Oral administration of caffeine during voluntary exercise markedly decreases tissue fat and stimulates apoptosis and cyclin B1 in UVB-treated skin of hairless p53-knockout mice

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Treatment of p53(-/-) mice orally with caffeine, voluntary exercise or their combination for 2 weeks prior to a single irradiation with UVB increased the level of the epidermal fat pads by 22, 40 and 56%, (ii) decreased the thickness of the dermal fat layer by 10, 26 and 42%, (iii) increased the number of apoptotic sunburn cells by 29, 100 and 489%, and (iv) increased the number of mitotic cells with cyclin B1-positive staining by 40, 210 and 510%, respectively. Pearson’s correlation coefficient indicated a statistically significant inverse relationship between the level of tissue fat and the number of mitotic cells with cyclin B1 in p53(-/-) mice but not in p53(+/-) littermates. Western blot analysis indicated that treatment of p53(-/-) mice with caffeine together with exercise increased the level of cyclin B1 significantly more than in p53(+/-) mice. p53(-/-) mice, but not p53(+/-) mice, treated with caffeine during exercise exhibited a dramatic decrease in the level of survivin. Our results suggest that voluntary exercise in combination with oral caffeine may exert a synergistic increase in UVB-induced apoptosis and that tissue fat may be a more important modulator of apoptosis and carcinogenesis in p53-deficient mice than in p53-normal mice. The stimulatory effects on apoptosis in p53(-/-) mice by the combination treatment might be associated with increased levels of cyclin B1 and decreased levels of survivin.

Introduction

We previously reported that oral administration of caffeine strongly inhibited UVB-induced skin tumor formation in hairless SKH-1 mice (1,2). Subsequently, our mechanistic studies showed that caffeine enhanced UVB-induced increases in apoptosis by p53-dependent and p53-independent pathways (3–5). Oral administration of caffeine inhibited the UVB-induced increase in ataxia-telangiectasia mutated (ATM)- and Rad3- related (ATR)-mediated phosphorylation of Chk1 and prematurely increased the number of mitotic cells with cyclin B1 resulting in enhanced early and inappropriate lethal mitosis (apoptosis) (6). In additional studies, we found that inhibition of tumorigenesis by orally administered caffeine was associated with a decrease in tissue fat and stimulation of locomotor activity (2,7). An increase in locomotor activity by voluntary exercise decreased fat stores, stimulated apoptosis and deceased skin tumor formation (8). Voluntary exercise also stimulated apoptosis in the epidermis of p53-knockout mice suggesting that the effects of exercise on apoptosis may be through a p53-independent mechanism (9). In a recent study, we found that treatment of mice with voluntary running wheel exercise (RW) in combination with oral caffeine had a greater than additive stimulatory effect in decreasing tissue fat and stimulating apoptosis in SKH-1 mice (10). 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 (10). In other studies, we found that decreasing tissue fat by surgical removal of the parametrial fat pads also enhanced UVB-induced apoptosis (9). The results of these studies suggest that RW, when combined with caffeine administration, may have a synergistic inhibitory effect on UVB-induced skin carcinogenesis.

Most skin cancers have mutant p53 but no wild-type p53; therefore, in the present study, we explored whether voluntary exercise in combination with oral administration of a low dose of caffeine (i.e. 0.1 mg/ml and yielding blood levels of caffeine equivalent to approximately one to two cups of coffee per day in humans) may be a more effective regimen for enhancing UVB-induced apoptosis in the epidermis of p53-knockout mice than in their p53 wild-type littermates. We also investigated whether the stimulatory effects on UVB-induced apoptosis by this regimen in p53-knockout mice is associated with a decreased level of tissue fat and an increased level of cyclin B1.

Materials and methods

Animals

Male haired p53(-/-) mice on a C57BL/6J genetic background (The Jackson Laboratory, Bar Harbor, ME) were mated with female hairless SKH-1 mice (p53 wild-type) to obtain male and female hairless congenic p53-deficient mice. The hairless p53(+/-) mice were intercrossed for 26 generations to obtain heterozygous and homozygous p53-deficient mice and their wild-type littermates (F26 generation). The animals were kept in our animal facility and were given water and Purina Laboratory Chow 5001 diet from the Ralston Purina Co. (St Louis, MO) ad libitum. They were kept on a 12 h light–12 h dark cycle. Mice were 8–10 weeks old at the start of the study.

