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Stimulatory Effect of Topical Application of Caffeine on UVB-Induced Apoptosis in the Epidermis of p53 and Bax Knockout Mice

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ABSTRACT

Shaved male or female p53(−/−) C57BL/6J mice and their wild-type littermates were irradiated once with UVB (60 mJ/cm^2). The UVB-induced increase in apoptotic sunburn cells in p53(−/−) mice at 6–10 h after exposure to UVB was only 10–30% of that observed after treatment of p53(+/+) mice with UVB. Topical applications of caffeine immediately after UVB irradiation in female p53(+/+) or p53(−/−) mice enhanced the UVB-induced increase in apoptotic sunburn cells 6 h later by 127% and 563%, respectively. In another study, shaved female Bax(−/−) C57BL/6J mice and their wild-type littermates were irradiated once with UVB (60 mJ/cm^2). The UVB-induced increase in apoptotic sunburn cells in Bax(−/−) mice at 6 h after exposure to UVB was only 14% of that observed after treatment of Bax(+/+) mice with UVB. Topical application of caffeine immediately after irradiation of Bax(+/+) or Bax(−/−) mice with UVB enhanced the UVB-induced increases in apoptotic sunburn cells at 6 h by 214% and 467%, respectively, and topical application of caffeine immediately after irradiation of Bax(+/+) or Bax(−/−) mice with UVB enhanced the UVB-induced increase in caspase 3 (active form) positive cells at 6 h by 253% and 750%, respectively. The results indicate that UVB-induced increases in apoptosis in the epidermis of wild-type mice are predominantly (but not entirely) by p53- and Bax-dependent pathways and that topical application of caffeine can enhance UVB-induced increases in apoptosis by p53- and Bax-independent pathways.

INTRODUCTION

Earlier studies in our laboratory demonstrated an inhibitory effect of oral administration of green or black tea on the formation of UVB-induced nonmalignant and malignant skin tumors (keratoacanthomas and squamous cell carcinomas) in SKH-1 hairless mice (1, 2). The regular teas were more effective inhibitors of carcinogenesis than the decaffeinated teas, and adding back caffeine to the decaffeinated teas restored their inhibitory activities (3, 4). These results indicated that caffeine is a major component of tea responsible for its inhibitory effect on UVB-induced tumor formation in mice.

Mechanistic studies indicated that oral administration of green tea or caffeine for 2 weeks before a single irradiation with UVB enhanced UVB-induced increases in wild-type p53 and apoptosis in the epidermis, but these treatments with green tea or caffeine had no effect in the absence of UVB (5). In addition, topical application of caffeine to the skin of mice immediately after a single irradiation with UVB also enhanced UVB-induced apoptosis, but topical application of caffeine had no effect on apoptosis in the absence of prior treatment with UVB (6). In another study, topical applications of caffeine once a day 5 days a week to tumor free "high-risk mice" treated previously with UVB for 20 weeks (initiated mice) inhibited the subsequent formation of nonmalignant tumors and squamous cell carcinomas by 44% and 72%, respectively, and these treatments had a selective apoptotic effect in the tumors but not in non-tumor areas of the epidermis (7). The presence of p53 mutations in most UVB-induced tumors suggested that topical application of caffeine may have enhanced apoptosis in the tumors by a p53-independent pathway.

In the present study, we investigated whether or not functional p53 and Bax were required for the stimulatory effect of caffeine on UVB-induced apoptosis in mouse epidermis. We describe here a stimulatory effect of topical applications of caffeine on UVB-induced apoptosis in the epidermis of p53(−/−) and Bax(−/−) knockout mice.

MATERIALS AND METHODS

Chemicals and Animals. Acetone (high performance liquid chromatography grade) and 10% phosphate-buffered formalin were obtained from Fisher Scientific (Springfield, NJ). Caffeine (>99% purity) was obtained from the Sigma Chemical Co. (St. Louis, MO), and female SKH-1 hairless mice (6–7 weeks old) were purchased from the Charles River Breeding Laboratories (Kingston, NY).

Male p53(−/−) mice on a C57BL/6J genetic background and their female wild-type control littermates (6–7 weeks old) or female Bax(−/−) knockout mice on a C57BL/6J genetic background and their wild-type female control littermates (6–7 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). The p53(−/−) mice were constructed to carry a germline disruption of the gene. This mutation removes approximately 40% of the coding capacity of p53 and completely eliminates synthesis of p53 protein. Animals homozygous for this p53 deletion mutation are viable but highly predisposed to malignancy. Heterozygous animals also have an increased cancer risk, although the distribution of tumor types in these animals differs from that in homozygous mutants. In most cases, tumorigenesis in heterozygous animals is accompanied by loss of the wild-type p53 allele (8).

C57BL/6J male p53(−/−) mice were mated with female wild-type C57BL/6J mice to obtain male and female p53(+/−) mice. We then mated male p53(−/−) mice with p53(+/−) female or p53(+/−) males with p53(+/−) females and genotyped the progeny.

The Bax(−/−) mice and control Bax(+/+) mice were used directly from The Jackson Laboratory after 2–3 weeks in our laboratory.

All 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*, and they were kept on a 12 h light/12 h dark cycle. Animals were studied when they were 8–10 weeks old.

Determination of p53 Genotype. Chromosomal DNA extractions from the tails of mice were performed according to standard procedures (9) with minor modification. We used the PCR protocol from The Jackson Laboratory with two pairs of primers in the same reaction. The DNA fragment (280 bp) from the neomycin resistance gene insert was amplified with 5′-CTTGGGTGGAGAGGCTATTC and 5′-AGGTGAGATGACAGGAGATC whereas the DNA fragment (648 bp) from the p53 gene (between exons 6 and 7) was amplified with 5′-ATAGGTCGGCGGTTCAT and 5′-CCCGAGTATCTGGAAGACAG. The PCR product from the p53(+/+) wild-type mouse is 648 bp, the PCR product from the p53(+/−) heterozygote is 648 and 280 bp, and the PCR product from the p53(−/−) homozygote is 280 bp.

