PDE2 Is a Novel Target for Attenuating Tumor Formation in a Mouse Model of UVB-Induced Skin Carcinogenesis

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Abstract

Our previous studies demonstrated that the topical application of caffeine is a potent inhibitor of UVB-induced carcinogenesis and selectively increases apoptosis in tumors but not in non-tumor areas of the epidermis in mice that are at a high risk for developing skin cancer. While this effect is mainly through a p53 independent pathway, the mechanism by which caffeine inhibits skin tumor formation has not been fully elucidated. Since caffeine is a non-selective phosphodiesterase inhibitor, we investigated the effects of several PDE inhibitors on the formation of sunburn cells in mouse skin after an acute exposure to ultraviolet light B (UVB). The topical application of a PDE2 inhibitor, erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA hydrochloride), stimulated epidermal apoptosis compared to control (P<0.01) and to a greater extent than caffeine whereas a PDE4 inhibitor attenuated the epidermal apoptosis compared to control (P<0.01). Since PDE2 hydrolyzes cyclic nucleotides, mainly cGMP, the effects of EHNA hydrochloride on epidermal apoptosis following UVB exposure may be mediated, in part, by increased cGMP signaling. Data demonstrated that the topical application of dibutyryl cGMP stimulated epidermal apoptosis (P<0.01) following an acute exposure to UVB. Treating UVB-pretreated mice topically with 3.1 μmole or 0.8 μmole of EHNA hydrochloride attenuated tumor formation to a greater extent than treating with 6.2 μmole caffeine when these compounds were applied once a day, five days a week for 18 weeks. These observations suggest a novel role for PDE2 in UVB-induced tumorigenesis and that PDE2 inhibitors that mediate cGMP signaling may be useful for the prevention and treatment of skin cancer.

Introduction

Sunlight-induced non-melanoma skin cancer is the most common cancer in the United States [1]. Wavelengths in the ultraviolet B light (UVB) range (290–320 nm) induce erythema, burns and are primarily responsible for these cancers. Although most skin cancers are squamous cell carcinomas and basal cell carcinomas that are easily cured if detected early, many people still die from these cancers, as well as from the more dangerous sunlight-induced melanomas. The development of strategies to prevent or cure UVB-induced cancers would have a major impact in decreasing the total load of human cancer.

Our previous studies demonstrated that the topical application of caffeine is a potent inhibitor of UVB-induced carcinogenesis by selectively increasing apoptosis in tumors but not in non-tumor areas of the epidermis in mice that are at a high risk for developing skin cancer [2–5]. While this effect is mainly through a p53 independent pathway [3], the mechanism by which caffeine inhibits skin tumor formation has not been fully elucidated.

Caffeine (1,3,7-trimethylxanthine) is a broad spectrum, non-selective phosphodiesterase (PDE) inhibitor that is metabolized into dimethylxanthines. Naturally occurring methylxanthines were the first inhibitors of cyclic nucleotide PDEs to be discovered. Caffeine acts by competing with adenine, the purine base of DNA, for access to the catalytic site of PDEs and prevents PDEs from breaking the diester bond that connects the 5 carbon to the 3 carbon of the ribose. Eleven families of PDE isoenzymes can be distinguished that differ in their biochemical properties, their localization and their affinities for cAMP, cGMP or both [6–8].

cAMP and cGMP are intracellular second messengers that modulate several signaling pathways and control several functions in cells. A novel approach to anti-tumor therapy is to modulate cAMP and cGMP with PDE inhibitors as cAMP and cGMP are negative regulators of cell growth and aberrant signaling has been shown to play an important role in various carcinomas and hematological malignancies [7,9]. Since caffeine is a non-selective PDE inhibitor, we hypothesized that caffeine inhibits UVB-induced skin cancer by inhibiting PDEs to modulate the intracellular cyclic nucleotides cAMP and/or cGMP.