Exposure to UVB

The UV lamps used (FS72T12-UVB-HO; National Biological Corp., Twinsburg, OH) emitted UVB (280–320 nm; 75–80% of total energy) and UVA (320–375 nm; 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, Newburyport, MA).

Caffeine administration, voluntary exercise and the preparation of skin samples

For studies on the effect of coffee administration, voluntary RW or their combination on UVB-induced apoptosis and cyclin B1, two groups each of hairless p53(-/-), p53(+/-) or their p53(+/-) wild-type littermates (six mice per group) were placed in cages with a running wheel (13.75 cm diameter; 7 cm width) with free access to the wheel 24 h/day for 2 weeks. A bottle containing water or caffeine (0.1 mg/ml) was attached at the top of the cage. Two other groups of mice (six mice per group) with matched body weights and age were placed in cages without running wheel and they were given water or caffeine (0.1 mg/ml) (control groups). 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. After 2 weeks of RW with or without caffeine administration, the treated mice and their controls were irradiated once with 30 mJ/cm² of UVB and killed 6 h later. Our previous studies showed that 6–10 h after a single irradiation with UVB is the peak time for the formation of UVB-induced apoptotic sunburn cells (3,4). The two epidermal 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 h. The skin samples (~20 mm long and 5 mm wide) taken from the middle of the back were dehydrated, cleared in xylene and embedded in Paraplast. Four micrometer serial sections of skin were made and used for regular hematoxylin and eosin or immunohistochemical staining. The thickness of the dermal fat layer

Abbreviation: ATR, ataxia-telangiectasia mutated- and Rad3- related; RW, running wheel exercise.

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was measured using an ocular micrometer with an Olympus BHTU light microscope under 100-fold magnification at 5–10 representative areas per slide and averaged (2). Apoptotic sunburn cells, caspase-3 (active form)-positive cells or mitotic cells with cyclin B1-positive staining were determined as described previously (4).

### Measurement of apoptotic sunburn cells
Identification of apoptotic sunburn cells was based morphologically on cell shrinkage and nuclear condensation as we have done previously (4). Apoptotic sunburn cells were identified by their intensely eosinophilic cytoplasm and small dense nuclei, which were observed in hematoxylin and eosin-stained histological sections of the skin using light microscopy. The percentage of apoptotic sunburn cells in the epidermis was calculated from the number of these cells per 100 epidermal cells counted from the entire 20 mm length of epidermis for each skin section.

### Caspase-3 immunostaining
Affinity-purified polyclonal rabbit antibody that reacts with the mouse p20 subunit of caspase-3 but does not react with the precursor form was purchased from Cell Signaling Technology (Danvers, MA, catalog no. 9661). Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol. The sections were incubated with a protein block for 1 h at room temperature. 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 (ABC Elite kit purchased from Vector Laboratory, Burlingame, CA) for 30 min. Color development was achieved by incubation with 0.02% 3,3′-diaminobenzidine tetrahydrochloride containing 0.02% hydrogen peroxide for 10 min at room temperature. The slides were then counterstained with hematoxylin and dehydrated. A positive reaction was shown as a brown precipitate in the cytoplasm and/or nuclei of the cells. The percentage of caspase-3-positive cells was calculated from the number of stained caspase-3-positive cells per 100 epidermal cells counted from the entire 20 mm length of epidermis.

### Cyclin B1 immunostaining and mitosis
Cyclin B1 immunostaining was described earlier (6). Briefly, skin sections were first treated with 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven at high setting for 15 min. The sections were then incubated with a protein block. The sections were then incubated with cyclin B1 antibody (purchased from Abcam, Cambridge, MA, catalog no. ab727) at 1:5000 dilution for 30 min at room temperature. The antibody for cyclin B1 is a mouse monoclonal antibody produced by immunizing the mouse with a His-tagged hamster cyclin B1 expressed in bacteria. This antibody is specific for cyclin B1 and is recommended by the manufacturer for the immunohistochemical detection of cyclin B1 in mouse tissues. The samples were then incubated with a biotinylated anti-rabbit secondary antibody for 5 min at 37°C, followed by incubation with conjugated streptavidin solution for 5 min at 37°C. Color development was achieved by incubation with 0.02% 3,3′-diaminobenzidine tetrahydrochloride containing 0.02% hydrogen peroxide for 10 min at room temperature. The slides were then counterstained with hematoxylin and dehydrated. A positive reaction was shown as a brown precipitate in the cells (staining in nucleus and cytoplasm).

