Downregulation of Nuclear Respiratory Factor-1 Contributes to Mitochondrial Events Induced by Benzo(*a*)pyrene

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ABSTRACT: Environmental carcinogen benzo(*a*)pyrene (BaP) has been shown to be a genotoxicant that affects both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). Nuclear respiratory factor 1 (NRF-1) is a transcriptional activator of nuclear genes that encode a range of mitochondrial proteins including mitochondrial transcription factor A (mtTFA). However, the role of NRF-1 in BaP-induced mitochondrial event is not clear. We investigated the change of NRF-1 and mtTFA in human bronchial epithelial cells (16HBE) elicited by BaP. The results indicated that BaP induced cell apoptosis, total mitochondrial enzymes activities and ATP levels decrease in dose- and time-dependent manners, respectively. The transcription and protein levels of NRF-1 and mtTFA decreased at 48 h after 16 μM BaP treatment. Our results indicated downregulation of NRF-1 and mtTFA is involved in BaP-induced mitochondrial events. © 2012 Wiley Periodicals, Inc. Environ Toxicol 00: 000–000, 2012.

Keywords: benzo(*a*)pyrene; nuclear respiratory factor 1; mitochondrial transcription factor A; mitochondrial events

INTRODUCTION

Benzo(*a*)pyrene (BaP) is a well-recognized environmental carcinogen and it belongs to a polycyclic aromatic hydrocarbon (PAH) family of environmental carcinogens that are present in air, water, soil, incomplete combustion of tobacco during smoking and automobile exhaust. To date, the mutagenic and carcinogenic properties of BaP *in vitro* have been demonstrated in many cell types (Walle et al., 2006; Kim et al., 2007). Hepalcla7 hepatoma cells have been used to study the mechanism of BaP-induced apoptosis (Lei et al., 1998). Progressive cellular architectural changes due to oxidative stress, LPO, and modulation of various cellular molecular pathways by reactive free radicals generated during cytochrome P450 dependent metabolism of BaP has been implicated during the pathogenesis of lung carcinogensis (Das et al., 2007). Mitochondrion is now gaining importance in cancer research because of its central role as a regulator of energy balance and mitochondria appears to be the primary target for oxidative stress induced damage during cancers as it has been suggested to be the main sources of free radical production. Some study showed that altered mitochondria regulation of apoptosis and cell metabolism, especially mitochondrial DNA(mtDNA) mutation, were observed in several types of cancer cells including lung cancer cells (Jakupciak et al., 2008; Dasgupta et al., 2009). Mitochondrion and

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mtDNA play crucial roles in the onset and progression of cancer. However, mitochondrion regulation of cell metabolism and mtDNA activity in tumor cells are not yet fully understood.

Mitochondrial transcription factor A (mtTFA) is a key regulator of mtDNA transcription and replication and it is a nuclear-encoded protein of 246 amino acids (25 kDa) with a mitochondrial targeting presequence of 42 amino acids. Mitochondrial transcription factor A is a nuclear-encoded gene whose transcription is regulated by nuclear respiratory factors (NRF-1) (Scarpulla., 2006). NRF-1 is a 68-kDa protein containing an N-terminal nuclear localization signal and a C-terminal transcriptional activation domain that is ubiquitously expressed. NRF-1 plays a critical role in integrating nucleo-mitochondrial interactions by initiating transcription of nuclear-encoded mtDNA-specific transcription factors including mtTFA (Scarpulla, 2006). One study reported increased expression of 218 nuclear-encoded mitochondrial genes including a number of NRF-1 targets for example, mtTFA and COX5B, as associated with poor prognosis in breast cancer (Klinge, 2008). Although mitochondrial gene expression depends on nuclear genome function and reciprocally by ''retrograde communication'' mitochondrial activity regulates nuclear gene expression, the mechanisms coordinating these events remain to be clarified.

In this article, we reported that downregulation of NRF-1 and mtTFA expression was involved in mitochondrial event induced by BaP.

MATERIALS AND METHODS

Cell Culture and Chemicals

Human bronchial epithelial cells (16HBE) were kindly given to us by Prof. Zongcan Zhou (Department of Toxicology, Peking University Health Science Center, Beijing, China). Cells were maintained in Dulbecco's modification of Eagle's medium (DMEM, Gibco, US), supplemented with 10% fetal calf serum (Gibco, US), 100 U/mL penicillin and 100 g/mL streptomycin and incubated in a humidified atmosphere with 5% CO_2 at 37°C. BaP and other chemicals used in this study were from Sigma unless specified. Primary antibodies of goat anti-NRF-1, anti-mtTFA, and rabbit anti-β-actin were purchased from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA).