The present study used several pharmacologically selective and non-selective PDE inhibitors to assess the role of PDE inhibition on epidermal apoptosis following an acute exposure to UVB. The presence of apoptotic epidermal cells (apoptotic sunburn cells)
indicated the potential for anti-cancer effects of a compound in vivo [2,10–14]. Comparing structurally similar PDE inhibitors to caffeine, we found differential effects on the percentage of UVB-induced apoptotic sunburn cells. The selective PDE2 inhibitor, EHNA hydrochloride [15], significantly increased the percentage of UVB-induced apoptotic sunburn cells compared with caffeine and control while the selective PDE4 inhibitor, ICI 63,197 [16], significantly decreased the percentage. PDEs hydrolyze cyclic nucleotides and, due differences in affinities, PDE inhibitors, specifically EHNA hydrochloride, have been shown to mainly increase cAMP signaling whereas PDE4 inhibitors have been shown to increase cGMP signaling. Despite the differences observed with EHNA hydrochloride and ICI 63,197, the topical application of exogenous dibutyryl cGMP or dibutyryl cAMP derivatives both increased epidermal apoptosis after an acute exposure to UVB. EHNA hydrochloride was then tested for its efficacy in attenuating UVB-induced skin carcinogenesis. Results demonstrated that EHNA hydrochloride potently inhibited UVB-induced skin tumor formation.

Materials and Methods

Animals

These studies were approved by the Rutgers IACUC. CO₂ and cervical dislocation were used as a method of euthanasia. The protocol approval number is 88-056. 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.

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). For PDE apoptosis studies, mice were exposed to a single dose of 30 mJ/cm² of UVB and then treated topically with PDE inhibitors. For the PDE carcinogenesis studies, mice were treated with UVB (30 mJ/cm²) twice a week for 20 weeks and UVB exposure was stopped. Following UVB, mice were treated topically with PDE inhibitors. More experimental details on treatments can be found in the two sections below.

PDE apoptosis studies

Female SKH-1 hairless mice were exposed to a single dose of 30 mJ/cm² of UVB. Immediately following UVB and at 30 and 120 minutes post-UVB exposure, mice were treated topically with MMPX, EHNA hydrochloride, Cilostamide, ICI 63,197, T0156, zaprinast, dipyridamole or caffeine in 100 μl acetone/water (9:1). The animals were killed at 6 hrs after UVB exposure (peak time point for UVB-induced apoptosis). Apoptotic sunburn cells in the epidermis were determined morphologically by cell shrinkage and nuclear condensation. The results showed that a selective cGMP-activated PDE2 inhibitor, EHNA hydrochloride had a more pronounced stimulatory effect than caffeine on UVB-induced apoptosis (Fig. 1A). Topical application of 3.1 μmol EHNA enhanced UVB-induced apoptosis by 267% (P<0.01), whereas topical application of same amount of caffeine (3.1 μmol) only enhanced apoptosis by 68% (P<0.01) compared with the acetone control group. Topical application of 3.1 μmol of EHNA hydrochloride induced 0.01% apoptotic sunburn cells in non-UVB irradiated mouse epidermis. The significant increase in apoptotic sunburn cells in EHNA hydrochloride-treated epidermis was validated with a dose-response experiment, where several doses of EHNA hydrochloride were compared to the same doses of caffeine. Except at the lowest dose (0.8 μmol), EHNA hydrochloride significantly stimulated UVB-induced apoptosis when compared to caffeine (Fig. 1C). EHNA hydrochloride at 0.8, 1.6, 3.1, and 6.2 μmol stimulated UVB-induced apoptosis 83, 134, 80, and 68% more than the same dose of caffeine (Fig. 1C).

Dipyridamole, a PDE 5, 6, 10, 11 inhibitor, also stimulated epidermal apoptosis 79% more than the acetone control (P<0.05)

Statistical analysis

The Student’s t-test was used for simple comparisons of two groups. The analysis of variance (ANOVA) model was used for comparisons of multiple treatment groups with a common control group using Microsoft Excel. For all statistical tests, a P value of < 0.05 was accepted as statistical significance. All data are means +/- SEM.

