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Ionizing radiation synergistic induction of cyclooxygenase-2 with benzo[a]pyrene diol-epoxide through nuclear factor of activated T cells in mouse epidermal Cl 41 cells

RONGHE ZHANG*, JINGXIA LI*, FREDRIC J. BURNS and CHUANSHU HUANG

Nelson Institute of Environmental Medicine, New York University School of Medicine, 57 Old Forge Road, Tuxedo, NY 10987, USA

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Abstract. Carcinogenic effects of ionizing radiation and benzo[a]pyrene-7,8-diol-9,10-epoxide (B[a]PDE), a major metabolite of benzo[a]pyrene (B[a]P), have been well demonstrated both *in vitro* and *in vivo*. Two-stage carcinogenesis results indicate that mouse skin is highly susceptible to both ionizing radiation and benzo[a]pyrene-7,8-diol-9,10-epoxide (B[a]PDE), a major metabolite of benzo[a]pyrene (B[a]P). It is believed that signaling pathways leading to the regulation of gene expression play a significant role in the development of skin cancers. The NFAT family of proteins are important transcription factors involved in the regulation of various target genes, such as IL-1 and TNF-α, which play key roles in the regulation of inflammation and carcinogenesis. Thus, the effect of ionizing radiation and B[a]PDE on COX-2 induction and NFAT3 activation, and their relationship, was investigated in mouse epidermal Cl 41 cells. We found that B[a]PDE exposure induced a very high level of NFAT activation in mouse epidermal Cl 41 cells. Ionizing radiation exhibited a synergistic effect with B[a]PDE on NFAT activation and COX-2 induction, while ionizing radiation alone had no effect. By stably knocking down NFAT3 protein expression by means of the specific interfering RNA (siRNA) technique, we found that COX-2 induction by B[a]PDE and the synergistic effect of ionizing radiation with B[a]PDE was totally blocked. These results indicate that ionizing radiation acts synergistically with B[a]PDE on COX-2 induction, and the synergism is dependent on the NFAT3 pathway.

Correspondence to: Dr Chuanshu Huang, Nelson Institute of Environmental Medicine, New York University School of Medicine, 57 Old Forge Road, Tuxedo, NY 10987, USA
E-mail: chuanshu@env.med.nyu.edu

*Contributed equally

Abbreviations: Cox-2, cyclooxygenase-2; B[a]P, benzo[a]pyrene; B[a]PDE, benzo[a]pyrene-7,8-diol-9,10-epoxide; NFAT, nuclear factor of activated T cells

Key words: cyclooxygenase-2, benzo[a]pyrene-7,8-diol-9,10-epoxide, ionizing radiation, nuclear factor of activated T cells

Introduction

Humans are exposed to environmental toxins throughout their lives. Biological consequences of the vast majority of these toxins are highly complicated because most exposures occur as mixtures, often creating interactive effects (1). There are several plausible scenarios for simultaneous exposure to B[a]PDE and ionizing radiation, such as the Chernobyl accident and underground hard rock mining. A retrospective cohort study in 15 countries to assess the risk of cancer after low does of ionizing radiation showed that 1-2% of deaths from cancer may be attributable to radiation (2). The carcinogenic properties of ionizing radiation were evident as an increased incidence of skin cancers among early radiation workers (3). This observation has been confirmed in a number of animal models, in which it has been shown that radiation could induce cancer development in most tissues of various mammalian species (3).

In vitro studies show that ionizing radiation induces genomic instability significantly more frequently than conventional gene mutations (4,5). DNA double-strand breaks (DSB) are often identified as the initiators of radiation-induced genomic instability (5). DSBs constitute a genomically dangerous type of DNA damage that, if not adequately repaired, can lead directly to cell death via lethal chromosomal aberrations or to increased apoptosis as a protective response (6). Radiation-induced DNA damage is repaired through non-homologous end-joining, single-strand annealing, and/or homologous recombination pathways (7). Although most DNA damage is repaired correctly, the repair process sometimes fails and the genome is disrupted, which may lead to increased mutation induction (7). Radiation-induced genomic instability can be transmitted through many cell generations after irradiation (4,7). Radiation-induced genomic instability provides a driving force for accumulating genetic alterations, including those responsible for the initiation of multi-step carcinogenesis (7).