Mitotic cells were determined as described earlier (6,11) by observing (i) chromosome condensation together with breakdown of the nuclear envelope, (ii) alignment of the chromosomes on the spindle equator, (iii) separation of sister chromatids and (iv) movement of their respective spindle poles. Two separate nonadjacent skin sections from each mouse were analyzed, and an average value of the percentage of total epidermal cells that were mitotic with cyclin B1-positive staining was analyzed.

### Measurement of cyclin B1 and survivin by western blots
Measurement of cyclin B1 in the epidermis by western blots was described previously (6). Briefly, dorsal skin samples were removed and immediately placed in a buffer solution containing 50 mM potassium phosphate (pH 7.7) at 52°C for 20 s. The samples were then submerged immediately in an ice bath containing the same buffer for 40 s and the epidermis was scraped from the dermis and placed in 1 ml of tissue lysis buffer (catalog no. 9803 from Cell Signaling) containing 1 mM phenylmethylsulfonyl fluoride. The epidermis was sonicated five times (each for 5 s at 4°C at 5 s intervals). Samples were centrifuged at 17 800g for 10 min at 4°C. Equal amounts of protein (20 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (4% stacking gel and 15% gradient) and electrophoretically transferred onto a polyvinylidene difluoride membrane. The blots were blocked in Odyssey blocking solution (catalog no. 927-40000 from LI-COR Biosciences, Lincoln, NE) for 1 h at room temperature and incubated with cyclin B1 (1:150 dilution) or survivin antibody (1:1000 dilution) at 4°C overnight. The cyclin B1 antibody obtained from Santa Cruz Biotechnology, Santa Cruz, CA (catalog no. sc-752) is a rabbit polyclonal raised against amino acids 1–433 representing full-length cyclin B1 of mouse origin. The survivin antibody obtained from Novus Biologicals, Littleton, CO (catalog no. NB500-201) is a rabbit polyclonal raised against full length of recombinant human survivin. Blots were then incubated with a 1:1500 dilution of IRDye 800 conjugated affinity-purified goat anti-rabbit secondary antibody (catalog no. 611-132-122 from Rockland Immunomchemi-
cals, Gilbertsville, PA) for 1 h at room temperature and visualized by an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

### Results
#### Effects of exercise in combination with caffeine on body weight, food and fluid consumption
Treatment of p53(+/+) and p53(+/-) mice with oral caffeine (0.1 mg/ml in the drinking water), RW or caffeine + RW for 2 weeks did not have a statistically significant effect on initial body weight (Figure 1A) or final body weight (Figure 1B). Oral caffeine together with RW resulted in 20–25% more average daily food consumption (Figure 1C) and 37–55% more daily fluid consumption than in control mice receiving water alone (Figure 1D). However, there was no statistical difference between mouse genotypes.

Although it was difficult to determine the distance run by individual RW mice because there were six mice per cage and sometimes more than one mouse at a time was on the running wheel, we estimate that the RW mice ran ~2 miles/day. Over the course of the 2 weeks study, the addition of a low dose of caffeine to the voluntary exercise regimen in p53(+/-) mice increased running activity 60% more than that in p53(+/+) mice with RW alone; the caffeine + RW-treated p53(−/−) mice ran 13% more than the p53(−/−) mice with RW alone and the caffeine + RW-treated p53(+/-) mice ran 9% more than the p53(+/+) mice with RW alone.

#### Effect of exercise in combination with caffeine on tissue fat
Treatment of p53(+/+) mice with caffeine, RW or caffeine + RW for 2 weeks decreased the weight of the epididymal fat pads by 14, 20 and 53% (P < 0.01; Figure 2A), and the thickness of the dermal fat layer was decreased by 13, 36% (P < 0.05) and 67% (P < 0.01), respectively (Figure 2B). Treatment of p53(−/−) mice with caffeine, RW or caffeine + RW decreased the weight of the epididymal fat pads by 22, 40% (P < 0.05) and 56% (P < 0.01; Figure 2A), and the thickness of the dermal fat layer was decreased by 10, 26% (P < 0.05) and 42% (P < 0.01), respectively (Figure 2B). Treatment of p53(+/-) mice with caffeine, RW or caffeine + RW decreased the weight of the epididymal fat pads by 25% (P < 0.05), 49% (P < 0.01) and 51% (P < 0.01) (Figure 2A), and the thickness of the dermal fat layer was decreased by 0, 33 and 57% (P < 0.01), respectively (Figure 2B).