Results

A PDE2 inhibitor stimulates and a PDE4 inhibitor attenuates epidermal apoptosis after an acute exposure to UVB

Previous studies determined that caffeine, a non-specific phosphodiesterase (PDE) inhibitor, attenuated UVB-induced carcinogenesis [4] therefore, we tested the effect of several different selective and non-selective PDE inhibitors on epidermal apoptosis following an acute exposure to UVB. The presence of apoptotic epidermal cells (apoptotic sunburn cells) was determined to be an indicator for the anti-cancer effects of a compound in vivo [2,10–14]. Immediately following UVB and at 30 and 120 minutes post-UVB exposure, female SKH-1 mice were treated topically with MMPX, erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA hydrochloride), Cilostamide, ICI 63,197, T0156, zaprinast, dipyridamole or caffeine in 100 μl acetone/water (9:1) at a concentration of 3.1 μmol (Fig. 1A) or 6.2 μmol (Fig. 1B). The animals were then killed at 6 hrs following UVB exposure (peak time point for UVB-induced apoptosis). Apoptotic sunburn cells in the epidermis were determined morphologically by cell shrinkage and nuclear condensation. The results showed that a selective cGMP-activated PDE2 inhibitor, EHNA hydrochloride had a more pronounced stimulatory effect than caffeine on UVB-induced apoptosis (Fig. 1A). Topical application of 3.1 μmol EHNA enhanced UVB-induced apoptosis by 267% (P<0.01), whereas topical application of same amount of caffeine (3.1 μmol) only enhanced apoptosis by 68% (P<0.01) compared with the acetone control group. Topical application of 3.1 μmol of EHNA hydrochloride induced 0.01% apoptotic sunburn cells in non-UVB irradiated mouse epidermis. The significant increase in apoptotic sunburn cells in EHNA hydrochloride-treated epidermis was validated with a dose-response experiment, where several doses of EHNA hydrochloride were compared to the same doses of caffeine. Except at the lowest dose (0.8 μmol), EHNA hydrochloride significantly stimulated UVB-induced apoptosis when compared to caffeine (Fig. 1C). EHNA hydrochloride at 0.8, 1.6, 3.1, and 6.2 μmol stimulated UVB-induced apoptosis 83, 134, 80, and 68% more than the same dose of caffeine (Fig. 1C).

Dipyridamole, a PDE 5, 6, 8, 10, 11 inhibitor, also stimulated epidermal apoptosis 79% more than the acetone control (P<0.05)
although not to the same extent as the same dose of caffeine (6.2 μmole) (Fig. 1B). Conversely, topical application of a selective cGMP-insensitive, cAMP-mediated PDE4 inhibitor, 2-amino-6-methyl-4-propyl-[1,2,4]triazolo[1,5-a]pyrimidin-5(4H)-one (ICI 63,197), almost completely inhibited UVB-induced apoptosis (96% inhibition) when compared with the acetone control group (P<0.01, Fig. 1A). These data demonstrate that UVB-induced apoptosis is dependent which PDEs are inhibited.

Effects of phosphodiesterase inhibitors and cyclic nucleotides on epidermal apoptosis after an acute exposure to UVB

To mimic a more physiologically relevant model of skin cancer, we repeated this study utilizing congenic p53 knockout (−/−) hairless mice since most UVB-induced skin tumors are characterized by p53 mutations. p53 wild-type (+/+) littermates were used as a control. The dose of caffeine and EHNA hydrochloride was reduced to 1.6 and 3.1 μmole as the previous experiment indicated that EHNA hydrochloride was still able to significantly stimulate epidermal apoptosis at these doses (Fig. 2A). Topical application of EHNA hydrochloride dose-dependently induced
apoptotic sunburn cells in the UVB-irradiated mouse epidermis in p53 (+/+) (224 and 367%) and p53 (−/−) (200 and 350%) mice similar to that which was observed in the SKH-1 mice (Fig. 2A). Interestingly, EHNA hydrochloride significantly stimulated apoptotic sunburn cells compared with caffeine in the p53 (+/+) mice \( (P<0.05) \) but not in the p53 (−/−) mice indicating that the number of epidermal apoptotic cells may have plateaued in the p53 (−/−) which are more sensitive to UVB (unpublished observation). These data suggest that a PDE2 inhibitor will attenuate tumor formation independently of p53 expression.