Intercellular and extracellular signals can be critical to the suppression of neoplastic cellular behavior (8). Disruption of cell-cell interactions are implicated in tumor promotion and progression (9). Radiation exposure may disrupt the normal

cell-cell interaction and induce activation of signaling pathways leading to the activation of transcription factors that consequently alter gene expression pathways relevant to carcinogenesis (8). However, the signaling pathways involved in the cell response to ionizing radiation or other carcinogens are far from understood at present.

Polycyclic aromatic hydrocarbons (PAHs) are environmental toxins found in the soil, air, water and food (10). B[a]P is one of many PAHs found in the complex mixture of chemicals in cigarette smoke (10). B[a]PDE, an ultimate metabolite of B[a]P (12), has been found in cigarette smoke, charred foods, and the output from internal combustion engines and power plants (10,11). B[a]PDE, which has been extensively studied for mutagenicity (12), interacts with nucleophilic sites on cellular DNA, RNA and protein (13). Following exposure to air pollution and elevated levels of oxidative DNA damage, including bulky DNA adducts, 8-hydroxy-2'-deoxyguanosine(8-Oxo-dG) and 5-methylcytosine were found in various tissues and lymphocytes (14). The carcinogenic and mutagenic activity of these compounds has been convincingly proven by studies *in vivo* and *in vitro*, and are proven factors in smoking associated cancers (10).

The COX enzyme controls the rate-limiting step in the conversion of arachidonic acid into prostaglandins (PGs) (15), which are thought to contribute to tumor growth and metastasis. Previous studies show that the overexpression of COX-2 could lead to cancer cell proliferation (16,17), promote angiogenesis (15), inhibit apoptosis (17), and increase metastatic potential (18,19). COX-2 is thought to play an important role in carcinogenesis (20), and protect cancer cells from radiation-induced cell death (15). COX-2 is also reported to be up-regulated in many types of human cancer and can be induced by cytokines, growth factors, and tumor promoters (21). Overexpression of COX-2 in a transgenic mouse model was sufficient to induce tumors with increased concentration of PGs (20). Thus, COX-2 could play an essential role in cancer development.

In the present study, we investigated the combined effects of B[a]PDE and ionizing radiation on NFAT activation and its role in COX-2 induction in JB6 Cl 41 cells, a well-characterized and widely used mouse cell line for tumor promotion studies (22).

Materials and methods

Cell culture, reagents and ionizing radiation. JB6 P^+ mouse epidermal cell line Cl 41 and its transfectants were cultured in Eagle's minimum essential medium (MEM; Calbiochem San Diego, CA) supplemented with 5% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 2 mM L-glutamine (Life Technologies, Inc., Rockville, MD). The cells were cultured at 37˚C in a humidified atmosphere containing 5% CO_2.

B[a]P and B[a]PDE were purchased from Eagle-Picher Industries, Inc., Chemsyn Science Laboratories (Lenexa, KS) and dissolved in dimethysulfoxide at a 2 mM stock concentration. The substrate for the luciferase assay was from Promega (Madison, WI). COX-2 (murine) polyclonal antibody was purchased from Cayman Chemical, and monoclonal anti ß-actin antibody was purchased from Sigma. Rabbit anti-