Exposure of Mice to UVB and the Preparation of Skin Sections. The mice were shaved, and 3 days later animals without any sign of hair re-growth were irradiated with UV lamps that emit UVB (280–320 nm; 75–80% of total energy) and UVA (320–375 nm; 20–25% of total energy). The UV lamps used

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(FS72T12-UVB HO; National Biological Corp., Twinsburg, Ohio) emitted little or no radiation below 280 nm or above 375 nm. The dose of UVB was quantified with a UVB Spectra 305 dosimeter (Daevlin Co., Byran, OH). The radiation was further calibrated with a model IL-1700 research radiometer/photometer (International Light Inc., Neburgport, MA).

Skin samples, about 25-mm length and 5-mm width, were taken from the middle of the back and placed in 10% phosphate-buffered formalin at 4°C for 18–24 h. The skin samples were then dehydrated in ascending concentrations of ethanol (80, 95, and 100%), cleared in xylene, and embedded in Paraplast (Oxford Labware, St. Louis, MO). Four-μm serial sections of skin containing epidermis and dermis were made, deparaffinized, rehydrated with water, and used for regular H&E staining or immunohistochemical staining. All immunohistochemical determinations were made with 400-fold magnification using a light microscope.

Measurement of Apoptotic Sunburn Cells. Identification of apoptotic sunburn cells was based morphologically on cell membrane shrinkage and nuclear condensation attributable to fragmentation of the cells (10, 11). Earlier studies demonstrated that sunburn cells are a hallmark of apoptosis (12, 13). Sunburn cells were identified in the epidermis as cells with a homogeneous, densely staining glossy eosinophilic cytoplasm and a small hyperchromatic condensed pyknotic nucleus that can readily be seen with routine H&E-stained histological sections of the skin using light microscopy. The percentage of apoptotic sunburn cells in the epidermis (basal plus suprabasal layers) was calculated from the number of these cells/100 cells counted from the entire 25-mm length of epidermis for each skin section as done routinely in this laboratory (14).

Caspase 3 Immunostaining. Caspases play a pivotal role in the initiation and execution of apoptosis. Caspase 3 (active form) is found in cells undergoing apoptosis by proteolysis. 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 R&D, Inc. (Minneapolis, MN). Skin sections used for the measurement of caspase 3 were stained by the horseradish peroxidase-conjugated-avidin method (15) with some modification as described in our laboratory (6). Briefly, endogenous peroxidase was blocked by incubating tissue sections in 3% hydrogen peroxide in methanol for 30 min at room temperature. Sections were then treated with 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven at high setting and temperature for 10 min. The sections were incubated with a protein block (normal goat serum) for 10 min, followed by avidin D for 15 min and biotin blocking solution for 15 min (Avidin-Biotin blocking kit; Vector Laboratory, Burlingame, CA) at room temperature. The sections were incubated with caspase 3 primary antibody (1:500 dilution) for 30 min at room temperature followed by incubation with a biotinylated antirabbit secondary antibody for 30 min and 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, dehydrated, and coverslips were added for permanent mounting. A positive reaction was shown as a light brown to dark brown precipitate in the cytoplasmic and/or perinuclear portion of the cells. The percentage of caspase 3 positive cells in the epidermis was calculated from the number of caspase 3 stained cells divided by the total number of epidermal cells counted from the entire length (about 25 mm) of epidermis ×100 for each skin section.

We have used sunburn cells and caspase 3 positive cells in our laboratory for many years and have validated them as two relevant biomarkers for apoptosis (6). It should be noted that the use of morphological and immunohistochemical methods for our studies allows us to make multiple measurements on the same tissue samples that have been embedded in paraffin blocks.

Bromodeoxyuridine Incorporation into DNA. BrdUrd, a thymidine analog that is incorporated into proliferating cells during the S-phase, is detected by a biotinylated monoclonal anti-BrdUrd antibody and visualized using streptavidin peroxidase and 3,3′-diaminobenzidine, which stains BrdUrd-containing nuclei a dark brown (staining kit from Oncogene Research Products, Cambridge, MA; Refs. 16 and 17). Briefly, all animals were injected with BrdUrd (50 mg/kg) i.p. and killed 1 h later. Endogenous peroxidase was blocked by incubating the tissue sections in 3% hydrogen peroxide in methanol for 10 min at room temperature. The tissue sections were then incubated in a moist chamber with 0.125% trypsin for 10 min at 37°C, rinsed in deionized water, and incubated at room temperature for 30 min with denaturing solution (Oncogene Research Products). The sections were incubated with blocking solution for 10 min at room temperature and covered with biotinylated mouse monoclonal anti-BrdUrd antibody (Oncogene Research Products) at room temperature for 90 min. Sections were rinsed with PBS and incubated with streptavidin peroxidase for 10 min. Color development was achieved by incubation for 5 min at room temperature with a substrate solution containing 0.02% 3,3′-diaminobenzidine tetrahydrochloride and 0.02% hydrogen peroxide. The slides were weakly counterstained with Mayer's hematoxylin (Sigma Chemical Co.) for 2 min, cleared with xylene, mounted with a coverslip, and scored under a light microscope. The percentage of BrdUrd-labeled cells in the basal layer of the epidermis was calculated from the number of stained BrdUrd-positive cells/100 basal cells counted from the entire 25-mm length of epidermis for each section as described previously (6).

RESULTS

Number of Female and Male p53(−/−) Homozygous and p53(+/−) Heterozygous Mice Obtained from Appropriate Matings. Although the mating of male p53(−/−) with female p53(+/−) or male p53(+/−) with female p53(+/−) C57BL/6J mice provided us with an approximately equal number of male and female p53(+/+) or p53(+/−) mice, we obtained very few female p53(−/−) mice as compared with male p53(−/−) mice. Mating male p53(−/−) mice with female p53(+/−) mice or male p53(+/−) with female p53(+/−) mice produced 138 male p53(−/−) mice but only 15 female p53(−/−) mice. This large difference in the number of male and female p53(−/−) mice obtained from the matings was because a significant proportion of female p53(−/−) mice died during embryogenesis or shortly after birth because of the sex-specific failure of normal neural tube closure as other investigators described previously (18). In addition to obtaining p53(−/−) mice, we also obtained 208 female p53(+/−) and 195 male p53(+/−) heterozygous mice, as well as 102 female p53(+/+) wild type and 98 male p53(+/+) littermates. Using these p53-deficient mice together with their p53 wild-type control littermates, we evaluated the effects of topical application of caffeine immediately after a single dose of UVB on apoptosis by using morphologically distinct apoptotic sunburn cells and caspase 3 (active form) immunoreactive-positive cells as indices of apoptosis.