BaP Treatment

16HBE cells were treated with BaP plus rat Liver S9 mixture (Sigma) for 4 h. Then, the culture media containing BaP were discarded and complete DMEM was added. After washed with PBS for three times, the cells were cultured in the complete DMEM for 24, 48, or 72 h.

Cytotoxicity Assay

The neutral red (NR) assay was performed according to the recommended method (ICCVAM, 2001). Individual well of a tissue culture 96-well plate was inoculated with 0.2 mL medium containing 2×10^4 cells, and incubated for 24 h. Thereafter, growth medium was replaced with the exposure medium, consisting of 5% FBS-medium with different concentrations of BaP (with S9 mixture). Eight replicated wells were used per concentration. After a 24, 48, or 72 h exposure, cytotoxicity was assessed by NR assay, which was based on the uptaking and lysosomal accumulation of supravital dye. All experiments were performed at least three times.

Mitotic Index

The cells were seeded in 25 cm^2 dishes, 24 h later 16 μM (with S9) for 4 h, then, the culture medium containing BaP was discarded. The cells were incubated in the complete DMEM for 24, 48, or 72 h. At the end of the incubation periods, the cells were harvested by centrifugation and treated with 0.075 M KCl at 37°C for 15 min, then centrifuged and fixed in 1:3 (v/v) acetic acid:methanol. Mitotic indexes were determined from triplicate slides stained with Giemsa; 1000 cells/slide were counted. The total mitoses per culture were calculated from the mitotic index and cell survival data.

Flow Cytometry

Cells (5×10^5) were seeded in 25 cm^2 culture flask. At 24 h after exposure to different concentrations of BaP (with S9 mixture) (0, 4, 8, 16, 32, or 64 μM), 16HBE cells were washed with PBS and were harvested by centrifugation at $800 \times g$ for 5 min. The cell pellet was fixed in 70% (v/v) ethanol and stored at 4°C overnight. The cells were then pelleted and resuspended in 1 mL of PBS containing 20 μg/mL RNase A. After 30 min at 37°C, the cells were stained with propidium iodide (20 μg/mL in PBS) and analyzed on a flow cytometry (Coulter Epics, USA). A total of 10 000 cells were counted for each sample. The percentage of apoptosis was quantified from sub-G_1 events.

Total Mitochondrial Enzymes Activities

Cells were treated with different concentrations of BaP (with S9) for 24, 48, or 72 h in 6-well plate and add 200 μL MTT to each well at 5 mg/mL, and then the plate were incubated at 37°C for 4 h, finally, the formazan crystal products were dissolved in acid isopropyl alcohol and stirred for 15 min, and 100 μL supernatant were added into 96-well plate for four repeated wells, optical density (OD) was read on Microplate Reader at 570 nm. The results were expressed as inhibition ratio of mitochondrial enzyme

activities. The inhibition ratio of mitochondrial enzyme activities were calculated by ([OD]control − [OD] test)/[OD]control.

RT-PCR

Total RNA was extracted from 16HBE cells using a Nuclospin® RNA II kit (MN, Germany) after treatment with 16 μM BaP (with S9) for 24 or 48 h. cDNA was synthesized by a RevertAid™ Frist Strand cDNA Synthesis Kit (Fermentas International, Canada).

The primer sequences of NRF-1 are as follows:

- upstream primer, 5′-CCAGTTTAGTGGGTGGTAGG-3′;
- downstream primer, 5′-CGGGAGCTTTCAAGACAT TC-3′.

The primer sequences of mtTFA are as follows:

- upstream primer, 5′-GTCACTGCCTCATCCACC-3′;
- downstream primer, 5′-CCGCCCTATAAGCATCTT-3′.

The primer sequences GAPDH are as follows:

- upstream primer, 5′-TGC (A/C)TCCTGCACCACCAA CT-3′
- downstream primer, 5′-(C/T)GCCTGCTTCACCACCT TC-3′

Levels of NRF-1 and mtTFA were compared with those of the GAPDH respectively. The fluorescent intensity of each band was measured with Quantity One (Bio-Rad), and the expression of mRNA was determined relative to that obtained for GAPDH.