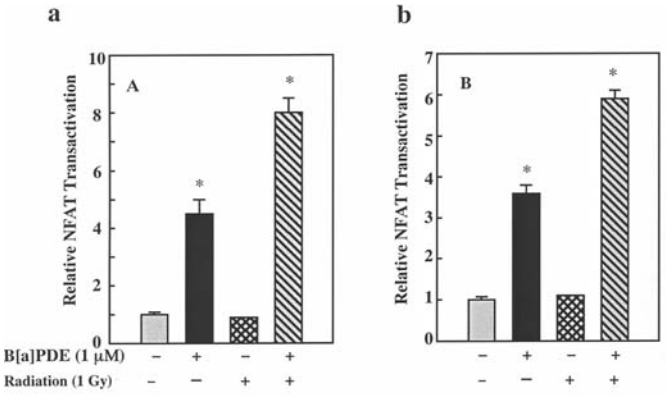

Figure 1. Effects of B[a]PDE and/or X-ray on NFAT activity in mouse epidermal Cl 41 cells (a), and mouse embryonic fibroblast PW cells (b). Cl 41 NFAT mass1 or PW NFAT mass1 C1 41 cells ($8x10^3$) were seeded into each well of 96-well plates. After being cultured at 37˚C overnight, cells were irradiated with a 1.0 Gy X-ray immediately followed by 1 μM of B[a]PDE for 12 h. The luciferase activity was measured and the results were then presented as NFAT transcription activity relative to medium control containing 0.1% DMSO (relative NFAT activity). Each bar indicates the standard deviation of triplicate assay wells. *Indicates a significant increase from control ($p<0.05$).

mouse NFAT3, c-Jun, and GAPDH polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-rabbit-AP-linked IgG and a Western blotting system were purchased from Amersham Biosciences (Piscataway, NJ).

The X-ray beam was generated by an unfiltered 100 kVp Grenz ray unit operated at 5.0 mA at the Nelson Institute of Environmental Medicine, New York University School of Medicine. The dose rate was 7.4 Gy/min, and radiation was given to cells just before the B[a]PDE treatment began.

Construction of siRNA expression vector. The siRNA expression vector was constructed using the GeneSuppressor™ System (Imgenex Co., San Diego, CA). An inverted repeat DNA oligonucleotide, designed with the aid of siRNA Target Finder published on the website (Ambion Inc., Austin, TX), was synthesized, annealed, and inserted into the *Sal*I and *Xba*I sites of the vector. Sequences of the oligonucleotides were 5'-tcgaGCCATTGACTCTGCAGATGgagtactgCATCTGCAGAGTCAATGGCttttt-3' (forward), and 5'-ctagtttttGCCATTGACTCTGCAGATGcagtactcCATCTGCAGAGTCAATGGC-3' (reverse), where capital letters show the target sequence of mouse NFAT3, lowercase show the 5 nt spacers, five t indicate terminal signal, and *Xho*I and *Xba*I sites show compatible restriction overhangs. The construct was named NFAT3/pSuppressor.

Generation of stable cotransfectants. The COX-2-luciferase reporter plasmid contained a fragment of the upstream 5' flanking region of the human COX-2 gene linked to the luciferase reporter gene as described in previous studies (23). Cl 41 cells were cultured in a 6-well plate until reaching 85-90% confluence. COX-2-luciferase reporter plasmid DNA (5 μg), 1 μg of NFAT3/pSuppressor or empty pSuppressor vector, and 20 μl of lipofectamine reagent (Gibco BRL, Rockville, MD) were used to transfect each well in the absence of serum. After 10-12 h, the 5% FBS MEM was replaced without penicillin/streptomycin. At 36-48 h after the beginning of transfection, the medium was replaced with