There were no statistical differences in tissue fat between mouse genotypes except p53(+/+) mice treated with caffeine + RW had thinner dermal fat layers than p53(−/−) mice treated with caffeine + RW (P < 0.01). The results indicate that oral administration of a low dose of caffeine together with RW had a greater effect on lowering tissue fat than either treatment alone.

#### Effect of exercise in combination with caffeine on apoptosis
A previous study on the time course for UVB-induced apoptosis in hairless p53(−/−) C57BL/6J black mice as well as their wild-type p53(+/-) littermates indicated that the peak effects occurred at 6–10 h post-UVB, and topical application of caffeine enhanced apoptosis at 6–10 h post-UVB (12). Accordingly, we studied the effects of oral caffeine, RW or their combination for 2 weeks on UVB-induced apoptosis at 6 h post-UVB in the epidermis of the male congenic hairless p53-deficient mice.

Treatment of p53(+/-) mice with caffeine, RW or caffeine + RW for 2 weeks prior to a single dose of UVB stimulated UVB-induced increases in apoptotic sunburn cells by 9, 75 and 241%, respectively, at 6 h post-UVB. Treatment of p53(−/−) mice with caffeine, RW or caffeine + RW stimulated apoptotic sunburn cells by 29, 100 and 241%, respectively, at 6 h post-UVB.
Caffeine plus exercise stimulates apoptosis and cyclin B1 in p53-knockout mice

RW or caffeine + RW stimulated apoptotic sunburn cells by 54, 146 and 317%, respectively (Figure 3A). Caspase-3 (active form) measurements have been widely used as an index of apoptosis. In order to further confirm these observations, we used an immunohistochemical method for caspase-3 staining. As expected, treatment of p53(+/+) mice with caffeine, RW or caffeine + RW for 2 weeks stimulated UVB-induced increases in caspase-3 (active form)-positive cells by 12, 77 and 296%, respectively, at 6 h post-UVB. Treatment of p53(−/−) mice with caffeine, RW or caffeine + RW stimulated UVB-induced increases in caspase-3 (active form)-positive cells by 33, 117 and 667%, respectively, and treatment of p53(+/−) mice with caffeine, RW or caffeine + RW stimulated caspase-3 (active form)-positive cells by 78, 211 and 422%, respectively (Figure 3B). The total percent apoptotic sunburn cells and caspase-3-positive cells in UVB-irradiated p53(−/−) mice treated with water, caffeine, RW or caffeine + RW were all significantly lower than that in their control p53(+/+) mice. The results indicate that a low dose of caffeine in combination with RW markedly amplified the UVB-induced increase in apoptosis in the epidermis of p53(−/−) mice through a p53-independent mechanism.

Effect of exercise in combination with caffeine on mitotic cells with cyclin B1

The cyclin B1 protein is a regulatory protein involved in mitosis. The cyclin B1 product complexes with p34(cdc2) to form the maturation-promoting factor that is expressed predominantly during the G2/M phase of the cell cycle. The activity of cyclin B1 throughout the cell cycle can be monitored by immunocytochemistry. During the G1, S and G2 phases, an antibody to cyclin B1 labels the cytoplasm. In prophase, the labeling translocates from the cytoplasm to the nucleus. The nuclear labeling then dissipates during the later stages of mitosis. In this study, we determined the effects of caffeine, RW or their combination on UVB-induced changes on the percentage of total epidermal cells that were mitotic with cyclin B1-positive staining. We evaluated the possibility that oral caffeine, RW or their combination enhanced UVB-induced apoptosis through abrogation of the cell cycle blockade at the G2/M checkpoint by elevating epidermal cyclin B1 level prematurely thereby causing lethal mitosis in the epidermis of UVB-treated animals.

