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Oral caffeine during voluntary exercise markedly inhibits skin carcinogenesis and decreases inflammatory cytokines in UVB-treated mice

YouRong Lou¹, QingYun Peng¹, Tao Li¹, Bonnie Nolan², Jamie J. Bernard¹, George C. Wagner^{2,3}, Yong Lin^{4,5}, Weichung Joe Shih^{4,5}, Allan H Conney^{1,5,6}, and YaoPing Lu^{1,5,7}

¹Susan Lehman Cullman Laboratory for Cancer Research, Department of Chemical Biology, Ernest Mario School of Pharmacy

²Department of Neuroscience, The State University of New Jersey, Piscataway, NJ 08854

³Department of Psychology, Rutgers, The State University of New Jersey, Piscataway, NJ 08854

⁴Department of Biostatistics, School of Public Health, University of Medicine and Dentistry of New Jersey, New Brunswick, NJ

⁵Cancer Institute of New Jersey, New Brunswick, NJ

⁶Guangdong University of Technology, Guangzhou, China

Abstract

UVB-pretreated SKH-1 mice were treated with water, caffeine (0.1 mg/ml), voluntary running wheel exercise (RW) or caffeine together with RW for 14 weeks. Treatment of the mice with caffeine, RW, or caffeine plus RW decreased skin tumors per mouse by 27, 35 and 62%, and the tumor volume per mouse was decreased by 61, 70 and 85%, respectively. In mechanistic studies, mice were treated with water, caffeine, RW, or caffeine plus RW for 2 weeks prior to a single irradiation with UVB. Caffeine plus RW increased RW activity by 22% when compared with RW alone. Caffeine ingestion was not significantly different between groups. Treatment of mice with caffeine plus RW for 2 weeks decreased the weight of the parametrial fat pads and stimulated the formation of UVB-induced apoptosis to a greater extent than treatment with caffeine or RW alone. An antibody array revealed that caffeine plus RW administered to mice fed a high fat diet and irradiated with UVB decreased the epidermal levels of LIX, sTNFR1 and MIP-1. Overall, caffeine during RW exerts a stronger effect than either treatment alone for decreasing tissue fat, increasing UVB-induced apoptosis, lowering the levels of cytokines associated with inflammation and for inhibiting UVB-induced carcinogenesis.

Introduction

We previously reported that orally administered caffeine decreased tissue fat as measured by the size of the parametrial fat pads, stimulated UVB-induced apoptosis, increased locomotor activity and inhibited UVB-induced carcinogenesis⁽¹⁻⁴⁾. Subsequently, we found that increased locomotor activity by voluntary running wheel exercise (RW) also decreased tissue fat stores, stimulated UVB-induced apoptosis and decreased UVB-induced skin tumor formation^(5,6). In a recent study, we found that treatment of SKH-1 mice with RW in

⁷To whom correspondence should be addressed: Dr. YaoPing Lu, Susan Lehman Cullman Laboratory for Cancer Research, Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, 164 Frelinghuysen Road, Piscataway, NJ 08854-8020. Phone: (732) 445-3400, Fax: (732) 445-0687; sago@pharmacy.rutgers.edu.

combination with a low dose of oral caffeine (i. e., 0.1 mg/ml drinking fluid and yielding blood levels of caffeine equivalent to approximately 1–2 cups of coffee per day in humans) had a greater than additive stimulatory effect in decreasing tissue fat and in stimulating UVB-induced apoptosis in SKH-1 mice than the individual treatments alone^(7,8). There was a good inverse correlation between the level of tissue fat and apoptosis suggesting that decreasing tissue fat has a role in enhancing apoptosis and inhibiting skin tumor formation⁽⁷⁾. In other studies, we found that decreasing tissue fat by surgical removal of the parametrial fat pads also enhanced UVB-induced apoptosis and inhibited UVB-induced carcinogenesis^(9,10).

In the present study, we determined whether RW in combination with caffeine administration (0.1 mg/ml drinking water) has a greater inhibitory effect on UVB-induced skin carcinogenesis than either caffeine administration or RW alone. We also investigated whether the inhibitory effect of RW in combination with oral administration of caffeine on UVB-induced carcinogenesis is associated with decreased levels of inflammatory cytokines in the epidermis of SKH-1 mice fed a high-fat diet. Since most skin cancers have mutant p53, we also explored whether caffeine administration and RW decreased inflammatory cytokines in the epidermis of p53 knockout mice and their wild-type littermates irradiated with UVB and fed a high-fat diet.

Materials and Methods

Animals and diet

Female SKH-1 hairless mice (6–7 weeks old) were purchased from Charles River Breeding Laboratories and kept in our animal facility for 1 week before use. Mice were maintained on a 12 h light/12 h dark cycle and provided food (Laboratory Chow 5001 from the Ralston Purina company) and water *ad libitum*. The 40% kcal high fat diet rich in omega-6 fatty acids as described by Reddy et al.⁽¹¹⁾ was purchased from Dyets Inc. (Bethlehem, PA) and stored at –20°C until use.