Sensitivity of C57BL/6J Black Mice and SKH-1 Hairless Mice to UVB-Induced Increases in Epidermal Apoptotic Sunburn Cells. Previous studies in our laboratory on the time course for UVB-induced apoptosis indicated that treatment of female SKH-1 hairless mice with 30 mJ/cm^2 UVB induced apoptotic sunburn cells in the epidermis with a maximum effect at 6–10 h after UVB irradiation (5, 6). In the present study, shaved female C57BL/6J black mice or female SKH-1 hairless mice (7–8 weeks old, five mice/group) were irradiated with different doses of UVB, and the animals were killed 6 h later. We found that exposure of C57BL/6J black mice to 60 mJ/cm^2 UVB resulted in about the same number of apoptotic sunburn cells in the epidermis as occurred after exposure of SKH-1 hairless mice to 30 mJ/cm^2 UVB (Table 1). These results indicate that C57BL/6J black mice have about half the sensitivity to UVB-induced apoptosis as SKH-1 hairless mice.

UVB-Induced Apoptosis in p53(+/+) and p53(−/−) C57BL/6J Black Mice. Treatment of shaved female p53(+/+) C57BL/6J mice with 60 mJ/cm^2 UVB increased the number of apoptotic sunburn cells to about the same extent as was observed in male p53(+/+) mice (Fig. 1, *A* and *C*). Treatment of female or male p53(+/−) C57BL/6J mice with UVB increased the number of apoptotic sunburn cells at 6–10 h to about the same extent or to a slightly lower extent than in female or male p53(+/+) mice (data not shown).

The UVB-induced increase in apoptotic sunburn cells in female or male p53(−/−) acetone-treated control mice at 6–10 h after exposure

Table 1 *Dose response for UVB irradiation-induced increase in apoptotic sunburn cells in the epidermis of C57/BL6J mice and SKH-1 hairless mice*

Shaved female C57BL/6J mice or SKH-1 hairless mice (7 weeks old, five per group) were irradiated with different doses of UVB. The animals were killed 6 h later. Apoptotic sunburn cells in the epidermis were determined morphologically. Each value represents the mean ± SE.

UVB dose	Percent apoptotic sunburn cells	
	C57BL/6J	SKH-1
No UVB	0.02 ± 0.02	0.01 ± 0.01
30 mJ/cm^2	0.21 ± 0.08	0.39 ± 0.08
60 mJ/cm^2	0.44 ± 0.10	0.88 ± 0.18
90 mJ/cm^2	0.95 ± 0.17	1.28 ± 0.28
180 mJ/cm^2	0.40 ± 0.05	2.35 ± 0.46

to 60 mJ/cm^2 was only 13–39% of that after UVB treatment of female or male p53(+/+) control mice (Fig. 1, *B* and *D*) indicating that most (but not all) of the UVB-induced increase in apoptosis in p53 wild-type mice was p53-dependent. Earlier studies showed strong inhibition of UVB-induced apoptosis in p53(−/−) mice (11).

The effect of dose of UVB on the formation of apoptotic sunburn cells was studied in male p53(−/−) mice. It was found that 60–180 mJ/cm^2 UVB increased apoptotic sunburn cells in the epidermis at 6 h from almost non-detectable levels in non-UVB-treated animals to 0.18–0.21% apoptotic sunburn cells (Table 2). In contrast to the lack of a dose response for UVB-induced apoptosis at 6 h after UVB exposure, treatment of the mice with 60, 90, or 180 mJ/cm^2 UVB resulted in 0.18%, 0.66%, or 1.33% apoptotic sunburn cells, respectively, in the epidermis at 24 h after UVB irradiation (Table 2). These results indicate dose-dependent differences in the time course for UVB-induced apoptosis in p53(−/−) mice and provide further evidence for a strong p53-independent pathway for UVB-induced apoptosis. Time-course studies indicated that treatment of male p53(−/−) mice with a 60 mJ/cm^2 dose of UVB caused a small but prolonged increase in apoptosis (approximately 0.15–0.20% apoptotic cells) between 6 and 48 h after UVB (Fig. 1*D*).

Stimulatory Effect of Topical Applications of Caffeine on UVB-Induced Increases in Epidermal Apoptosis in p53(+/+) and p53(−/−) Mice. Very few apoptotic sunburn cells (0.01–0.03% of the epidermal cells) were detected in control female or male p53(+/+), p53(+/−), or p53(−/−) mice in the absence of exposure to UVB, and treatment of the mice with caffeine in the absence of UVB did not increase the number of apoptotic cells in the epidermis (data not shown). Topical applications of 1.2 mg of caffeine in 100 μl of acetone to the dorsal skin of female p53(+/+) wild-type mice immediately after irradiation with 60 mJ/cm^2 UVB and another two applications of caffeine 0.5 and 2 h later increased UVB-induced apoptosis at 6 and 10 h by 127% and 143%, respectively (Fig. 1*A*). The response of female p53(+/−) mice to UVB alone or together with caffeine was similar to that observed in p53(+/+) mice (data not presented).

Treatment of female p53(−/−) knockout mice with 60 mJ/cm^2 UVB resulted in only a small increase in apoptotic sunburn cells at 6 h (0.08% of the epidermal cells were apoptotic; Fig. 1*B*). However, topical applications of caffeine to female p53(−/−) homozygous mice immediately after UVB stimulated the UVB-induced increase in apoptotic sunburn cells by 563% at 6 h after UVB (Fig. 1*B*). These results indicated that topical applications of caffeine markedly amplified the UVB-induced increase in apoptotic sunburn cells in p53(−/−) mice through a p53-independent mechanism.

Treatment of control male p53(+/+) mice with UVB (60 mJ/cm^2) increased apoptosis to about the same extent as was observed in control p53(+/+) female mice (Fig. 1, *A* and *C*). Although topical applications of caffeine immediately after UVB to the male mice enhanced UVB-induced apoptosis, the effect of caffeine was somewhat less than that observed in female mice (Fig. 1, *A* and *C*). Additional experiments would be needed to determine whether or not there is a sex difference in the caffeine response. The effect of UVB and UVB plus caffeine on apoptotic sunburn cells in male p53(+/−) mice was similar to that observed in male p53(+/+) mice (data not presented).