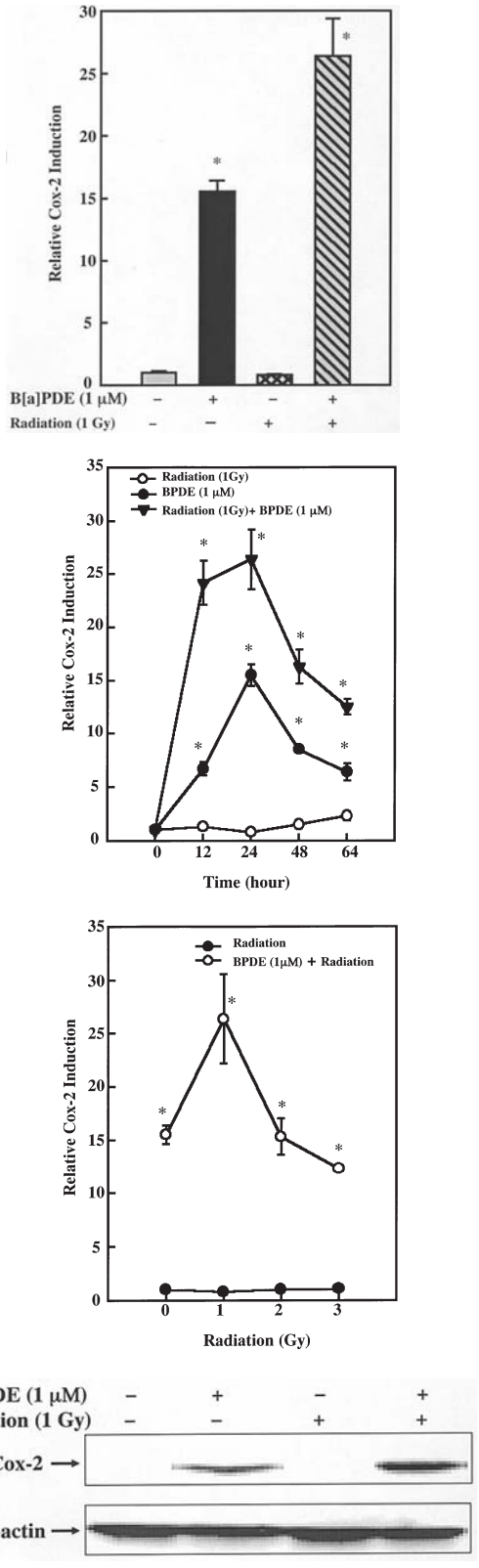

Figure 2. Effects of B[a]PDE and/or X-ray on COX-2 induction in mouse epidermal Cl 41 cells. C1 41 COX-2 mass1 cells were seeded into each well of a 96-well plate. After being cultured at 37˚C overnight, cells were irradiated with a 1.0 Gy X-ray and immediately followed by 1 μM of B[a]PDE for (a) 12 h, (b) at various time points indicated, or (c) with 1 μM of B[a]PDE and/or various doses of ionizing radiation as indicated for 12 h. The luciferase activity was then measured, and the results were presented as relative COX-2 induction. Each bar indicates the mean and standard deviation of triplicate assay wells. *Indicates a significant increase from medium control ($p<0.05$). (d) C1 41 cell COX-2 mass1 protein was extracted with SDS sample buffer. Western blot analysis was carried out as described in Materials and methods using antibodies specific against COX-2 and ß-actin.

5% FBS MEM containing 400 μg/ml G418 (Gibco BRL, Rockville, MD). After selection for 28-35 days with G418, the stable transfectants were identified by measuring the basal levels of luciferase activity and NFAT3 protein expression. Stable transfectants, Cl 41 siNFAT COX-2 mass1, and Cl 41 COX-2 mass1, were established and cultured in G418-free 5% FBS MEM for at least two passages.

Western blot analysis. The Cl 41 transfectants ($3x10^5$/well) were seeded in 6-well plates, and collected by adding 100 μl 1X SDS sample buffer until reaching 80-90% confluence. Western blot analysis was performed using standard procedures. Briefly, 15 μl of the prepared sample was loaded on 8% SDS-polyacrylamide gels (SDS-PAGE) and transferred to a PVDF membrane. For immunodetection, COX-2 (murine) polyclonal antibody and monoclonal anti-ß-actin antibody, rabbit anti-mouse NFAT3, c-Jun, and GAPDH polyclonal antibodies (Santa Cruz Biotechnology, Inc., CA) were used. The protein band bound to the primary antibody was detected by using an AP-linked anti-rabbit IgG and the ECF Western blotting system (Amersham Biosciences).

Assays for COX-2 induction. Confluent monolayers of the transfectant cells were trypsinized, and $8x10^3$ viable cells were suspended in 100 μl of MEM supplemented with 5% FBS and added to each well of 96-well plates. Plates were incubated at 37˚C in a humidified atmosphere of 5% CO_2. After the cell density reached 80-90%, the culture medium was replaced with 100 μl of MEM supplemented with 0.1% FBS, and 2 mM L-glutamine. After 12 h, the cells were exposed to ionizing radiation and/or B[a]PDE for the times indicated in the figure legends. The luciferase activity was determined by using a luminometer (Wallac 1420 Victor 2 multilable counter system) after adding 50 μl of the luciferase assay lysis buffer for 30 min at 4˚C. The results were expressed as COX-2 induction relative to controls containing the same concentration (0.1%) of DMSO only (relative COX-2 induction).