Irradiation of p53(+/+) mice treated with caffeine, RW or caffeine + RW for 2 weeks prior to a single dose of UVB resulted in a 0, 123 and 255% increase in the number of mitotic cells with cyclin B1-positive staining at 6 h post-UVB. Irradiation of p53(−/−) mice treated with caffeine, RW or caffeine + RW with UVB resulted in a 40, 210 and 247% increase in the number of mitotic cells with cyclin B1, and irradiation of p53(+/−) mice treated with caffeine, RW or caffeine + RW with UVB resulted in a 21, 58 and 247% increase in the number of mitotic cells with cyclin B1 at 6 h post-UVB. The total percent epidermal cells that are mitotic with cyclin B1 in each treatment of UVB-irradiated p53(−/−) mice were significantly lower than that in p53(+/+) mice. An example of cyclin B1 staining present in the cytoplasm of a cell (left) and an example of cyclin B1 staining present in the nucleus of a cell undergoing mitosis (right) in the epidermis of p53(−/−) mice is shown in Figure 4.

Our results indicate that oral administration of caffeine, RW or their combination overcame the prolonged UVB-induced decrease in the percentage of mitotic cells with cyclin B1 that normally occurred after UVB resulting in an enhanced early and inappropriate mitosis in the epidermis. Our results also indicate that the combination treatment increased the number of mitotic cells with cyclin B1 in p53(−/−) mice greater than that in their p53(+/+) wild-type littermates.

Relationship between epididymal fat pads and apoptosis

We used the Pearson’s correlation coefficient for evaluating the relationship between the weight of epididymal fat pads and UVB-induced increase in apoptosis in individual control mice and mice treated with caffeine, RW and caffeine + RW in each genotype. We found a statistically significant inverse relationship between the
weight of epididymal fat pads and the UVB-induced increase in apoptosis. For apoptotic sunburn cells, in p53(+/+)/ mice, the $r = 0.474$ ($P < 0.05$); in p53(+/-) mice, the $r = 0.601$ ($P < 0.01$) and in p53(-/-) mice, the $r = 0.554$ ($P < 0.05$). For caspase-3 (active form)-positive cells, in p53(+/-) mice, the $r = 0.587$ ($P < 0.05$); in p53(+/-) mice, the $r = 0.732$ ($P < 0.01$) and in p53(-/-) mice, the $r = 0.537$ ($P < 0.05$). Interestingly, a statistically significant inverse relationship between the weight of epididymal fat pads and the percent epidermal cells with mitotic cells with cyclin B1-positive staining was observed only in p53(-/-) mice ($r = 0.623$, $P < 0.01$) but not in p53(+/-) or p53(+/-) mice. A similar effect was also observed for the thickness of dermal fat layer ($r = 0.591$, $P < 0.01$). Our results suggest that tissue fat may be a more important modulator of UVB-induced apoptosis and carcinogenesis in p53-deficient mice than in p53-normal mice. The relatively low $r$ values indicate that other factors, in addition to tissue fat, may also play a role in modifying UVB-induced apoptosis.

Effects of exercise in combination with caffeine on cyclin B1 and survivin—western blot

Oral administration of caffeine, voluntary RW or their combination on tissue fat. p53(+/-), p53(-/-) or p53(+/-) mice were treated with oral caffeine (0.1% in the drinking water), voluntary RW or a combination of caffeine + RW for 2 weeks. The epididymal fat pads were weighted (A) and the thickness of dermal fat layer was measured using a light microscope with an ocular micrometer under 100-fold magnification (B). Bars in Figure 2 use the same key as in Figure 1.

Fig. 2. Effect of oral administration of caffeine, voluntary RW or their combination on tissue fat. p53(+/-), p53(-/-) or p53(+/-) mice were treated with oral caffeine (0.1% in the drinking water), voluntary RW or a combination of caffeine + RW for 2 weeks. The epididymal fat pads were weighted (A) and the thickness of dermal fat layer was measured using a light microscope with an ocular micrometer under 100-fold magnification (B). Bars in Figure 2 use the same key as in Figure 1.