Male haired p53(–/–) mice on a C57BL/6J genetic background (The Jackson Laboratory, Bar Harbor, Maine) were mated with female hairless SKH-1 mice (p53 wild type) to obtain hairless congenic p53 deficient mice. The hairless p53(+/-) mice were intercrossed to obtain heterozygous and homozygous p53 deficient mice and their wild type littermates⁽¹²⁾.

Exposure to UVB

The UV lamps used (FS72T12-UVB-HO; National Biological Corp., Twinsburg, Ohio) emitted UVB (280–320 nm; 75–80% of total energy) and UVA (320–375 nm; 20–25% of total energy). The dose of UVB was quantified with a UVB Spectra 305 dosimeter (Daavlin Co., Byran, OH). The radiation was further calibrated with a model IL-1700 research radiometer/photometer (International Light Inc., Newburyport, MA).

Effect of oral caffeine, voluntary exercise, and their combination on UVB-induced skin carcinogenesis

One hundred and sixty female SKH-1 mice (7–8 weeks old) were exposed to UVB (30 mJ/cm²) once a day, twice a week for 20 weeks. UVB irradiation was then stopped. Half of the mice were then placed in cages with a RW (13.75-cm diameter; 7 cm width) with free access to the wheel 24 h/day (ten mice per cage). Other mice with matched body weights and age were placed in cages without a RW and served as controls. We pre-measured the running activity of each individual mouse for 3 consecutive days. Mouse ran at or below half of the average exercise levels were excluded from our study. The wheels were attached to a permanent magnetic switch that activated a digital counter to count wheel revolutions. Total

wheel revolutions were recorded daily, with total distance run per day determined by multiplying the number of wheel rotations by the circumference of the wheel. A water bottle containing water or caffeine (0.1 mg/ml) was attached at the top of the cage unit. During the experiment, food consumption (Laboratory Chow 5001) was measured twice a week, fluid consumption was measured three times a week, and running wheel activities were measured daily. Body weight and skin tumor formation (percent mice with tumors, number of tumors per mouse and tumor volume per mouse) were measured every 2 weeks. The counting of all tumors and measurement of tumor size were performed blind with respect to treatment group. Tumor volume was determined by measuring the three-dimensional size (height, length, and width) of each mass. The average of the three measurements was used as the diameter. The radius (r) was determined, and the tumor volume was calculated by: $\text{volume} = \frac{4}{3} r^3$. After 14 weeks of voluntary RW exercise with or without caffeine administration, all mice were sacrificed, and the parametrial fat pads were removed and weighed.

Time course for the effects of oral caffeine, voluntary exercise, and their combination on UVB-induced apoptosis and cell proliferation

For studies on the time course for the effects of caffeine administration, RW, or their combination on UVB-induced apoptosis, half of the SKH-1 mice (5 mice per time point, 11 time points) were placed in cages (5 mice per cage) with a running wheel with free access to the wheel 24 hr/day for 2 weeks. A bottle containing water or caffeine (0.1 mg/ml) was attached at the top of the cage unit. The other half of the mice (5 mice per time point, 11 time points with matched body weights and age) were placed in cages without a running wheel and they were given water or caffeine (0.1 mg/ml drinking water).

After two weeks of running wheel exercise with or without caffeine administration, the mice were irradiated once with 30 mJ/cm² of UVB and killed at various times post-UVB. All animals were injected with bromodeoxyuridine (BrdU, 50 mg/kg) intraperitoneally and killed 1 hour later. The parametrial fat pads were removed and weighed. The dorsal skin was taken, stapled flat to a plastic sheet and placed in 10% phosphate-buffered formalin at 4°C for 18–24 hrs. The skin samples (about 20 mm long and 5 mm wide) taken from the middle of the back were dehydrated, cleared in xylene, and embedded in Paraplast. Four- μ m serial sections of skin were made and used for regular hematoxylin-eosin (H&E) or immunohistochemical staining. Apoptotic sunburn cells, caspase 3 (active form) positive cells and BrdU positive cells were determined.

Measurement of apoptotic sunburn cells, caspase 3 immunostaining and bromodeoxyuridine incorporation into DNA

Identification of apoptotic sunburn cells was based morphologically on cell shrinkage and nuclear condensation as we have done previously⁽¹³⁾. Apoptotic sunburn cells were identified by their intensely eosinophilic cytoplasm and small, dense nuclei, which were observed in H&E-stained histological sections of the skin using light microscopy.