Western Blot Analysis

16HBE cells were treated with 16 μM BaP (with S9 mixture) for 24 or 48 h respectively and then lysed in cell lysis buffer for 30 min on ice. Soluble protein was recovered after centrifugation at 4°C for 30 min at 12 000 × *g*. Samples were assayed for protein concentration using the Bradford method. Equal amounts of sample were separated on 12% SDS-PAGE gels and then transferred to PVDF membrane. Membranes were blocked for 1 h with 5% nonfat milk in Tris buffered saline containing 0.05% Tween-20 (TBST) at room temperature and incubated with primary antibodies (NRF-1 and mtTFA, Santa Cruz) overnight at 4°C. After rinsed with TBST for 30 min, the membranes were incubated with peroxidase-labeled secondary antibody for 1 h at room temperature. Immunoreactive bands were visualized using ECL reagents. The images were analyzed by Quantity One software (Bio-Rad, Hercules, CA).

ATP Measurement

ATP level was measured using a Cell-Titer-Glo® Luminescent Cell Viability Assay (Promega, USA). Briefly, equal numbers of cells that had been treated with 0, 4, 8, 16, 32, or 64 μM BaP (with S9 mixture) or DMSO (control) for 24, 48, or 72 h were trypsinized and incubated in 96-well plate in eight replicates for 1 h. 100 μL of the reaction reagent was added to each well, after 10 min the luminescence signal was detected using an ELISA reader at 562 nm.

Statistical Analysis

All statistical analysis was performed using SPSS 14.0 (SPSS, Chicago, IL). Results are expressed as mean ± S.D. Experimental data were analyzed with one-way analysis of variance (ANOVA) followed by Tukey's multiple range test for significant differences.

RESULTS

Cytotoxicity

Cytotoxicity of a 24-, 48-, or 72-h exposure to BaP was evaluated in 16HBE cells using the NR assay. 16HBE cells were treated with increasing concentrations of BaP (with S9) for 4 h, and cells were harvested at the various time points for analyses of cytotoxicity. As shown in Figure 1(A), slight inhibition effects were observed in proliferation among cells exposed to 16 μM BaP for 72 h. Cell proliferation was markedly inhibited for cells treated with 32 or 64 μM BaP for more than 24 h. The data of mitotic index indicated that 16 μM BaP inhibited cell proliferation at both 48 and 72 h [Fig. 1(B)]. These data indicate that BaP inhibited 16HBE cells proliferation in a dose- and time-dependent behavior.

Induction of Apoptosis

The time course of apoptosis in continuous treatment with various concentrations of BaP (0, 4, 8, 16, 32, or 64 μM) was exhibited in Figure 2. Only 64 μM BaP was observed inducing apoptosis in 16HBE cells after treatment with BaP for 24 h; however, cell apoptosis increased with the prolong of the treatment time and cell apoptosis was observed even if at 72 h after 16 μM BaP treatment. From Figure 2, we could see that 6.60% ± 1.19%, 9.92% ± 0.63%, and 14.26% ± 0.83% cell apoptosis were detected at 24, 48, or 72 h after 64 μM BaP treatment, respectively, suggesting that BaP induced apoptosis in dose- and time-dependent manners.

Mitochondrial Enzymes Activities

The amount of formazan reaction product formed in the MTT assay reflects the total mitochondrial enzymatic