Since the PDE2 inhibitor EHNA hydrochloride, which can enhance cGMP expression, induced epidermal apoptosis after an acute exposure to UVB, we hypothesized that the topical application of cGMP would also induce apoptotic sunburn cells. To test this hypothesis, immediately following UVB and at 30 and 120 minutes post-UVB exposure, female SKH-1 mice were treated topically with either cAMP or cGMP at 6.2 µmole to mimic the dose of caffeine that stimulated epidermal apoptosis. cAMP or cGMP had no significant effect on epidermal apoptosis (Fig. 2B) and therefore, dibutyryl cAMP or dibutyryl cGMP (6.2 µmole) were applied. These are membrane permeable analogs of cAMP and cGMP that are used experimentally to mimic the intracellular actions of cAMP and cGMP. Dibutyryl cGMP increased the percentage of apoptotic sunburn cells by 146%, similar to that of EHNA hydrochloride \( (P<0.01) \) (Fig. 2B). Interestingly, dibutyryl cAMP also increased the percentage of apoptotic sunburn cells (86%), but less significantly than dibutyryl cGMP \( (P<0.05) \) suggesting that intracellular cGMP is the critical mediator of the apoptotic sunburn response induced by EHNA hydrochloride (Fig. 2B). These results suggest that the cGMP-activating PDE2 inhibitor, EHNA hydrochloride, and caffeine may inhibit UVB-induced skin carcinogenesis by regulating the levels of cGMP.

**A PDE2 selective inhibitor attenuates UVB-induced carcinogenesis**

Since the apoptotic studies indicated that the cGMP-activating PDE2 inhibitor, EHNA hydrochloride, induced more epidermal apoptosis when compared to caffeine, a non-specific PDE inhibitor we next determined the effect of different doses of EHNA hydrochloride on UVB-induced carcinogenesis. Female SKH-1 hairless mice were treated with UVB (30 mJ/cm²) twice a week at 28 weeks. Acetone was used as the vehicle control and acetone treatment at 28 weeks \( (P<0.05) \) but not in the p53 (−/−) mice indicating that the number of epidermal apoptotic cells may have plateaued in the p53 (−/−) which are more sensitive to UVB (unpublished observation). These data suggest that a PDE2 inhibitor will attenuate tumor formation independently of p53 expression.

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**Figure 2. Effects of phosphodiesterase inhibitors and cyclic nucleotides on epidermal apoptosis after an acute exposure to UVB.** A. Female p53 wild-type or p53 knockout SKH-1 hairless mice (7 to 8 weeks old, 5 per group) were treated topically with caffeine or different PDE inhibitors (in 100 µl acetone:water (9:1) right after a single dose of 30 mJ/cm² of UVB and at 30 and 120 min later. The animals were killed at 6 hrs after UVB. Apoptotic sunburn cells in the epidermis were determined morphologically. Value is percent increase compared with acetone control (*P<0.05, **P<0.01). In the p53 wild-type mice only, EHNA hydrochloride significantly induces apoptotic sunburn cells compared with caffeine \( (P<0.05) \). All data are mean ± SD. B. Female SKH-1 mice (8 to 9 weeks old, 5 per group) were treated topically with 100 µl of acetone:water (9:1) or 6.2 µmole of compounds in 100 µl of acetone:water (9:1) immediately after 30 mJ/cm² of UVB irradiation and at 30 and 120 min later. The animals were killed 6 hours later. Apoptotic sunburn cells in the epidermis were determined morphologically. Value is percent increase compared with acetone control \( (*P<0.05, **P<0.01) \). doi:10.1371/journal.pone.0109862.g002

and \( P<0.01 \) for the 3.1 µmole dose). In addition, the tumor volume per mouse increased quickly in the EHNA hydrochloride-
treated mice compared with the acetone-treated mice (Fig. 3C). These results indicated that EHNA hydrochloride treatment significantly reduced tumor volume per mouse compared with acetone treatment at 28 weeks ($P < 0.01$).