For caspase 3 staining, affinity-purified polyclonal rabbit antibody that reacts with the mouse p20 subunit of caspase 3 was purchased from Cell Signaling Technology (Danvers, MA, cat, no: 9661). Endogenous peroxidase was blocked with 3% hydrogen peroxide. The sections were incubated with a protein block for 1 h. The sections were incubated with caspase 3 primary antibody (1:300 dilution) overnight at 4°C followed by incubation with a biotinylated anti-rabbit secondary antibody for 30 min, followed by incubation with conjugated-avidin solution for 30 min. Color development was achieved with 0.02% 3,3'-diaminobenzidine tetrahydrochloride containing 0.02% hydrogen peroxide.

For BrdU staining, endogenous peroxidase was blocked with 3% hydrogen peroxide. The tissue sections were incubated with 0.125% trypsin for 10 min at 37°C, followed by incubation with denaturing solution for 30 min. The sections were incubated with blocking solution, and covered with biotinylated mouse monoclonal anti-BrdU antibody (Life Technologies, Grand Island, NY) at room temperature for 90 min. Sections were incubated with streptavidin-peroxidase. Color development was achieved with 0.02% 3,3--diaminobenzidine tetrahydrochloride containing 0.02% hydrogen peroxide.

The percentage of apoptotic sunburn cells, caspase 3 and BrdU positive cells was calculated from the number of these cells per 100 epidermal cells counted from the entire 20 mm length of epidermis as we have done previously^(13,14).

Oral caffeine, voluntary exercise, and their combination on inflammatory cytokine levels - mouse inflammation antibody array

Effects of caffeine, RW, and their combination on inflammatory cytokine levels in the epidermis of UVB-treated mice fed a 40% kcal high fat diet for 2 weeks were determined using a RayBio® Mouse Inflammation Antibody array kit (detection of 40 proteins associated with inflammation, purchased from RayBiotech, Inc. Norcross GA).

In experiment 1, twenty-four female SKH-1 mice were given the high fat diet for 4 weeks. Half of the mice (3 mice per group) were then treated with: water (control), 0.1 mg/ml of caffeine in the drinking fluid, RW, or 0.1 mg/ml caffeine plus RW for 1 week. The other half of the mice (3 mice per group) were treated with water, caffeine, RW, or caffeine plus RW for 2 weeks. During the treatment, all the mice were exposed to UVB (30 mJ/cm²) once a day for 7 or 14 consecutive days and killed 2 hours after last UVB exposure

In experiment 2, twelve male congenic hairless p53 knockout mice and twelve of their p53 wild-type littermates were given the high fat diet for 4 weeks. These mice were then treated with water, 0.1 mg/ml of caffeine, RW, and 0.1 mg/ml of caffeine plus RW for 1 week. During treatment, all the mice were exposed to UVB (30 mJ/cm²) once a day for 7 consecutive days and killed 2 hours after last UVB exposure.

Epidermal lysates were prepared as described previously⁽¹⁵⁾. The antibody array was performed following the instructions provided by the manufacturer. Briefly, membranes were treated with 2 ml of Blocking Buffer and incubated overnight with 1 ml of tissue lysate (150 µg protein) at 4°C. After washing, 1 ml of biotin-conjugated antibodies were added for 1 hr and incubated with 2 ml of HRP-conjugated streptavidin at room temperature for 2 hrs. The membranes were then treated with 500 µl of Detection Buffer and, finally, exposed to x-ray film.

By comparing the signal intensities, relative expression levels of cytokines were determined and quantified by densitometry. Positive controls were used to normalize the results from the different membranes being compared.

All experimental procedures were approved by IACUC of the Rutgers (protocol No: 88-056).

Statistics analysis

The ANOVA model with Dunnett's adjustment was used for comparisons of multiple treatment groups with a common control group. For the analyses of tumor distributions (percent of mice with tumors vs. time) between the treatments, the Kaplan–Meier method was used for estimations, and the log-rank test was used to test homogeneity. The repeated measurement models were used for the analyses of the treatment effects on body weight,

tumor number per mouse, and tumor volume per mouse between the groups, and the cubic root for tumor volume per mouse was used in the analyses. The treatment effects were assessed on the basis of the comparisons of slope of the regression lines. The comparisons were also performed at the last time point (week 14). A 5% significance level was used for all of the tests.