Assay for NFAT transactivation. Confluent monolayers of $8x10^3$ viable Cl 41 NFAT mass1 transfectants were trypsinized, suspended in 100 μl medium and added to each well of a 96-well plate as described in previous studies (24,25). Plates were incubated at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were exposed to ionizing radiation and/or followed by B[a]PDE for 12 h at the indicated dosages, then extracted with the luciferase assay lysis buffer, and the luciferase activity was measured. Results were expressed as NFAT activity relative to untreated controls (24).

Statistical analysis. The Student's t-test was used to determine the significance of differences of NFAT activity or COX-2 induction among various groups. Differences were considered significant at $p\leq0.05$.

Results

Ionizing radiation was synergistic with B[a]PDE on NFAT activation. Our previous studies have shown that NFAT activation is involved in cellular response to environmental

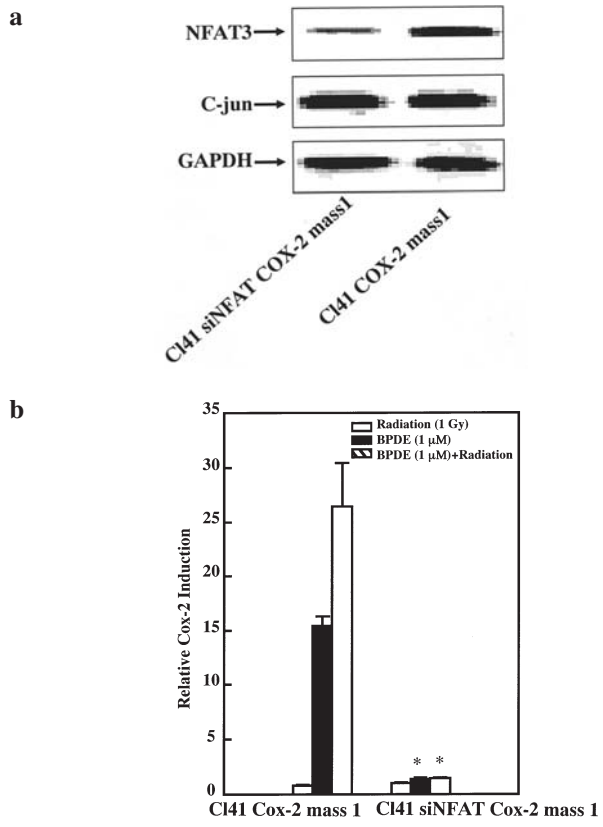

Figure 3. Inhibition of B[a]PDE or B[a]PDE plus ionizing radiation-induced COX-2 expression by knockdown of NFAT3 expression. (a) Cl 41 transfectant cells ($2x10^5$), including Cl 41 COX-2 mass1, and Cl 41 siNFAT COX-2 mass1 protein were extracted with SDS sample buffer. Western blot analysis was carried out as described in Materials and methods using antibodies specific against NFAT3, c-Jun, and GAPDH; (b) Cl 41 COX-2 mass1 or Cl 41 siNFAT COX-2 mass1 were seeded into each well of 96-well plates. After being cultured at 37˚C overnight, the cells were irradiated with a 1.0 Gy X-ray and immediately followed by 1 μM of B[a]PDE for 24 h. The luciferase activity was then measured, and the results were presented as relative COX-2 induction. Each bar indicates the mean and standard deviation of triplicate assay wells. *Indicates a significant decrease compared with that from Cl 41 COX-2 mass1 cells ($p<0.05$).

carcinogenic factors, such as UV radiation, hydrogen peroxide and vanadium exposure (24-26). To study the potential involvement of NFAT activation in cells exposed to ionizing radiation, a mouse epidermal Cl 41 NFAT-luciferase reporter transfectant, Cl 41 NFAT mass1 was used. A clinically relevant dose of 1.0 Gy ionizing radiation was applied (27), and 1 μM B(a)PDE was chosen as the culture concentration according to a study by Weng *et al* (28). The results showed that 1.0 Gy ionizing radiation alone did not affect NFAT activation in the Cl 41 cell line at any time point (Fig. 1 and data not shown), while B[a]PDE, an ultimate carcinogenic metabolite of B[a]P, induced marked activation of NFAT in Cl 41 cells (Fig. 1).