Treatment of male p53(−/−) mice with 60 mJ/cm^2 UVB resulted in a small increase in apoptotic sunburn cells that persisted for 48 h (Fig. 1*D*). The area under the UVB-induced apoptosis curve for p53(−/−) male mice was substantially less than that for p53(+/+) mice (Fig. 1, *C* and *D*). Topical applications of caffeine to the male p53(−/−) mice immediately after irradiation with UVB enhanced the UVB-induced increase in apoptotic sunburn cells by 207–418% at 6–10 h after UVB, and caffeine-induced increases in apoptosis were also observed at 16 and 24 h after UVB irradiation (Fig. 1*D*).

As indicated above, topical applications of caffeine (1.2 mg at zero time and at 0.5 and 2 h) to female or male p53(+/+) wild-type mice, female or male p53(+/−) mice or male p53(−/−) mice had no effect on apoptosis at 6 h after caffeine administration in non-UVB-irradiated epidermis.

Treatment of male or female p53(−/−) mice with UVB increased the number of caspase 3 (active form) positive cells in the epidermis, and this response was enhanced in mice treated with caffeine immediately after UVB (Fig. 1, *E* and *F*). The stimulatory effect of caffeine on UVB-induced apoptosis in the epidermis as measured morphologically by increased numbers of apoptotic sunburn cells was similar to that observed immunohistochemically by the number of caspase 3 (active form) positive cells. Topical applications of caffeine to male p53(−/−) knockout mice did not exert any effect on the number of caspase 3 (active form) positive cells in the absence of UVB treatment (data not shown).

UVB-Induced Changes in Epidermal Cell Proliferation in p53(+/+) and p53(−/−) Mice. Irradiation of female or male p53(+/+) mice with UVB (60 mJ/cm^2) resulted in a marked decrease in BrdUrd incorporation into epidermal DNA by 2 h after UVB followed by a gradual recovery and then somewhat enhanced BrdUrd incorporation into DNA at 24–48 h after UVB exposure (Fig. 2, *A* and *C*). Treatment of these mice with caffeine immediately after UVB had little or no effect on UVB-induced changes in BrdUrd incorporation into epidermal DNA (Fig. 2, *A* and *C*) except for the possible more rapid return to control values at 48 h in male p53(+/+) mice (Fig. 2*C*). Similar results were observed in p53(+/−) mice (data not shown). Treatment of male p53(−/−) mice with UVB (60 mJ/cm^2) decreased BrdUrd incorporation into epidermal DNA at 2–6 h, but the effect of UVB appeared to be somewhat less in p53(−/−) mice than in p53(+/+) and p53(+/−) mice. At later times (10–24 h after UVB) there was a greatly enhanced BrdUrd incorporation into the epidermal DNA of p53(−/−) mice that was not seen in p53(+/+) or p53(+/−) mice (Fig. 2, *C* and *D*, and data not presented). Treatment of female or male p53(−/−) mice with caffeine immediately after UVB (60 mJ/cm^2) had little or no effect on UVB-induced changes in BrdUrd incorporation into DNA (Fig. 2, *B* and *D*).

Stimulatory Effects of Topical Applications of Caffeine on UVB-Induced Increases in Apoptosis in Bax(+/+) and Bax(−/−) Mice. Apoptotic sunburn cells or cells that stain with an antibody to caspase 3 (active form) were not detected in the epidermis of untreated or acetone-treated female C57BL/6J mice. Treatment of female Bax(+/+) or Bax(−/−) mice with UVB (60 mJ/cm^2) resulted in 0.22% or 0.03% apoptotic sunburn cells and 0.15% or 0.02% caspase 3 (active form) positive cells, respectively, in the epidermis at 6 h after irradiation (Fig. 3, *A* and *B*). The UVB-induced increase in epidermal apoptotic sunburn cells in Bax(−/−) mice was only 14% of that in Bax(+/+) mice, and the UVB-induced increase in epidermal caspase