Results

Effects of caffeine, exercise or their combination on body weight, food and fluid consumption, running wheel activity and tissue fat in UVB-pretreated high-risk mice

One hundred and sixty female SKH-1 mice were exposed to UVB (30 mJ/cm²) once a day, twice a week for 20 weeks. UVB irradiation was stopped. These UVB-pretreated mice have no tumors but they develop tumors in the absence of further UVB irradiation over the next several months. The mice were then equally divided into four groups (40 mice per group) and treated with: water (control), caffeine (0.1 mg/ml in the drinking water), or caffeine (0.1 mg/ml) plus RW for 14 weeks. The results showed that although treatment of SKH-1 mice with oral caffeine, RW or caffeine + RW for 14 weeks did not have an effect on body weight (Fig. 1A), oral caffeine together with RW resulted in a 24% increase in the daily food consumption ($p<0.01$) (Fig. 1B), and a 31% increase in fluid consumption ($p<0.01$) (Fig. 1C) than in control mice receiving water alone. Over the course of the 14 weeks study, the addition of caffeine to the voluntary exercise regimen in SKH-1 mice increased running activity by 40% compared with RW alone ($p<0.01$) (Fig. 1D), and the combined caffeine + RW group ingested about 8% more caffeine than the group treated only with caffeine (Fig. 1C).

Treatment of the high-risk mice with caffeine, RW or caffeine + RW for 14 weeks decreased the weight of the parametrial fat pads by 20%, 27% and 44%, respectively ($p<0.01$) (Fig. 1E). These results indicate that oral administration of caffeine together with RW had a greater effect for lowering tissue fat than either treatment alone.

Effect of caffeine, exercise or their combination on skin carcinogenesis in UVB-pretreated high-risk mice (14 week treatment)

Treatment of UVB-pretreated high-risk mice with caffeine together with RW during 14 weeks had no effect on body weight (Fig. 2A), but this treatment decreased the percentage of mice with tumors during 6–12 week time intervals of the study (Fig. 2B). The medium tumor-free survival time in the combination treatment group was significantly longer than that in other treatment groups ($p=0.0097$, log-rank test). The tumors per mouse and tumor volume per mouse in combination of caffeine and RW group were decreased substantially at all time intervals when compared to that in water control group (Fig. 2C, 2D). After 14 weeks of treatment, the water-treated control group had 5.47 tumors per mouse, caffeine-treated, RW-treated and caffeine + RW-treated mice had 4.00, 3.53, and 2.07 tumors per mouse, respectively (Fig. 2C). Treatment with caffeine, RW or caffeine together with RW decreased the number of skin tumors per mouse by 27, 35 ($p<0.05$) and 62% ($p<0.01$), respectively, in the UVB-pretreated, high-risk mice (Fig. 2C). Treatment with caffeine, RW or caffeine together with RW decreased UVB-induced skin tumor volume per mouse by 61, 70 ($p=0.05$) and 85% ($p<0.01$), respectively (Fig. 2D).

Effects of caffeine, exercise or their combination for two weeks on body weight, food and fluid consumption, running wheel activity and tissue fat

Mechanistic studies indicated that treatment of female SKH-1 mice with oral caffeine, RW or caffeine + RW for 2 weeks did not have an effect on body weight (Fig. 3A). Oral caffeine together with RW resulted in a 12% increase in daily food consumption ($p<0.01$) (Fig. 3B),

and a 15% increase in fluid consumption ($p<0.01$) (Fig. 3C) when compared with control mice receiving water alone. Over the course of the two-week study, the addition of caffeine to the voluntary exercise regimen in SKH-1 mice increased running activity by 22% compared with RW alone ($p<0.01$) (Fig. 3D), and the combined caffeine + RW group ingested about 5% more caffeine than the group treated only with caffeine (Fig. 3C).

Treatment of SKH-1 mice with caffeine, RW or caffeine + RW for 2 weeks decreased the weight of the parametrial fat pads by 30%, 56% and 63%, respectively ($p<0.01$) (Fig. 3E). Our results further demonstrated that oral administration of caffeine together with RW had a greater effect in lowering tissue fat than either treatment alone.

Effects of caffeine, exercise or their combination for two weeks on the time courses for UVB-induced apoptosis and proliferation

Treatment of SKH-1 mice with a single irradiation of UVB (30 mJ/cm^2) resulted in increased apoptotic sunburn cells and caspase 3 (active form) staining cells in the epidermis starting at 4 hrs after UVB, and the peak was at 6–10 hrs after UVB (Fig. 4A). Treatment of SKH-1 mice with caffeine, RW or caffeine + RW for 2 weeks prior to UVB irradiation stimulated the UVB-induced increase in apoptotic sunburn cells by 21%, 124% and 298%, respectively, at 6 hr post-UVB (Fig. 4A), and these treatments stimulated the UVB-induced increase in caspase 3 (active form) positive cells by 30%, 164% and 333%, respectively, at 6 hr post-UVB (Fig. 4B). Our results indicate that a low dose of caffeine in combination with RW markedly amplified the UVB-induced increase in apoptosis in the epidermis of SKH-1 mice. These results are similar to what we observed earlier with a single 6 hr post-UVB time point⁽⁷⁾.