Since most human exposures occur as a result of multiple carcinogenic factors, we determined whether ionizing radiation had an effect on B[a]PDE-induced NFAT activation. The results indicated that 1.0 Gy of radiation had a synergistic effect on B[a]PDE-induced NFAT activation in Cl 41 cells (Fig. 1). These results indicated that ionizing radiation acted synergistically with B[a]PDE on NFAT activation, but did not induce NFAT activation when used alone in contrast to UV radiation, which does increase NFAT activation (24).

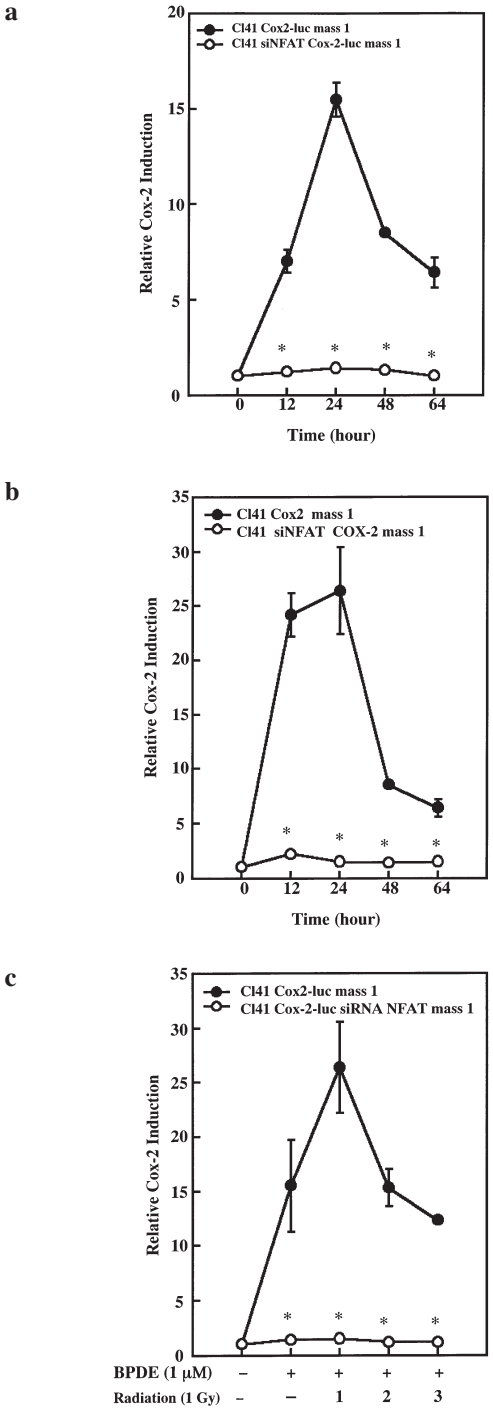

Figure 4. Time course and dose-response studies on effects of NFAT3 siRNA on B[a]PDE or B[a]PDE plus ionizing radiation-induced COX-2 expression. Cl 41 COX-2 mass1 or Cl 41 siNFAT COX-2 mass1 cells were seeded into each well of 96-well plates. After being cultured at 37˚C overnight, the cells were exposed to (a) 1 μM of B[a]PDE, (b) irradiation with a 1.0 Gy X-ray and immediately followed by 1 μM of B[a]PDE for the various time periods indicated, or (c) irradiation with a 1-3 Gy X-ray and immediately followed by 1 μM B[a]PDE for 24 h. The luciferase activity was then measured, and the results were presented as relative COX-2 induction. Each bar indicates the mean and standard deviation of triplicate assay wells. *Indicates a significant decrease compared with that from Cl 41 COX-2 mass1 cells ($p<0.05$).