**EHNA hydrochloride attenuates both malignant and non-malignant tumor formation to a greater extent than caffeine**

Histological examination of tumors demonstrated that caffeine-treatment and EHNA-hydrochloride-treatment yielded 46% and 63% less mice with non-malignant tumors (squamous cell papillomas and keratoacanthomas) than acetone-treatment and 45% and 67% less mice with malignant tumors (squamous cell carcinomas) at the 6.2 µmole dose (Table 1). Even at the lower dose of EHNA hydrochloride (3.1 µmole), 19% less mice had non-malignant tumors and 43% less mice had malignant tumors than in the acetone control group. Caffeine-treated mice and EHNA-hydrochloride-treated mice had 67% and 76% less non-malignant tumors per mouse and 20% and 72% less malignant tumors per mouse compared to acetone-treated mice at the 6.2 µmole dose. EHNA-hydrochloride-treated mice (3.1 µmole) had 30% less non-malignant tumors and 52% less malignant tumors per mouse.

EHNA hydrochloride reduced tumor volume per mouse (non-malignant and malignant) by 79% and 94% at the low and high doses whereas caffeine decreased tumor volume by only 29% (Table 2). Overall, EHNA hydrochloride at both the low and high doses reduced the number of malignant tumors and total tumor volume per mouse to a greater extent than caffeine. The difference
Table 1. Effect of topical applications of EHNA on the incidence and multiplicity of histologically characterized skin tumors in high risk SKH-1 mice previously treated with UVB.

| Treatment       | No. of mice | Squamous cell papillomas | Keratoacanthomas | Total nonmalignant tumors | Squamous cell carcinomas | Total tumors |
|-----------------|-------------|--------------------------|------------------|---------------------------|--------------------------|-------------|
|                 |             | % mice with tumors       | % mice with tumors | % mice with tumors        | % mice with tumors       | % mice with tumors |
| Acetone         | 28          | 0                        | 0                | 89                        | 3.32 ± 0.66              | 21          |
| CF              | 25          | 4.0 (0)                  | 0.04 ± 0.04 (0)  | 48 (46)                   | 1.08 ± 0.35 (67)         | 12 (43)     |
| EHNA (0.8 μmole)| 25          | 3.7 (0)                  | 0.04 ± 0.04 (0)  | 30 (60)                   | 0.81 ± 0.31 (76)         | 7 (67)      |
| EHNA (3.1 μmole)| 27          | 3.7 (0)                  | 0.04 ± 0.04 (0)  | 72 (19)                   | 2.28 ± 0.48 (31)         | 12 (43)     |

Female SKH-1 mice (7–8 weeks old) were irradiated with UVB (30 mJ/cm²) twice weekly for 20 weeks, and UVB irradiation was stopped. These tumor-free mice with a high risk of developing skin tumors were treated topically with 3.1 μmole or 0.8 μmole of erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA hydrochloride) or 6.2 μmole caffeine in 100 μl acetone:water (8:2) once a day five days a week for 16 weeks. These mice were killed at 36 weeks after the last dose of UVB and all tumors were characterized by histopathology and the size of each tumor was determined. Each value is the mean ± SE, and the numbers in parentheses represent percent decrease.