BrdU-labeling of epidermal cells was utilized as a measure of DNA synthesis. BrdU was injected into SKH-1 mice 1 h before sacrifice, and the BrdU labeling index was determined. Irradiation of SKH-1 mice with UVB (30 mJ/cm^2) resulted in a rapid doubling in the number of BrdU-positive cells mainly localized in the basal layer of the epidermis with the peak at 0.1 h post UVB (within 0 – 1 h), and this was followed by a >100% inhibition in BrdU-positive cells at 2–6 h after irradiation of the mice with UVB (Fig. 5). A large increase in the number of BrdU-positive cells (about 3-fold) occurred at 16–24 hrs post-UVB. Treatment of the mice with caffeine together with RW for 2 weeks prior to UVB irradiation decreased BrdU labeling in the epidermis between 0.1–0.5 hr post-UVB and between 2–6 hr post-UVB (34% decrease at 0.1 h after UVB and a 66% decrease at 6 hr after UVB, $p<0.01$).

Effects of caffeine, exercise or their combination on inflammatory cytokines in the epidermis: Studies with an antibody array

Previously, we reported that inflammatory cytokine levels were elevated in UVB-induced skin tumors in SKH-1 mice⁽¹⁶⁾. In the normal epidermis, only a few inflammatory cytokines were observed. Observable cytokines were IL-1 (interleukin-1), MIP-1 (macrophage inflammatory protein-1) and sTNF RI (soluble TNF alpha receptor-1). Among 40 cytokines examined, 3 cytokines were strikingly increased in tumors. They were: TIMP1 (tissue inhibitor of metalloproteinases-1), LIX (lipopolysaccharide-induced CXC chemokine) and sTNF RI. TIMP1 was not detected in normal epidermis, whereas, high levels of TIMP1 were observed in keratoacanthomas and carcinomas. In addition to TIMP1, the levels of LIX, sTNF RI and MIP-1 were also significantly increased in tumors. Besides these four cytokines, small amounts of several other cytokines associated with inflammation were observed in tumors, but they were not found in the normal epidermis⁽¹⁶⁾. These results suggest that TIMP1, LIX, sTNF RI, MIP-1 and other inflammation-related cytokines may play an important role in UVB-induced skin tumor formation.

In the present study, an antibody array was used to compare the levels of inflammatory cytokines in the epidermis of SKH-1 mice fed a high-fat diet and treated with UVB together with caffeine, RW or caffeine + RW. Our initial studies showed that oral administration of caffeine during running wheel exercise had little observable effects in the levels of inflammatory cytokines in low-fat chow diet fed animals exposed to UVB once a day for 1–2 weeks (data not shown). In experiment 1, SKH-1 mice were given a high-fat diet rich in omega-6 fatty acids for 4 weeks. This diet was continued, and the animals were treated with UVB (30 mJ/cm²) once a day for 7 or 14 days together with water, caffeine, RW, or caffeine + RW. Treatment with caffeine, RW or caffeine + RW for 1 week resulted in little or no change in the levels of these four cytokines (LIX, sTNF RI, MIP-1 and TIMP1), but there was a lowering of a few faintly observed cytokines associated with inflammation (Fig. 6A). After 2 weeks of treatment with UVB together with caffeine, RW or caffeine + RW, the level of LIX was decreased by 10%, 50% and 92%, respectively; the level of sTNF RI was decreased by 23%, 63% and 61% respectively, and the level of MIP-1 was decreased by 69%, 49% and 71%, respectively. Barely detectable TIMP1 was observed in water-treated control mice, but TIMP1 was not detected in the epidermis of caffeine-, RW-, or caffeine + RW-treated mice. In addition, low levels of 11 other cytokines (BLC, CD30 L, GM-CSF, IL-4, IL-12p40p70, IL-12p70, lymphotactin, MCP-1, MCSF, SDF-1 and TCA-3) were observed in the epidermis from water treated mice, but only 6 of these cytokines were observed in caffeine-treated mice, two were observed in RW-treated and none of them were seen in caffeine + RW treated mice (Fig. 6B). These results indicated that UVB-induced inflammation was enhanced by the high-fat diet, and treatment of SKH-1 mice given the high-fat diet together with UVB irradiation with caffeine + RW resulted in a significant decrease in the levels of several cytokines that are associated with inflammation.

Our results in experiment 2 indicated that hairless p53 wild-type mice given a high-fat diet rich in omega-6 fatty acids for 4 weeks and then treated with UVB together with water, caffeine, RW or caffeine + RW for 7 days resulted in similar changes in the levels of inflammatory cytokines as observed in the SKH-1 mice treated with caffeine, RW, or caffeine + RW for 2 weeks (Fig. 6C). Treatment of p53 wild-type mice with caffeine, RW or caffeine + RW, for 7 days, decreased the level of LIX by 44%, 47% and 49%, respectively; the level of sTNF RI was decreased by 19%, 18% and 43%, respectively, and the level of MIP-1 was decreased by 45%, 42% and 58%, respectively. Barely detectable TIMP1 was also observed in the epidermis of water-treated control mice but not in the epidermis from caffeine-, RW- or caffeine + RW- treated mice (Fig. 6C). In addition, 6 other cytokines, such as IL-4, IL-12p40p70, lymphotactin, MCP-1, MCSF, and TCA-3 were barely detectable in water-treated epidermis, but only 4 of them were observed in caffeine-treated mice, and 2 were observed in RW-treated or in caffeine + RW treated mice (Fig. 6C).