p53(+/-) control mice that received only UVB irradiation (Figure 5B). Treatment of p53(-/-) mice with caffeine, RW or caffeine + RW increased the level of cyclin B1 by an average of 14, 66 and 223%, respectively, when compared with the level of cyclin B1 in p53(-/-) control mice that received only UVB irradiation. Treatment of p53(-/-) mice with UVB alone (control mice) significantly increased the level of cyclin B1 by an average of 48% when compared with the level of cyclin B1 in UVB-treated p53(+/-) control mice ($P < 0.05$). Treatment of p53(-/-) mice with caffeine + RW increased the level of cyclin B1 by an average of 141%, which is significantly higher than the level of cyclin B1 in p53(+/-) mice treated with caffeine + RW ($P < 0.01$; Figure 5A and B).
The mice were then exposed to a single dose of UVB (30 mJ/cm²) and killed 6 h later. The skin epidermis was taken and cyclin B1 or survivin was determined by western blot analysis (A). Each blot was from the pooled epidermis of three mice. Densitometry results for cyclin B1 (B) or survivin (C) were from three independent experiments.

In another study, we found that oral caffeine, RW or caffeine + RW to p53(+/–) mice increased the level of cyclin B1 by an average of 16, 66 and 178%, respectively, at 6 h post-UVB, when compared with the p53(+/–) mice treated with UVB alone. The caffeine + RW increased the level of cyclin B1 significantly higher than RW alone (P < 0.05). There was little or no effect with oral caffeine, RW or caffeine + RW on the level of cyclin B1 in non-UVB-treated epidermis of the p53(+/-) mice (data not shown). These results demonstrate that caffeine + RW increased the level of cyclin B1 mainly by a p53-independent mechanism.

Survivin is an inhibitor of apoptosis and a mitotic regulator and is selectively overexpressed in tumors (13,14). Manipulation of this pathway may facilitate the elimination of cancer cells at mitosis. Treatment of p53(−/−) mice but not p53(+/-) mice with RW or caffeine + RW for 2 week prior to exposure to UVB decreased the level of survivin at 6 h post-UVB (Figure 5A). Densitometry measurement in three separate experiments indicate that treatment of p53(+/-) mice with caffeine, RW or caffeine + RW had no effect on the level of survivin when compared with the level of survivin in p53(+/+) control mice that received only UVB (Figure 5C). However, treatment of p53(−/−) mice with RW or caffeine + RW decreased the level of survivin by an average 59 or 80% when compared with the level of survivin in p53(−/−) control mice that received only UVB. Interestingly, treatment of p53(−/−) mice with oral administration of caffeine had no effect on the level of survivin. The results suggest that survivin may play an important role on apoptosis in the epidermis of p53(−/−) mice treated with RW or caffeine + RW, and the effects of caffeine administration and voluntary exercise on apoptosis in UVB-treated p53(−/−) mice may be through a different mechanism.

**Specificity of cyclin B1 antibody**

We determined that the antibody to cyclin B1 was selective for dephospho-cyclin B1 but not for phospho-cyclin B1. Lysates of cultured colon cancer HT-29 cells, which are known to contain both dephospho-cyclin B1 and phospho-cyclin B1 (Ser147), were made. The lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (4% stacking and 4–15% gradient). The samples were then electroblotted onto a polyvinylidene difluoride membrane. The membrane was probed with antibody to cyclin B1 (1:125 dilution), phospho-cyclin B1 (Ser147) (1:333 dilution) or a combination of the two antibodies. Our results showed that in the absence of phosphatase treatment, probing with antibody to cyclin B1 gave a single band at 55 kDa, whereas probing with the antibody to phospho-cyclin B1 gave a single band at 60 kDa. Probing with a combination of antibodies to cyclin B1 and phospho-cyclin B1 gave two bands (at 55 and 60 kDa; Figure 6A). In an additional experiment, after electroblotting of proteins, the polyvinylidene difluoride membrane was treated with buffer alone or protein phosphatase 1 (PP1, 60 U/ml, 0.25 ml/cm²) in buffer for 3 h at 37°C and then probed with antibody to cyclin B1 or phospho-cyclin B1. Treatment with phosphatase followed by probing with phospho-cyclin B1 antibody showed that a decrease of the phospho-cyclin B1 band. Whereas, probing a parallel sample with the cyclin B1 antibody indicated a lack of effect of phosphatase treatment on the cyclin B1 band (Figure 6B). The results of these studies indicate that the cyclin B1 antibody recognizes dephospho-cyclin B1 but not phospho-cyclin B1 (Ser147) and that the antibody to phospho-cyclin B1 recognizes phospho-cyclin B1 (Ser147) but not dephospho-cyclin B1.