### Discussion

Skin is the barrier between the environment and internal organs. Important for the preservation of body homeostasis through a highly regulated cutaneous neuroendocrine system [17]. Skin carcinogenesis and the expression of these cyclic nucleotides have been well characterized [2]. The huge commercial success of the PDE5 inhibitor, Viagra, has stimulated research and development of PDE -based therapies. New classes of PDE inhibitors have been developed, and the uses of ICI 63,197 and EHNA hydrochloride as a tool to examine the role of PDE4 for calcium signaling is a new therapeutic strategy for chemoprevention and chemotherapy [7,18–20] however, the effects of PDE inhibitors on skin carcinogenesis have never been reported. This manuscript demonstrates that the PDE2 selective inhibitor, EHNA hydrochloride, induced epidermal apoptosis and skin tumorigenesis in a high risk mouse model. The structure of EHNA hydrochloride, ICI 63,197 and caffeine are all very similar. However, this is the first manuscript to test the use of ICI 63,197 and EHNA hydrochloride compared to caffeine in a model of carcinogenesis.
Our observations that the PDE4 inhibitor, ICI 63,197, which stimulates cAMP signaling, attenuated UVB-induced apoptosis while the PDE2 inhibitor, EHNA hydrochloride, which stimulates cGMP signaling, enhanced UVB-induced apoptosis and attenuated tumor formation in a mouse model of UVB-induced carcinogenesis are partly consistent with what has been reported for intracellular cyclic nucleotides in other types of epithelial cancers. For example, colorectal cancer cells have decreased basal levels of cGMP [29] and lung, bladder, ovarian, prostate, breast and colon cancers overexpress the transporter which exports cGMP from cells [30,31]. This suggests that lower levels of cGMP are associated with epithelial cancers. In fact, several studies have demonstrated that cGMP signaling in epithelial cells stimulates apoptosis and inhibits proliferation to promote tumorigenesis [32–34]. In addition, cGMP signaling activates PKG which can then regulate other downstream targets that also have anti-proliferative and pro-apoptotic effects [35–37]. Conversely, cAMP signaling has been associated with oncogenic activity in epithelial tissues [38,39].

In our study, topically applied dibutyryl cAMP or dibutyryl cGMP to mouse skin following UVB exposure increased the percentage of apoptotic sunburn cells. However, dibutyryl cGMP increased the percentage of epidermal apoptotic cells (146%) to a greater extent than dibutyryl cAMP (83%). Based on our results with the PDE4 inhibitor, ICI 63,197, we expected dibutyryl cAMP to decrease the percentage of epidermal apoptotic cells. However, the topical application of exogenous intracellular cyclic nucleotide second messengers may have differential effects on epidermal apoptosis compared to that of endogenous intracellular cyclic nucleotide second messengers stimulated by PDE inhibitors. While the classical role of cGMP in skin is for vasodilation, the importance of cGMP and the differential roles of cAMP and cGMP signaling in skin cancer needs further exploration. Moreover, there is the potential for non-specific effects of EHNA hydrochloride and therefore future studies are planned to determine the effect of PDE2 knockdown or other PDE2 selective inhibitors such as IC 933 and BAY 60–7550 on skin cell transformation and tumor formation.

Herein, we demonstrated that PDE4 inhibition and PDE2 inhibition have opposing effects on the percentage of sunburn cells following an acute exposure to UVB. A PDE4 inhibitor attenuated apoptosis and a PDE2 inhibitor enhanced apoptosis. While caffeine is a broad-spectrum PDE inhibitor, it was not as potent as EHNA hydrochloride and therefore future studies are planned to determine the effect of PDE2 knockdown or other PDE2 selective inhibitors such as IC 933 and BAY 60–7550 on skin cell transformation and tumor formation. This may be due to the ability of caffeine to inhibit not only PDE2, but also PDE4. Overall, these data suggest that PDE2 may be a novel target for inhibition of human skin tumors after years of sun exposure.

Author Contributions
Conceived and designed the experiments: YPL JJB. Performed the experiments: JJB YRL TL QYP. Analyzed the data: YPL JJB. Contributed reagents/materials/analysis tools: YPL JJB. Contributed to the writing of the manuscript: YPL JJB.
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