Ionizing radiation synergistically increased COX-2 induction by B[a]PDE in Cl 41 cells. As shown in Fig. 2, and analogous to the NFAT activation, there was no effect by ionizing radiation on COX-2 induction, whereas 1 μM B[a]PDE

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n a 15-fold increase in COX-2 induction (Fig. 2a). radiation synergistically induced COX-2 in combination with B[a]PDE (Fig. 2a). The COX-2 synergism appeared to be time-dependent (Fig. 2b), and was found at 1-3 Gy radiation plus 1 μM B(a)PDE (Fig. 2c). Fig. 2d shows Western blotting results of Cl 41 cells after the same treatment. A dose of 1.0 Gy radiation did not increase COX-2 expression compared with control cells, and 1.0 Gy of radiation together with 1 μM B[a]PDE significantly increased COX-2 expression compared with 1 μM B[a]PDE only. These data indicate that ionizing radiation is able to increase COX-2 induction by B[a]PDE, even though it has no effect on COX-2 expression when used alone.

The NFAT3 is required for COX-2 induction by B[a]PDE or ionizing radiation plus B[a]PDE. The above results show that ionizing radiation and/or B[a]PDE have a similar effect on both NFAT and COX-2 induction, suggesting a possible association between transcription factor NFAT activation and COX-2 transcription. To determine whether NFAT plays a role in the regulation of COX-2 transcription, the NFAT3/pSuppressor siRNA expression vector and COX-2 luciferase reporter plasmids were co-transfected into Cl 41 cells. After transfection and selection with G418, Cl 41 siNFAT COX-2 mass1 and its vector control transfectant Cl 41 COX-2 mass1 were established. Western blot analysis showed that transfection of NFAT3/pSuppressor siRNA in Cl 41 siNFAT COX-2 mass1 resulted in a significant decrease in the expression of NFAT3 compared with Cl 41 COX-2 mass1 (Fig. 3a), while c-Jun, a major component of transcription factor AP-1, was not affected (data not shown), suggesting that the NFAT3 gene was specifically knocked down by NFAT3/pSuppressor siRNA. COX-2 induction by B[a]PDE or ionizing radiation plus B[a]PDE in Cl 41 siNFAT COX-2 mass1 was impaired in NFAT3 knockouts compared with Cl 41 COX-2 mass1 cells (Fig. 3b). These findings were further confirmed by the time course and dose-response studies (Fig. 4). These results demonstrate that NFAT3 is required for either COX-2 induction by B[a]PDE or synergistic COX-2 induction by ionizing radiation plus B[a]PDE.

Discussion

Exposure to combinations of carcinogens, including PAH-containing substances and ionizing radiation, occurs frequently in everyday life. The risk of cancer is often difficult to assign to a specific causality agent. There are previous reports that B[a]PDE up-regulated COX-2 expression through NF-κB in rat astrocytes (28). In the present study, the combined effects of B(a)PDE and X-rays on the induction of COX-2 were studied in the mouse JB6 cell line. The main finding is that X-ray exposure and B[a]PDE synergistically activated NFAT and induced COX-2 expression in mouse JB6 Cl 41 cells. A second finding is that NFAT-3 activation is required for COX-2 induction by B[a]PDE or ionizing radiation plus B[a]PDE. Since COX-2 is associated with tumor development, tumor growth, invasion and metastasis (15-21), we speculate that the COX-2 induction might be related to tumor promotion and progression induced by those carcinogenic factors.

NFAT is a transcription factor family that was first found in T lymphocytes (29), and consists of at least five isoforms (30). Three different early stages of NFAT activation have been defined: dephosphorylation, nuclear translocation and increase DNA binding affinity (31,32). NFAT activation is initiated by dephosphorylation, and increased intracellular calcium activates calcineurin (33). Activated calcineurin subsequently dephosphorylates the cytoplasmic NFAT regulatory domain, which leads to NFAT nuclear translocation and increases in affinity for DNA binding to its target gene promoter region, subsequently initiating its target gene transcription (33,34). Our previous studies have shown that exposing cells to carcinogenic factors, such as UV radiation and vanadium compounds, may lead to NFAT activation in a calcium-calcineurin-dependent manner (24,25). Since both B[a]PDE and ionizing radiation have been well-demonstrated to have strong carcinogenic effects on mouse skin (35), we determined whether both agents could induce NFAT activation in mouse epidermal Cl 41 cells. The results indicate that 1.0 Gy of radiation does not induce NFAT activation, while B[a]PDE induces marked NFAT activation in Cl 41 cells.