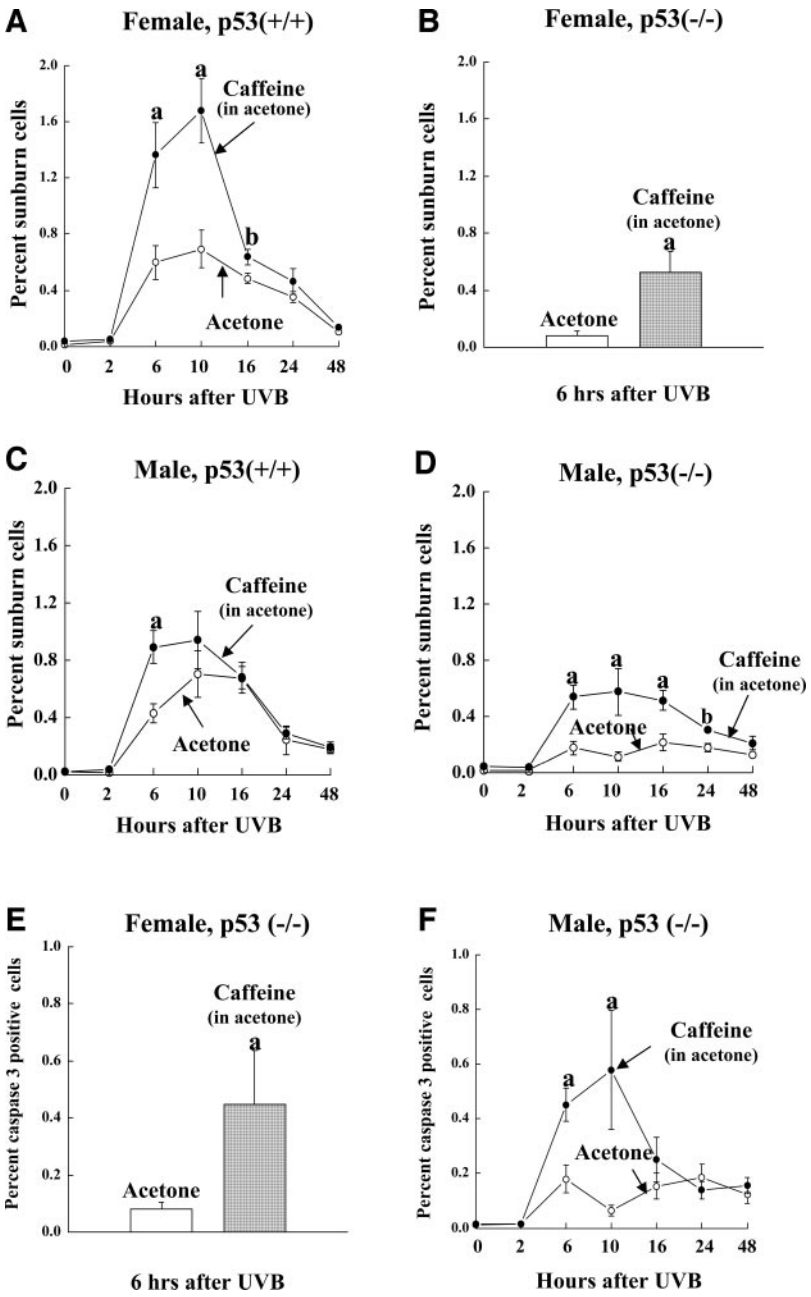

Fig. 1. Effect of topical application of caffeine on UVB-induced increases in apoptotic sunburn cells and caspase 3 (active form) positive cells in the epidermis of female or male p53(+/+) and p53(−/−) mice. Female or male p53(+/+) and p53(−/−) mice (7–8 weeks old, five mice for each time interval) were treated with 100 μl of acetone or caffeine (1.2 mg; 6.2 μmol) in 100 μl of acetone immediately after UVB (60 mJ/cm^2) and 0.5 and 2 h later. The animals were killed at the indicated times. Morphologically distinct apoptotic sunburn cells or caspase 3 (active form) positive cells were counted. Each value represents the mean ± SE. Statistically significant differences at individual times after UVB exposure were observed for the percentage of apoptotic sunburn cells between the acetone-treated control animals and the caffeine-treated animals. ($^a P < 0.01$; $^b P < 0.05$)

3 (active form) positive cells was only 13% of that in Bax(+/+) mice (Fig. 3, *A* and *B*). These results indicate the importance of Bax for UVB-induced apoptosis in the epidermis. Topical applications of caffeine to female Bax(+/+) mice immediately after exposure to UVB increased the number of apoptotic sunburn cells or caspase 3 (active form) positive cells by 214% and 253%, respectively, at 6 h after irradiation when compared with the effects of UVB treatment alone (Fig. 3, *A* and *B*). Topical applications of caffeine to female Bax(−/−) mice immediately after exposure to UVB increased the number of apoptotic sunburn cells by 467% and the number of caspase 3 (active form) positive cells by 750% at 6 h after irradiation when compared with the effect of UVB treatment alone (Fig. 3, *A* and *B*). These results demonstrate that caffeine can stimulate a Bax-independent pathway of apoptosis in the epidermis of mice treated with UVB.

Table 2 *Dose response for UVB irradiation-induced increase in apoptotic sunburn cells in the epidermis of male C57/BL6J p53(−/−) knockout mice*

Shaved male p53(−/−) knockout mice (five per group) were irradiated with different doses of UVB. The animals were killed at 6 or 24 h later. Apoptotic sunburn cells in the epidermis were determined morphologically. Each value represents the mean ± SE.

	Percent apoptotic sunburn cells	
UVB dose	6 h	24 h
No UVB	0.00 ± 0.00	
60 mJ/cm^2	0.18 ± 0.05	0.18 ± 0.03
90 mJ/cm^2	0.18 ± 0.04	0.66 ± 0.08
180 mJ/cm^2	0.21 ± 0.06	1.33 ± 0.19

DISCUSSION

Because substantial amounts of caffeine are ingested by people drinking coffee, tea, or caffeinated soft drinks, an understanding of the biological effects of caffeine is of considerable importance. In earlier studies, we demonstrated that oral administration or topical