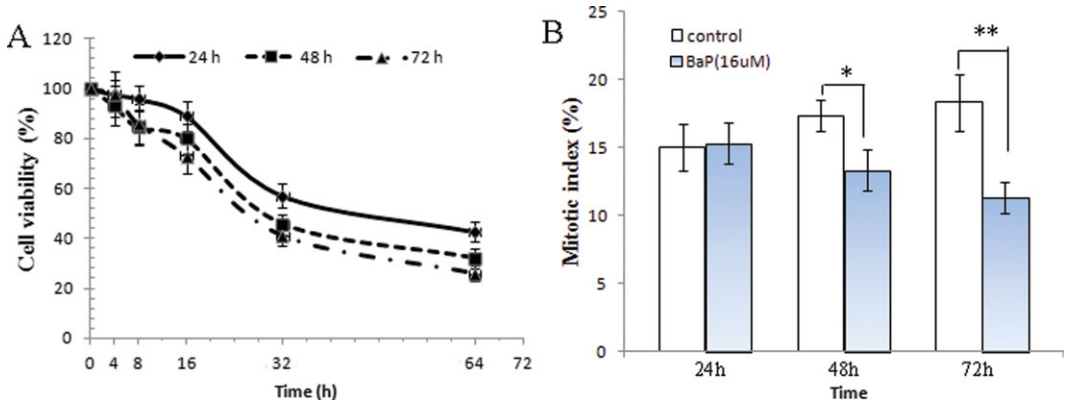

Fig. 1. Effect of BaP on cytotoxicity in 16HBE cells. (A) Cytotoxicity was estimated by NR assay. Results are expressed as a mean ± S.D. of three independent experiments. (B) Cells were treated with 16 μM of BaP for 24, 48, or 72 h. At the end of incubation period, mitotic index was measured. Results are expressed as a mean ± S.D. of three independent experiments. *$P < 0.05$, **$P < 0.01$ versus control group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

activity in each well (Jang et al., 2012). 16HBE cells were treated with or without BaP for 24 h before the MTT assay. Exposure to BaP decreased the MTT absorbance.

After treatment for 24 h, all selected concentrations of BaP could decrease total mitochondrial enzymes activities and inhibition ratio of mitochondrial enzyme showed a dose-dependent relationship with BaP concentrations [Fig. 3(A)]. 16 μM BaP treated 16HBE cells for 24, 48, or 72 h, time-dependent total mitochondrial enzymes activities decreasing was observed [Fig. 3(B)].

NRF-1 and mtTFA Transcription and Protein Levels

NRF-1 is a nuclear transcription factor that regulates of mtDNA transcription factors including mtTFA. The increased expression of mtTFA subsequently entered mitochondria to increase the expression of mtDNA-encoded genes, mitochondrial biogenesis, and oxidative phosphorylation (Mattingly et al., 2008). RT-PCR was performed to know the change of NRF-1 and mtTFA mRNA levels at 24 or 48 h after BaP treatment. At 24 or 48 h, NRF-1 mRNA expression in extracts from BaP treated cells were decreased significantly compared to the control (DMSO-treated cells) ($P < 0.01$) [Fig. 4(A,B)]. While at 24 h mtTFA mRNA expression in extracts from BaP treated cells were not changed compared to the control ($P > 0.05$) [Fig. 4(A,B)], and only at 48 h, mtTFA mRNA expression were detected decreasing significantly compared to the control ($P < 0.01$). The results suggest that transcription of NRF-1 and mtTFA are affected by BaP; moreover, mtTFA mRNA expression decreasing lagged behind NRF-1 mRNA expression decreasing.

To verify whether protein levels of NRF-1 and mtTFA were affected by BaP. We investigated the change of NRF-1and mtTFA protein expression at different time after 16 μM BaP treatment. As shown in Figure 4(A), at 24 h after BaP treatment, the NRF-1protein level was obviously decreased, whereas the mtTFA protein level did not show any change. At 48 h, the obviously decreased mtTFA protein level was also detected [Fig. 4(B)].

One possible mechanism by which insulin-simulated glucose uptake might be impaired in mtTFA knockdown cells would be through decreased ATP levels, which would in these cells lead to impairment in insulin signal transduction (Hresko et al., 1998). Next we further investigated the change of ATP level followed by mtTFA downregulation caused by BaP.

ATP Level

Steady-state ATP levels in mtTFA knockdown cells were indeed decreased was also confirmed by Xiarong and her

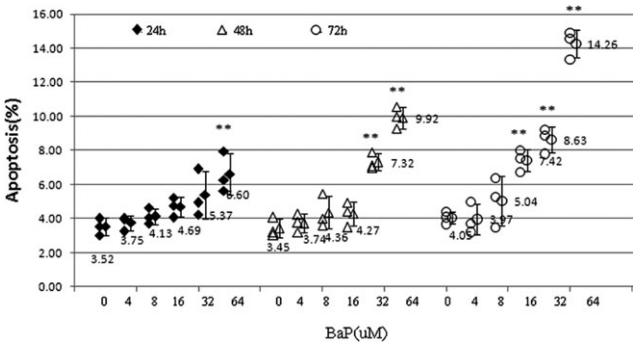

Fig. 2. Effect of BaP on apoptosis in 16HBE cells. Apoptosis results are expressed as a percentage of sub-G1 population obtained from the cells of flow cytometry data of propidium iodide fluorescence. Each value represents a means ± S.D ($n = 6$). *$P < 0.05$, **$P < 0.01$ versus control group.