**Discussion**

In earlier studies, we showed that RW or oral caffeine for 2 weeks stimulated UVB-induced apoptosis in the epidermis of hairless SKH-1 mice, and that combined RW and oral caffeine resulted in a greater than additive increase in apoptosis when compared with the effects of RW or caffeine alone (10); RW or oral caffeine had no effect on apoptosis in non-UVB-treated mouse epidermis (3,9). In the present study, as has been known, p53 is important for UVB-induced...
apoptosis (15) and this is demonstrated by the markedly reduced percent of apoptotic sunburn cells and caspase-3-positive cells in UVB-irradiated p53−/− mice compared with p53+/+ mice. In addition, p53−/− mice treated orally with caffeine in combination with RW for 2 weeks markedly amplified the UVB-induced increase in apoptosis in the epidermis. The increased magnitude of the combination treatment on mitotic cells with cyclin B1 by immuno-histochemical analysis or the level of cyclin B1 by western blot in p53−/− mice was greater than that in their p53 wild-type littermates. Interestingly, a statistically significant inverse relationship between the level of tissue fat and the UVB-induced increase in mitotic cells with cyclin B1 was observed only in p53−/− mice but not in p53+/+ or p53−/− mice. The results suggest that the increase in UVB-induced apoptosis by caffeine, exercise and in particular, the large increase by the combination of caffeine + exercise is clearly p53 independent. p53−/− mice are more sensitive to the combination treatment than p53−/− or p53+/− mice and tissue fat in p53−/− mice may play a more important role in modifying apoptosis than in their p53+/+ littermates.

Previously, we reported that the stimulatory effect of oral administration of caffeine on UVB-induced apoptosis was by a p53-dependent pathway as well as by a p53-independent pathway (5,12), and the stimulatory effect of RW on apoptosis was predominantly by a p53-independent pathway (9). Our results suggest that caffeine treatment or RW may inhibit skin carcinogenesis by enhancing apoptosis selectively in UVB-induced DNA-damaged cells or in tumors, but not in normal cells (4,8,9,16). The selectivity of caffeine to enhance apoptosis was also reported in cultured cells that are p53 deficient. Russell demonstrated that DNA damage-induced cell cycle arrest at the G1 checkpoint is highly dependent on wild-type p53, whereas the G2 checkpoint is still functioning even in p53-deficient cells (17). Treatment with caffeine abrogated the G2 checkpoint arrest and caused apoptosis mainly in p53-inactivated cells, and the radiosensitivity of the G1 checkpoint-deficient cells treated with caffeine was comparable with the p53-wild-type parental cell line (17). Powell et al. (18) showed in mouse, embryo fibroblasts derived from p53-wild-type and p53-knockout littermates that caffeine abrogated G2 arrest only in p53-deficient cells in response to irradiation. Treatment of p53-inactive Hela cells with caffeine abrogated etoposide-induced G2 arrest and concomitantly enhanced its cytotoxic effects (17,19). Recently, caffeine was found to potentiate the activation of cdc2 kinase (21). Caffeine prevented irradiation-induced inactivation of cyclin B1 and G2 arrest, which caused apoptosis preferentially in p53-mutated cells (21). Nghiem et al. (22) reported that ATR-mediated Chk1 phosphorylation is a potentially important proapoptotic target for caffeine in human osteosarcoma cells. Loss of p53 function sensitizes cells to premature chromatin condensation caused by ATR inhibition by caffeine (22,23).