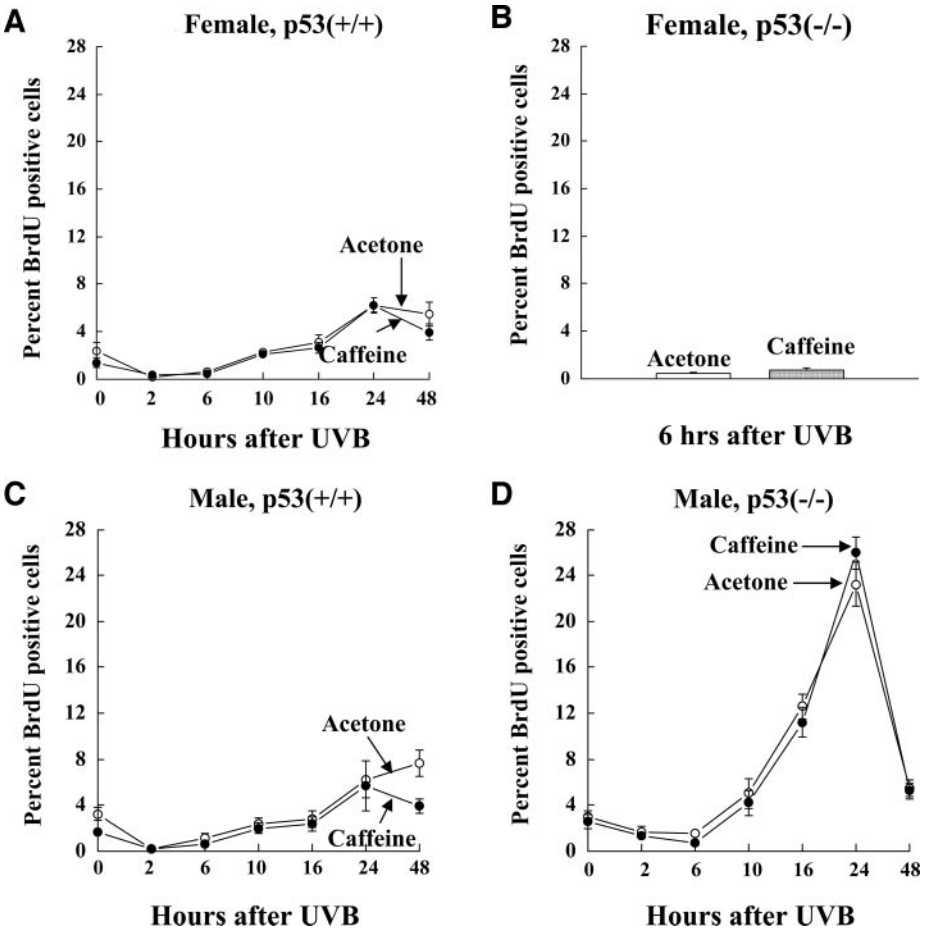

Fig. 2. Effect of topical application of caffeine on UVB-induced changes in the formation of BrdUrd-positive cells in the epidermis of female or male p53(+/+) and p53(−/−) mice. Female or male p53(+/+) and p53(−/−) mice (five mice for each time interval) were exposed to UVB (60 mJ/cm^2) followed immediately by topical application of caffeine (1.2 mg; 6.2 μmol) in 100 μl of acetone. Additional applications of caffeine (1.2 mg) were at 0.5 and 2 h after UVB. Control animals received topical applications of acetone. BrdUrd (BrdU; 50 mg/kg) was injected i.p. 1 h before sacrifice. Each value represents the mean ± SE.

application of caffeine enhanced UVB-induced apoptosis in mouse epidermis, but treatment of mice with caffeine had no effect on apoptosis in normal epidermis in the absence of UVB (5, 6). In the present study, we demonstrated that UVB-induced apoptosis in mouse skin is largely but not entirely dependent on wild-type p53 and Bax (Table 2; Figs. 1 and 3), and we also found that topical applications of caffeine can enhance UVB-induced apoptosis by p53- and Bax-independent mechanisms (Figs. 1 and 3). The impaired UVB-induced apoptosis in p53 knockout mice described here is similar to the results of earlier studies by Ziegler *et al.* (11), and both studies demonstrate the presence of a p53-independent pathway for UVB-induced apoptosis in mouse skin (in addition to the major p53-dependent pathway). In other studies, topical application of caffeine to "high-risk mice" previously initiated with UVB inhibited skin tumorigenesis, and selectively increased apoptosis in skin tumors but not in non-tumor areas of the epidermis (7). Our animal studies suggest that caffeine has a selective apoptotic effect on DNA-damaged cells *in vivo* (UVB-treated epidermis or UVB-induced tumors) but that treatment of mice with caffeine does not have an apoptotic effect in normal epidermal cells.

Although to the best of our knowledge the above studies are the only investigations on the effects of caffeine administration on apoptosis after DNA damage in an animal model or in tumors of tumor-bearing animals *in vivo*, there are several studies on the effects of caffeine on apoptosis in cultured cells *in vitro*. The results of cell

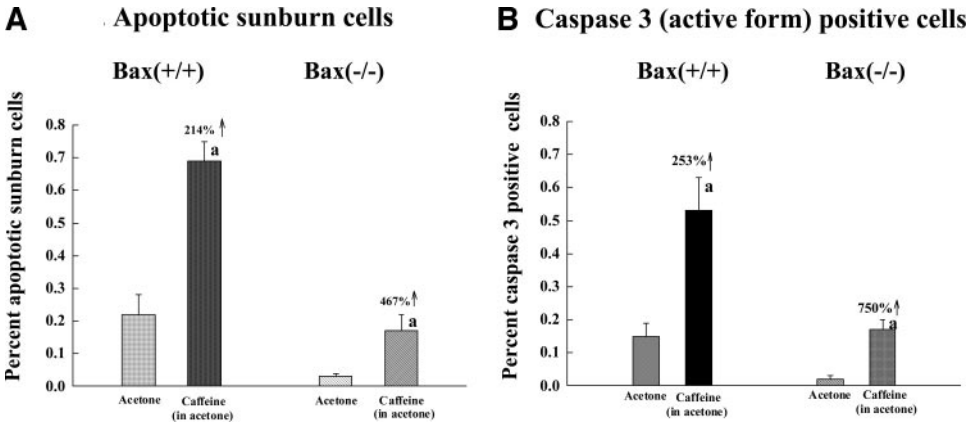

Fig. 3. Effects of topical application of caffeine on UVB-induced increases in the formation of apoptotic sunburn cells and caspase 3 (active form) immunoreactive-positive cells in the epidermis of Bax knockout mice. Female Bax(−/−) mice (eight mice) were exposed to UVB (60 mJ/cm^2) followed immediately by topical application of caffeine (1.2 mg; 6.2 μmol) in 100 μl of acetone. Additional applications of caffeine (1.2 mg) were at 0.5 and 2 h after UVB. Control animals (seven mice) received topical applications of acetone. The animals were sacrificed at 6 h after exposure to UVB. Apoptotic sunburn cells were counted morphologically and caspase 3 (active form) positive cells were counted immunohistochemically. Each value represents the mean ± SE. Statistically significant differences were observed for the percentage of apoptotic sunburn cells or the percentage of caspase 3 positive cells between the acetone-treated control animals and the caffeine-treated animals ($^a P < 0.01$).

culture studies concerning the role of p53 for caffeine-induced apoptosis are contradictory. Although most studies indicate the importance of a p53-independent pathway for caffeine-induced apoptosis, some studies indicate the importance of wild-type p53 for caffeine-induced apoptosis.

Dubrez *et al.* (19) reported that caffeine sensitizes the human non-small cell lung cancer H358 cell line to p53-mediated apoptosis by inducing mitochondrial translocation and a conformational change in the Bax protein. It was found that expression of a wild-type p53 expression vector in p53-null H358 cells restored p53 levels, and Bax protein expression was increased without inducing apoptosis. Addition of caffeine to these Bax-expressing cells induced a conformational change in the Bax protein, translocation of Bax to the mitochondria and apoptosis (19).

He *et al.* (20) reported recently that the induction of apoptosis by caffeine in serum-starved JB6 CL41 cells is mediated by p53, Bax, and caspase 3, but caffeine did not enhance apoptosis in p53(−/−) fibroblast cells. Several other investigators using cell culture systems reported that caffeine induced apoptosis in a p53-independent manner and preferentially in p53-deficient or p53-mutated cells. Caffeine (2 mM) increased gamma radiation-induced cell death in rat yolk sac tumor cell lines with or without functional p53 (21). Combinations of caffeine and gamma radiation caused a synergistic effect on apoptosis in p53-defective cells via a p53-independent pathway (21). Russell *et al.* (22) demonstrated that treatment of A549 human lung adenocarcinoma cells with caffeine caused nuclear fragmentation and apoptosis mainly in p53-inactivated cells, and the radiosensitivity of G_1 checkpoint-deficient cells treated with caffeine was comparable with the p53-wild type parental cell line.