Treatment of p53 knockout mice given a high-fat diet rich in omega-6 fatty acids for 4 weeks and treated with UVB together with water, caffeine, RW, or caffeine + RW for 7 days resulted in a slightly different pattern in the level of inflammatory cytokines when compared with their wild-type littermates. Treatment of p53 knockout mice with caffeine, RW or caffeine + RW, decreased the level of LIX by 19%, 24% and 60%, respectively; but the levels of sTNF RI and MIP-1 were not changed. TIMP1, which was barely detectable in the water-treated control p53 knockout mice, was not detected in the epidermis of RW- or caffeine + RW-treated mice. Interestingly, low levels of 14 other cytokines (BLC, CD30 L, Eotaxin-2, GM-CSF, IL-2, IL-4, IL-12p40p70, IL-12p70, lymphotactin, MCP-1, MCSF, RANTES, SDF-1 and TCA-3) were detected in the epidermis from water treated mice, but only 8 of these cytokines were observed in caffeine-treated mice, 5 of these cytokines were detected in RW-treated mice and 6 were observed in caffeine + RW treated mice (Fig. 6D).

Discussion

There have been numerous reports about the metabolic effect of caffeine ingestion during physical activity in humans. Caffeine ingestion during exercise increases the plasma concentration of epinephrine^(17–21), free fatty acids^(22–30), glycerol^(17,22,31), glucose^(26–28,32–34), insulin^(31,34), and lactate^(22,26–28,32–34). However, the effects of oral caffeine during physical exercise in decreasing skin tumor formation have not been previously investigated.

In earlier studies, we showed that RW or oral caffeine for 2 weeks decreased tissue fat and stimulated UVB-induced apoptosis in the epidermis of hairless SKH-1 mice^(9,35) and that combined RW and oral caffeine resulted in a greater than additive effect when compared with RW or caffeine alone⁽⁷⁾. Administration of caffeine or RW alone inhibited UVB carcinogenesis. In the present study, we report that UVB-pretreated, high-risk mice given a combination of caffeine and RW for 14 weeks had greater effects for decreasing tissue fat, stimulating UVB-induced apoptosis, decreasing cell proliferation and for inhibiting skin tumor formation than by treating the mice with caffeine or RW alone. These effects of the various treatments were associated with decreased tumor incidence, decreased number of tumors per mouse, and decreased tumor volume per mouse. In addition, this study showed that there were no differences in body weight between the various groups of mice from the beginning to the end of the study. These results suggest that the effects of caffeine and RW on tissue fat as well as on apoptosis may play a key role in inhibiting UVB-induced skin carcinogenesis. The results of the present study indicated that treatment of UVB-pretreated, high-risk mice with a low dose of caffeine, RW or the combination of caffeine and RW for 14 weeks, inhibited tumor formation, and the combination of caffeine and RW was more effective than either treatment alone. The enhanced effects of combined RW + caffeine, when compared with RW or caffeine alone may be partly a consequence of caffeine administration enhancing RW activity by 22% for the 2 week experiment and 40% for the 14 week experiment and RW increasing caffeine intake by $\approx 8\%$. For these experiments, mice were group housed (10 mice per cage), so we cannot rule out the possibility for the effects of environmental enrichment. As reported by Cao's group at Ohio State University, environmental enrichment influences carcinogenesis {Cao, 2012 #627}; Therefore, the mechanisms in the present study might be different if animals were housed individually.

In a previous study, we explored the effects of two types of 40% kcal high-fat diet (rich in either omega-3 or omega-6 fatty acids) on UVB-induced carcinogenesis and inflammatory cytokines in the epidermis by an antibody array (before tumor formation)⁽¹⁶⁾. UVB-induced carcinogenesis was enhanced by feeding SKH-1 mice the high fat diet rich in omega-6 fatty acids, compared to the high fat diet rich in omega-3 fatty acids. We found that when compared with normal epidermis in mice fed a low fat chow diet (without UVB exposure), the level of LIX in the epidermis of the mice fed the high fat diet rich in omega-6 fatty acids was increased by 196% and sTNF R1 was increased by 200%. TIMP-1 was not detected in the normal epidermis from chow fed mice, whereas a small amount of TIMP-1 was observed in the epidermis of mice fed the high fat diet rich in omega-6 fatty acids diet. In addition, small amounts of 18 other cytokines, such as CD30L, Eotaxin-2, GM-CSF, IFN-c, IL-4, IL-12p40p70, IL-12p70, IL-13, IL-17, lymphotactin, MCP-1, M-CSF, MIG, MIP-1a, RANTES, SDF-1, and sTNF RII, were observed in the epidermis from mice fed the high fat diet, but only three of the cytokines were seen in normal epidermis from chow fed mice⁽¹⁶⁾.