Molecular targets for the selectivity of caffeine have been investigated in certain cell lines. It was reported that caffeine-mediated abrogation of DNA damage-induced G2 arrest was initially linked to the activation of cdc2 kinase (21). Caffeine prevented irradiation-induced inactivation of cyclin B1 and G2 arrest, which caused apoptosis preferentially in p53-mutated cells (21). Nghiem et al. (22) reported that ATR-mediated Chk1 phosphorylation is a potentially important proapoptotic target for caffeine in human osteosarcoma cells. Loss of p53 function sensitizes cells to premature chromatin condensation caused by ATR inhibition by caffeine (22,23). Caffeine was also reported to directly disrupt the checkpoint pathway containing Chkl (24,25). Inhibition of Chk1 but not Chk2 in a p53−/− colon carcinoma cell line caused a greater abrogation of the G2 block induced by ionizing radiation than in the parental cell line with wild-type p53 (26). Therefore, the ATR/Chk1/cyclin B1 pathway may function as a molecular target for the selectivity of caffeine. In recent studies, we found that oral administration of caffeine inhibited the UVB-induced increase in phospho-Chk1 (Ser345) and prematurely increased the level of cyclin B1 as well as the number of mitotic cells with cyclin B1 in SKH-1 mice (6). These observations suggested that caffeine abrogated the G2/M checkpoint thereby causing lethal mitosis and apoptosis in UVB-treated animals. In the present study, we found that a combination treatment of caffeine and exercise was more effective for prematurely increasing the level of epidermal cyclin B1 in p53−/− mice than in p53+/+ mice (P < 0.01). These results suggest that both caffeine administration and exercise may modulate the effect of UVB by the p53-independent and cyclin B1-mediated pathway. The greater than additive effects of RW and caffeine on UVB-induced apoptosis may result from an increased level of a premature increase in cyclin B1 that results in an override of the G2/M checkpoint to cause catastrophic lethal mitosis and apoptosis. Further mechanistic studies on the biomarkers of upstream of cyclin B1, such as wee1, 14-3-3 and Chk1, are needed.

Survivin is a recently described inhibitor of apoptosis and mitotic regulator, which is selectively overexpressed in tumors. Activation of survivin has been identified by the main mitotic kinase p34cdc2/cyclin B1 and suggests that survivin may be required to preserve cell viability at cell division (13,14). Manipulation of this pathway may facilitate the elimination of cancer cells at mitosis. Our present results indicated that treatment of p53−/− mice with RW or RW + caffeine, but not caffeine, caused a dramatic decrease in the level of survivin suggesting that survivin may play an important role on apoptosis in the epidermis of p53−/− mice. The effects of caffeine administration and voluntary exercise on enhanced apoptosis in UVB-treated p53−/− mice may be through a different pathway. Further mechanistic studies are needed.
In an earlier study, we reported that mice with a thick dermal fat layer had more tumors than mice with a thin dermal fat layer suggesting a tumor-promoting effect of tissue fat (27). In the present study, using a limited number of mice, we found that a statistically significant inverse relationship between the weight of epididyinal fat pads or thickness of dermal fat layer and UVB-induced increase in mitotic cells with cyclin B1-positive staining is observed only in p53(-/-) mice but not in p53(+/+) littermates. Although the mechanisms by which decreased tissue fat levels enhance apoptosis in UVB-caused DNA-damaged cells of p53(-/-) mice remains to be explored, our results suggest that tissue fat may be a more important modulator of UVB-induced apoptosis and carcinogenesis in p53-deficient mice than p53-normal mice.

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 (28–32), free fatty acids (33–41), glycerol (28,33,42), glucose (37–39,43–45), insulin (42,45) and lactate (33,37–39,43–45). However, the effects of caffeine in combination with exercise in decreasing tissue fat and increasing apoptosis in tumors have not been previously investigated.

In summary, we evaluated whether a combination of oral caffeine and exercise was more effective for decreasing tissue fat and for stimulating apoptosis in the skin of p53-knockout mice than in their wild-type littermates. The results presented here demonstrate that oral administration of a low physiologically relevant dose of caffeine (0.1 mg/ml in the drinking water) together with voluntary exercise was more effective in stimulating UVB-induced apoptosis and increasing the number of mitotic cells with premature cyclin B1 that are thus probably to go on to lethal mitosis (apoptosis) in the epidermis of p53-deficient mice than in their wild-type littermates. These effects in p53-knockout mice were associated with a decrease in tissue fat. Our results suggest a need for further studies to determine the effects of combinations of voluntary exercise and orally administered low dose levels of caffeine on UVB-induced skin carcinogenesis in animal models.

Funding
National Cancer Institute (RO1CA128997, RO1CA114442).

Acknowledgments
The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health.

Conflict of Interest Statement: None declared.

References
11. Lu,Y.-P. et al. (1997) Inhibitory effect of black tea on the growth of established skin tumors in mice: effects on tumor size, apoptosis, mitosis and bromodeoxyuridine incorporation into DNA. *Carcinogenesis*, 18, 2163–2169.

Received October 5, 2009; revised October 26, 2009; accepted November 12, 2009