to induce mitochondrial biogenesis and oxidative metabolism in muscle cells, adipocytes, and cardiomyocytes (Puigserver et al., 1998; Lehman et al., 2000; Borniquel et al., 2006). Our studies also provided evidence that the transcription factors NRF-1, NRF-2, and ERRα mediate the effects of PGC-1α on the expression of unclear genes encoding mitochondrial proteins (Choksi, 2008). We have further shown that PGC-1α expression *in vivo* also induces mitochondrial biogenesis. Importantly, mice fed an HC diet were consequently protected from the development of antioxidant activity when they were treated with PKL. Although most of our conclusions are based on pharmacological interventions in mice, the novel association between genetic variation in the SirT-1 gene and energy homeostasis in man reveals a significant place for our work in the context of human pathophysiology.

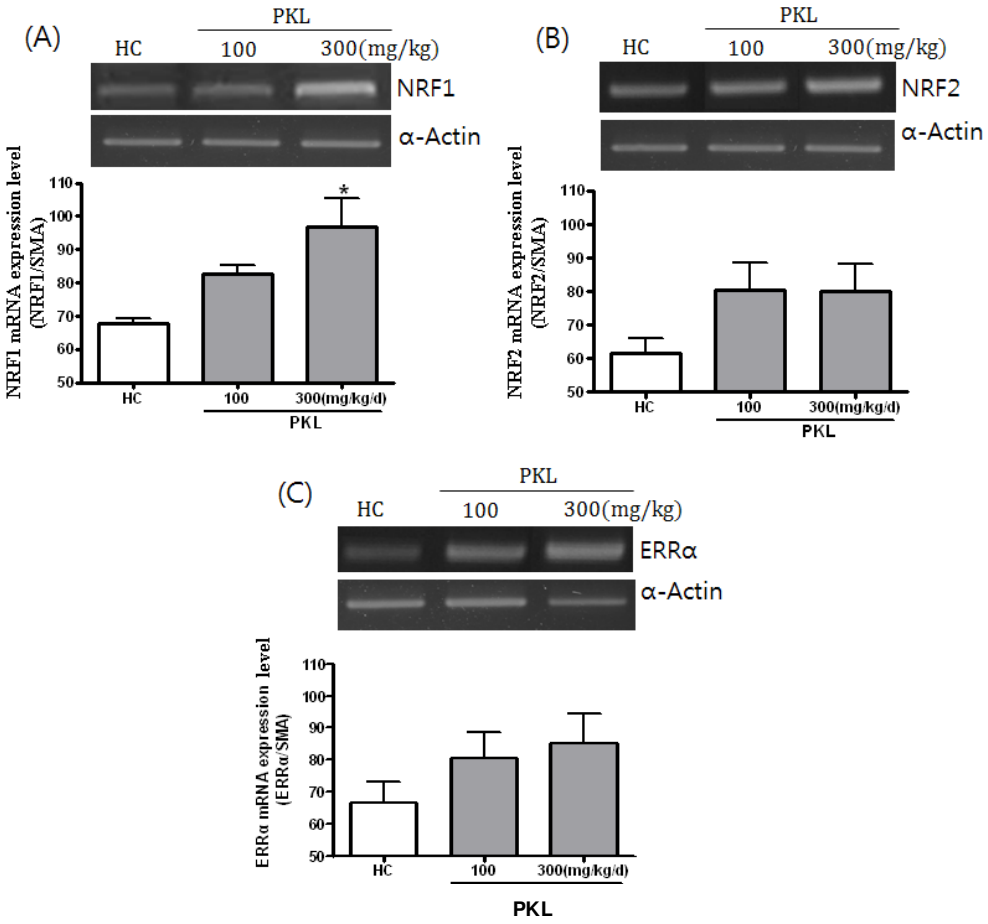

Figure 3. Enrichment of gene-expression in pathways related to mitocondrial biogenesis in skeletal muscle from PKL-treated mice. (A) nuclear respiratory factors 1 (NRF-1) and (B) 2 (NFR-2), (C) estrogen-related receptor alpha (ERR α) as determined by RT-PCR. Asterisks indicate significant differences from the corresponding control group (*$P < 0.05$, **$P < 0.01$).

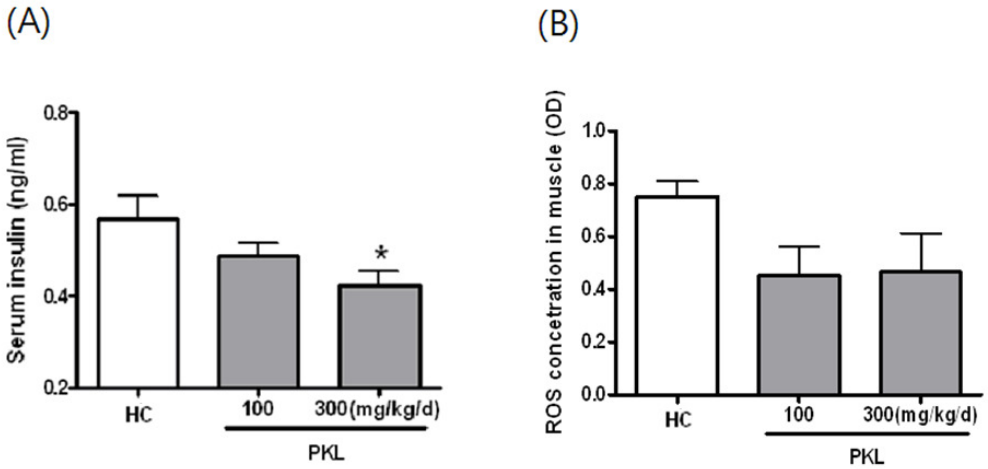

Figure 4. Increases in insulin sensitivity and ROS concentration from PKL-treated mice. (A) Plasma serum level from PKL-treated and non-treated mice. (B) ROS concentration in muscle. Asterisks indicate significant differences from the corresponding control group (*$P < 0.05$, **$P < 0.01$).

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