In the present study, we investigated the effects of caffeine in combination with exercise on UVB-induced inflammatory responses in the epidermis of mice fed the 40% kcal high fat diet rich in omega-6 fatty acids and irradiated with UVB for 1–2 weeks. Our data indicate that administration of caffeine + RW to SKH-1 mice receiving the high fat diet and UVB

irradiation resulted in greater than additive effects for decreasing the levels of LIX, sTNF RI, MIP-1, TIMP1 and several other cytokines which are associated with inflammation when compared with the effect of RW or caffeine alone. Tissue fat in the combination group was also markedly decreased (data not shown). These observations suggest that inflammatory cytokines were secreted by tissue fat and were increased by feeding the high fat diet and UVB irradiation. These increases were inhibited by caffeine and exercise.

LIX, sTNF RI, MIP-1 and TIMP1 are all pro-inflammatory cytokines. LIX has potent chemo-attractant activity for neutrophils *in vitro* and *in vivo* and amplifies a pro-inflammatory cytokine response via a phosphatidylinositol 3-kinase–nuclear factor-kappaB pathway⁽³⁶⁾. Tumor necrosis factor- (TNF-) is one of the most potent pro-inflammatory cytokines produced by activated macrophages in response to tissue injury or chronic inflammation. Its production leads to the shedding of soluble tumor necrosis factor receptors (sTNF RI and sTNF RII) from cell membranes into the circulation. Through its pro-inflammatory actions, TNF- may play a role in cancer growth and metastasis by inducing reactive oxygen species, which can cause DNA damage and inhibit DNA repair⁽³⁷⁾. An increase in the serum level of sTNF RI was significantly associated with a higher risk of endometrial cancer and the severity of multiple inflammatory-related symptoms in patients with colorectal and esophageal cancer⁽³⁷⁾. MIP-1 activates human granulocytes and lead to acute neutrophilic inflammation. MIP-1 also induces the synthesis and release of other pro-inflammatory cytokines such as IL-1, IL-6 and TNF- from fibroblasts and macrophages. TIMP-1 protein is able to promote cell proliferation and may have an anti-apoptotic function. TIMP-1 plays a significant role in regulation of angiogenesis in cancers^(38,39). TIMP1 may also play an important role in the pathogenesis of non-melanoma skin cancer and are useful indicators of cutaneous cancer invasion and progression⁽⁴⁰⁾. Our present studies provide evidence suggesting that decreasing the levels of LIX, sTNF RI, MIP-1 and TIMP1 by caffeine and exercise may be associated with decreased UVB-induced skin carcinogenesis.

In summary, our results demonstrate that oral administration of a low physiologically relevant dose of caffeine together with voluntary exercise was more effective in decreasing UVB-induced skin tumor formation when compared with caffeine or RW alone. Possible mechanisms for the inhibition may be associated with altered RW activity and caffeine intake as well as decreased tissue fat, resulting in UVB-induced apoptosis, and decreased cytokines that are associated with inflammation in the epidermis.

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FIG 1. Effect of oral administration of caffeine (CF), voluntary running wheel exercise (RW) or their combination (CF+RW) on body weight, food and fluid consumption, running wheel activity and tissue fat in SKH-1 mice previously treated with UVB

One hundred and sixty female SKH-1 mice (6–7 weeks old) were exposed to UVB (30 mJ/cm²) once a day, twice a week for 20 weeks. UVB irradiation was stopped, and the mice (40/group, 10 mice/cage) were then treated with water (control), CF (0.1 mg/ml in drinking water), RW or CF plus RW for 14 weeks in the absence of further irradiation with UVB. All mice were fed Laboratory Chow 5001 diet throughout the entire study. All animals were sacrificed and body weight (A), food consumption (B), fluid consumption (C), and running wheel activity (D) was measured from the beginning to the end of the study. The weight of

the parametrial fat pads (E) was measured at the end of the study. Each value is the mean $\pm$ SE from 40 mice (14 week study). *: Compared to water (control) for (B), (C), and (E), *: CF and RW compared to RW alone for (D), Dunnett's adjusted $p < 0.01$.