The mechanism by which caffeine enhances the apoptotic effect of agents inducing DNA double-strand breaks in p53-deficient cells is mainly through override of the G_2-M checkpoint block. Russell *et al.* (22) demonstrated that DNA damage-induced cell cycle arrest at the G_1 checkpoint is highly dependent on wild-type p53 whereas the G_2 checkpoint is still functioning in p53-deficient cells or tumors. Powell *et al.* (23) showed in mouse embryo fibroblasts derived from p53-wild type and p53-knockout littermates that caffeine abrogated G_2-M arrest only in p53-deficient cells in response to X-ray irradiation. Treatment of p53-inactive HeLa cells with caffeine abrogated etoposide-induced G_2-arrest and concomitantly enhanced its cytotoxic effects (22, 24). Recently caffeine was found to abrogate the G_2 checkpoint after DNA damage in several p53 mutant tumor cell lines (25). Bache *et al.* (26) reported that caffeine completely prevented the X-ray irradiation-induced G_2-M arrest in the US8–93 and LMS6–93 human sarcoma cell lines, which both have a p53 mutation.

The mechanisms for the abrogation of G_2-M arrest by caffeine preferentially in p53-deficient cell lines have been investigated. Normal functions of G_1-S and G_2-M cell cycle checkpoints are to protect cells from the deleterious consequences of replicating damaged DNA or segregating damaged chromosomes. P53-deficient cells retain a functional G_2 checkpoint, which is critical for cellular recovery from DNA damage. Abrogation of the G_2 checkpoint in cells with a defective G_1 checkpoint often leads to a marked increase in the sensitivity of these cells to chemotherapeutic agents (27). These observations suggest that inhibitors of the G_2 checkpoint signaling pathway may effectively sensitize cancer cells with a defective G_1 checkpoint to the lethal effects of drugs or radiation that induce DNA double-strand breaks. Indeed, two known inhibitors of cell cycle arrest at the G_2 checkpoint, caffeine and UCN-01 (7-hydroxystaurosporine), preferentially sensitize cells that lack functional p53 to radiation-induced cellular toxicity (28–30). The sensitizing actions of caffeine and UCN-01 have raised hopes that adjunctive therapy with G_2 checkpoint inhibitors will increase the therapeutic efficacies of radiation and other genotoxic therapies in the large population of cancer patients whose tumor cells lack functional p53.

The molecular target(s) for caffeine remain unclear. Because caffeine-treated cells display several of the phenotypic abnormalities of cells deficient in ataxia-telangiectasia-mutated (ATM) or ATM Rad3-related (ATR) function, ATM and ATR are suggested as relevant molecular targets for caffeine, and the results suggest that more potent and specific inhibitors of these phosphatidylinositol-3-kinase related kinase (PIKK) family members might be clinically useful chemosensitizing agents (31). ATM is believed to be the major kinase for the formation of Ser-15-phospho-p53 in X-ray-irradiated cells, whereas ATR plays a more prominent role in the phosphorylation of p53 at Ser-15 in response to UV light exposure (32).

Studies by Nghiem *et al.* (33) with cultured human osteosarcoma U20S cells suggested that caffeine-induced inhibition of ATR caused premature chromatin condensation and cell death, and these studies also suggested that cancer cells with a disrupted G_1 checkpoint (such as loss of p53 function) are sensitized to ATR inhibition and lethal premature chromatin condensation (33, 34). The concepts developed by Nghiem *et al.* as well as by others on the selectivity of ATR inhibitors for p53-defective cells may help explain why caffeine has a selective apoptotic effect in UVB-induced tumors (previously shown to have p53 mutations) but not in non-tumor areas of the epidermis (7).

Possible molecular targets for caffeine's action in abrogating the G_2 checkpoint are shown in Fig. 4. Although caffeine was thought to inhibit the G_2 checkpoint by inhibiting ATR-dependent phosphorylation of Chk1, and this does occur *in vitro*; a recent study by Cortez indicated that caffeine in combination with hydroxyurea unexpectedly stimulated ATR-dependent phosphorylation of Chk1 and Chk2 *in vivo* in cultured HCT116 cells (35). These results suggest that caffeine may inhibit the G_2-M checkpoint downstream from the ATR-dependent phosphorylation of Chk1 and Chk2. Additional cell culture studies suggested that caffeine abrogated radiation-induced G_2 arrest by inhibition of Cdc2 phosphorylation on Tyr-15 (36, 37) and by interfering with Cdc25C binding to 14-3-3 (38). Alternatively, it is also possible that caffeine abrogates the G_2-M checkpoint by inhibiting the p53- and ATR-independent p38 kinase-mediated Cdc25B signal transduction pathway or by decreasing the level of Wee 1, thereby decreasing the level of phospho-Cdc2 and decreasing the level of phosphorylated Cdc2/cyclin B1, which would be expected to up-regulate Cdc2/cyclin B1 activity and to enhance mitosis. A role of p38 kinase for the G_2-M checkpoint was described recently by Bulavin *et al.* (39). Further research is needed to identify molecular targets in mouse epidermis for the proposed effect of caffeine to abrogate the UVB-activated G_2-M checkpoint.

It is of interest that caffeine did not influence UVB-induced changes in BrdUrd incorporation into total epidermal DNA (Fig. 2). A caffeine-induced change in BrdUrd incorporation might have been expected in UVB-treated mice if caffeine had enhanced UVB-induced apoptosis by overriding the G_2-M checkpoint. A possible reason for this apparent inconsistency may be because caffeine abrogated the G_2-M checkpoint and enhanced apoptosis in only a small proportion of epidermal cells without altering BrdUrd labeling into total epidermal DNA. Alternatively, caffeine may have enhanced apoptosis by a mechanism that was independent of the ATR/Chk1 signal transduction pathway leading to the G_2-M checkpoint.

Wikonkal *et al.* (40) reported recently that E2f1 inactivation prevents apoptosis resistance to UVB irradiation in p53-deficient mice. These observations indicate that E2f1 inhibits UVB-induced apoptosis and suggest that caffeine may enhance UVB-induced apoptosis in p53 knockout mice by inhibiting E2f1 or by preventing the UVB-induced up-regulation of E2f1.