Although the exact mechanism by which B[a]PDE activates NFAT is unknown, participation of the calcium signaling pathway is anticipated. This notion was supported by the findings that PAHs increase cytosolic Ca^{2+} in lymphocytes, mammary epithelial cells (36) and human small airway epithelial (SAE) cells (37). For example, B[a]PDE has been reported to increase Ca^{2+} in SAE cells, and induce Ca^{2+} release from intracellular IP3-sensitive Ca^{2+} stores, such as endoplasmic reticulum (ER), via IP3R-gated Ca^{2+} channels (37).

NFAT activation is also reported to be regulated by several signaling pathways (29). For example, MAP kinases such as p38K and JNKs may promote NFAT1 and NFAT3 nuclear export, while JNK activation causes nuclear accumulation of $NFAT_4$ (38). Our previous studies demonstrate that B[a]PDE exposure results in marked activation of MAPKs, including ERKs, JNKs and p38K (22). The activation of these kinases might therefore be involved in the transactivation of NFAT3 in response to B[a]PDE. The synergistic activation of NFAT by ionizing radiation and B[a]PDE are currently under investigation in our laboratory.

NFAT has been reported to bind to the promoter sequences of cytokine genes or the early immune response gene, and up-regulate expression of these genes in T-cells during immune responses (39). Different isoforms of NFAT are distributed in various tissues and show different patterns of inducibility by various stimuli (39). COX-2 is induced in a variety of cell types by diverse stimuli including growth factors, mitogens, cytokines, and tumor promoters (40). The promoter regions of rat, mouse and human COX-2 have been isolated, sequenced, and shown to contain several consensus cis-acting regulatory sequences (41). These sequences were shown to be critical for COX-2 induction produced by various stimuli in different species and cell types (42). Two NFAT binding sites are present in the mouse COX-2 promoter region. Both distal (-105/-97) and proximal (-76/-61) NFAT response elements in the COX-2 promoter are essential for COX-2 induction in T lymphocytes (43).

It has been observed that prostaglandin E_2 generation and COX-2 protein expression were increased in a prostate

cancer cell line following radiation in the human androgen-dependent prostate cancer cell line, PC3 (44). However, the present study indicates that a 1-3 Gy X-ray alone does not induce COX-2 transcription, but shows synergistic effects on COX-2 transcription with B[a]PDE in mouse epidermal Cl 41 cells. The reason for the difference between the current and previous studies may be due to the cell-type specificity. Another reason why the X-ray alone did not induce COX-2 may be due to the radiation dose used here, which was low but clinically relevant.

COX-2, an important enzyme mediating the inflammatory process, is the target of many non-steroidal anti-inflammatory drugs (45). COX-2 is thought to contribute to tumor growth and metastasis (21). The association of up-regulated COX-2 expression with tumor development suggests that COX-2 metabolites play a role in the tumorigenesis process (20,21). More recently, COX-2 has been also associated with oncogenic transformation and angiogenesis (15,20), and studies using COX inhibitors support this notion (45). A COX-2 inhibitor has also been reported to increase the radiosensitivity of mice bearing a new fibrosarcoma (46). Strong evidence for a role of COX-2 and PGs in skin tumor promotion comes from initiation-promotion studies using COX-2 deficient mice, which showed a significant reduction in skin cancer development (47,48). PGE2 may play an important role in angiogenesis and invasion (15,18).

The results from this study demonstrate that B[a]PDE is able to affect COX-2 induction, and ionizing radiation has a synergistic effect on B[a]PDE-induced COX-2 transcription. This observation may have implications for the carcinogenic mechanisms of B[a]P/B[a]PDE and radiation exposure that commonly co-exist in environmental exposures. As a component of the COX-2 gene promoter, NFAT is essential for COX-2 induction in mouse JB-6 Cl 41 cells. Based on the data shown in this study, 1-3 Gy radiation did not increase COX-2 induction. More studies are needed to determine whether this finding applies to other cell lines. Overall, this study sheds light on the mechanisms involved in carcinogenic effects by B[a]PDE and/or ionizing radiation and also provides useful information on NFAT as a potential molecular target for cancer prevention and gene therapy.

Acknowledgements

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