FIG 2. Effect of oral administration of caffeine (CF), voluntary running wheel exercise (RW) or their combination (CF+RW) on UVB-induced skin carcinogenesis in SKH-1 mice

One hundred and sixty female SKH-1 mice (6–7 weeks old) were exposed to UVB (30 mJ/cm²) once a day, twice a week for 20 weeks. UVB irradiation was stopped. The mice (40/group, 10/cage) were then treated with water (control), CF (0.1 mg/ml in drinking water), RW or CF plus RW for 14 weeks. All mice were fed Laboratory Chow 5001 diet throughout the entire study. All animals were sacrificed and body weight (A), percent mice with tumors (B), number of tumors per mouse (C), and tumor volume per mouse (D) from the beginning to the end of the study were measured every 2 weeks. Each value is the mean $\pm$ SE from 40 mice.

FIG 3. Time course for the effects of oral administration of caffeine (CF), voluntary running wheel exercise (RW) or their combination (CF+RW) on body weight, food consumption, fluid consumption, running wheel activity and weight of the parametrial fat pads in SKH-1 mice
 Female SKH-1 mice (5 mice per time point, 4 treatments, total 220 mice) were treated with water (control), CF (0.1 mg/ml in drinking water), RW or 0.1 mg/ml of CF plus RW for 2 weeks. Laboratory Chow 5001 diet was fed to all mice *ad libitum*. The pretreated mice were then exposed to a single irradiation with UVB (30 mJ/cm²) and sacrificed at 0 (non-UVB), 5 min, 0.5, 1, 2, 4, 6, 10, 16, 24 and 48 hrs post UVB. Body weight (A), food consumption, (B) fluid consumption (C), and running wheel activity (D) were measured during the course of the study. The weight of the parametrial fat pads (E) was measured at the end of the

study. Each value is the mean $\pm$ SE from 55 mice (2 week study). *: Compared to water (control) for (B), (C), and (E), *: CF and RW compared to RW alone for (D), Dunnett's adjusted $p < 0.01$.

A

B

FIG 4. Time course for the effects of oral administration of caffeine (CF), voluntary running wheel exercise (RW) or their combination (CF+RW) on UVB-induced apoptosis in the epidermis of SKH-1 mice

Female SKH-1 mice (5 mice per time point, 4 treatments, total of 220 mice) were treated with: water (control), CF (0.1 mg/ml in drinking water), RW or 0.1 mg/ml of CF plus RW for 2 weeks. All mice were given Laboratory Chow 5001 diet *ad libitum*. The pretreated mice were then exposed to a single irradiation with UVB (30 mJ/cm²) and sacrificed at 0 (non-UVB) treated mice or at 5 min, 0.5, 1, 2, 4, 6, 10, 16, 24 and 48 hrs post-UVB. Apoptotic sunburn cells were determined morphologically (A), and caspase 3 (active form) positive cells were determined immunohistochemically (B). Each value is the mean $\pm$ SE from 5 mice.

FIG 5. Time course for the effects of oral administration of caffeine (CF), voluntary running wheel exercise (RW) or their combination (CF+RW) on UVB-induced cell proliferation in the epidermis of SKH-1 mice

Female SKH-1 mice (5 mice per time point, 4 treatments, total 220 mice) were treated as described in Fig. 4. Bromodeoxyuridine (BrdU) was injected at 1 hr prior to sacrificing the animals, and BrdU positive cells were determined immunohistochemically. Each value is the mean $\pm$ SE from 5 mice.

FIG 6. Effect of oral administration of caffeine (CF), voluntary running wheel exercise (RW) or their combination on the levels of inflammatory cytokines in the epidermis: an antibody array study

In experiment 1, twenty-four female SKH-1 mice were given a 40% kcal high fat diet rich in omega-6 fatty acids for 4 weeks. The high-fat diet was continued, and the mice were irradiated with UVB (30 mJ/ cm²) once a day for 7 or 14 days as described in the “Materials and Methods” section. Half of the mice (3 mice per group) were treated with: water (control), CF (0.1 mg/ml drinking fluid), RW, or CF + RW for 1 week (A). The other half of the mice were treated with: water, 0.1 mg/ml of CF, RW, or CF (0.1 mg/ml) + RW for 2 weeks (B). In experiment 2, twelve male congenic hairless p53 knockout mice and twelve of

their wild-type p53 littermates were given a high-fat diet rich in omega-6 fatty acids for 4 weeks as described in the “Materials and Methods” section. The high-fat diet was continued, and p53 wild-type mice (C) and p53 knockout littermates (D) (3 mice per group) were then treated with: water, CF (0.1 mg/ml), RW or CF+ RW for 1 week.

In experiments 1 and 2, all mice were irradiated with UVB (30 mJ/cm²) once a day for 7 or 14 consecutive days and killed 2 hours after the last UVB irradiation. The epidermis was collected, and epidermal lysates were prepared and subjected to antibody array as described in the “Materials and Methods” section. Each value in parenthesis is the average intensity of two parallel signals quantified by densitometry. Positive controls were used to normalize the results from different membranes being compared.