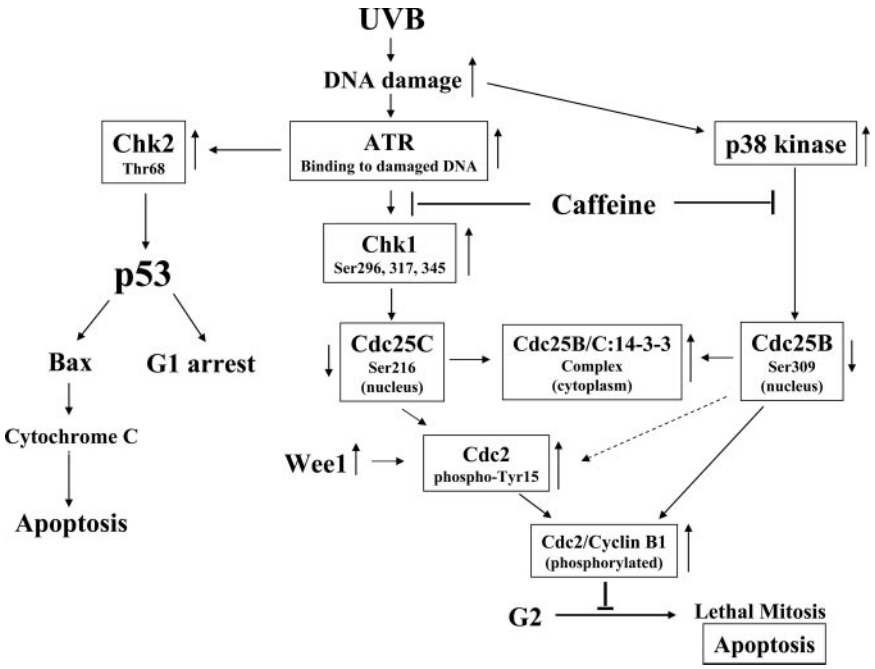

Fig. 4. Effect of UVB on Chk1 and p38 kinase signaling pathways leading to G_2 cell cycle arrest: Potential sites of action for caffeine in causing apoptosis by abrogating G_2 cell cycle arrest. UVB causes DNA damage that results in activation of ataxia-telangiectasia-mutated-Rad3 related (ATR) kinase and phosphorylation of Chk1 (on Ser-296, Ser-317, and/or Ser-345) and phosphorylation of Chk2 (on Thr-68). Enhanced phosphorylation of Chk2 results in an increased phosphorylation of p53, G_1 arrest, and apoptosis. This pathway of apoptosis is absent in p53-defective cells and in most UVB-induced tumors. Enhanced phosphorylation of Chk1 results in the inactivation of Cdc25C phosphatase by increased phosphorylation of Cdc25C (on Ser-216) that creates a binding site for complexation with the 14-3-3 protein, thereby increasing the transport of phospho-Cdc25C out of the nucleus and promoting its sequestration in the cytoplasm. Similar UVB-induced sequestration and transport into the cytoplasm occurs for Cdc25B (Ser-309). During the normal G_2-M phase of the cell cycle, Cdc25B/C-dependent dephosphorylation of phospho-Cdc2 and phospho-Cdc2/cyclin B1 kinase is a critical step for activating entry into mitosis. In the G_2-M phase of the cell cycle, Cdc2 is maintained in an inactive state in cytosol by phosphorylation on tyrosine-15 and threonine-14 residues. Inactivation of Cdc25B/C phosphatase activity by UVB results in decreased dephosphorylation of phospho-Cdc2 (Tyr-15 and Thr-14), thereby increasing the level of phospho-Cdc2 and phosphorylated Cdc2/cyclin B1, which inhibits proliferation and causes G_2 arrest. Activation of Cdc2 occurs by increased levels of Cdc25B/C phosphatase-mediated dephosphorylation of the inhibitory phosphates of Cdc2, which promotes the nuclear translocation of Cdc2 and the formation of an active dephosphorylated Cdc2/cyclin B1 complex and mitosis. Inactive phospho-Cdc2 (Tyr-15) is also maintained and mediated by the Wee1 kinase. Knockdown of Wee1 decreases the phosphorylation of Cdc2, increases the level of dephosphorylated Cdc2/cyclin B1, abrogates the G_2 checkpoint, and induces lethal mitosis (apoptosis). UVB- induced DNA damage also activates an ATR-independent p38 kinase pathway that enhances Cdc25B phosphorylation on Ser-309, thereby enhancing its complexation with 14-3-3 and its transport into the cytoplasm. Caffeine may abrogate the G_2 checkpoint and enhance apoptosis by (*a*) inhibiting ATR-mediated phosphorylation of Chk1 at Ser296, Ser317, and/or Ser345; (*b*) inhibiting Chk1-mediated phosphorylation of Cdc25C at Ser-216 thereby reducing the binding of Cdc25C phosphatase to 14-3-3 proteins and inhibiting the transport of Cdc25C out of the nucleus (increased level of active Cdc25C); (*c*) decreasing the level of Wee1 protein, resulting in decreased phosphorylation of Cdc2 at Tyr-15 and an increase in an active dephosphorylated Cdc2/cyclin B1 complex; (*d*) inhibiting p38-mediated phosphorylation of Cdc25B at Ser-309, thereby reducing the binding of Cdc25B phosphatase to the 14-3-3 protein and inhibiting the transport of Cdc25B out of the nucleus (increased level of active Cdc25B); and (*e*) increasing the level of the active dephosphorylated Cdc2/cyclin B1 complex.

In summary, the results of our studies in p53 and Bax knockout mice indicate a prominent role for p53 and Bax for UVB-induced apoptosis in mouse epidermis. The results also indicate that topical application of caffeine can enhance UVB-induced apoptosis by p53- and Bax-independent pathways. We are currently investigating the possibility that topical application of caffeine enhances UVB-induced apoptosis by inhibiting the ATR- or p38-mediated signal transduction pathways or by inhibiting E2f1. It will be of considerable interest to determine the effect of caffeine or caffeinated beverages on UVB-induced apoptosis and the risk of skin cancer in people with sunlight-induced keratoses or in individuals who have had a skin cancer removed and have a high risk of developing another skin cancer.

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