A

Cell lines	Concentrations (uM)	A570nm (x±s)	Inhibition ratio of mitochondrial enzyme(%)
16HBE	0	0.57±0.02	0
	4	0.51±0.01*	12.80±1.73
	8	0.43±0.01**	22.20±2.45
	16	0.37±0.01**	34.13±1.81
	32	0.32±0.02**	40.90±2.00
	64	0.30±0.01**	44.40±2.18

B

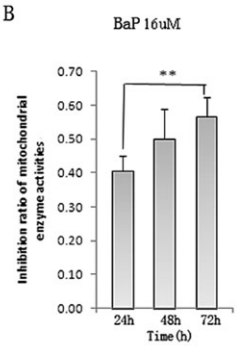

Fig. 3. Effect of BaP on total mitochondrial enzymes activities. Total mitochondrial enzymes activities were detected by MTT assay on Microplate Reader at 570 nm. The results were expressed as inhibition ratio of mitochondrial enzyme activities. The inhibition ratio of mitochondrial enzyme activities (%) was calculated by ([OD]control − [OD] test)/[OD]control. *$P < 0.05$, **$P < 0.01$ versus control group.

colleagues (Shi et al., 2008). To know whether BaP-induced NRF-1 decrease and subsequently mtTFA downregulation can decrease ATP levels or not, 16HBE cells were treated different concentrations of BaP or with DMSO (as control) for 24, 48, or 72 h, then ATP levels were detected using Cell-Titer-Glo® Luminescent Cell Viability Assay. The results showed that at 24 or 48 h after 16 μM BaP treatment, ATP did not change compared to the control ($P > 0.05$, Fig. 5), suggesting that ATP level was unaffected by BaP when mtTFA transcription or protein levels were decreased. ATP might be postregulated by mtTFA; moreover, there might be other ATP regulation pathways in BaP-induced mitochondrial event.

DISSUSSION

BaP is a known genotoxicant that affects both nDNA and mtDNA. BaP is metabolized by cytochrome P-450-mediated oxidation to produce anti-benzo[a]pyrene-7,8-diol-9,10-epoxide (anti-BPDE), and the formation of anti-BPDE-DNA adducts is considered to be critical in the carcinogenic process of BaP (Melikian et al., 1989; Barhoumi et al., 2009). The damage of nDNA can cause NRF-1 expression changed, NRF-1 regulates one of its target genes, mtTFA, and the change of mtTFA then induces a series of mitochondrial events occurring (Chou et al., 2007). BaP is a widely spread environmental pollutant. Exposure to BaP is not only related DNA damage but also cytotoxicity and mitochondrial dysfunction (Yang et al., 2011). Understanding the mechanism of BaP-induced mitochondrial dysfunction and the associated proteins would pave the way to a further understanding the anticancer mechanism and cancer therapy. Through PI staining, we detected the apoptotic ratio of 16HBE cells following BaP treating for 24, 48, or 72 h by flow cytometry. There was a significantly increase of apoptotic cells at the concentration of 16 μM BaP at 72 h. According to the scatterplot, the nonviable apoptotic cells increased in a dose-dependent manner which implied that BaP (16–64 μM) induced apoptosis in 16HBE cells.

In the earlier period BaP promoted the generation of excessive ROS and subsequently the mitochondrial depolarization (Yang et al., 2011). Hence, to understand BaP-induced mitochondrial events, total mitochondrial enzymes activities and ATP was detected in further experiments. A time- and dose-dependent total mitochondrial enzymes activities decreasing was observed. We investigated the change of ATP level at different time after various concentrations of BaP treatment. The result indicated there were no changes of ATP levels at 24 and 48 h after 16 μM BaP treatment, ATP level decrease was observed at 72 h after 16 μM BaP treatment, suggesting that mtTFA expression downregulation might contribute to ATP level decrease. This is similar to interference with mtTFA resulted in cells with decreased respiratory chain capacity, reflected by decreased basal oxygen consumption, and decreased mitochondrial ATP synthesis (Clayton, 2000; Lanza and Nair, 2009; Tsutsui et al., 2009). Here we also detected a time- and dose-dependent manner ATP level induced by ATP. However, other ATP regulation pathways could not be excluded in our assay. Previous studies have shown that BaP would elicit mitochondrial dysfunction in Hep3B cells, mouse hepatoma Hepa1c1c7 cells, and human fetal lung fibroblasts (Ko et al., 2004; Jiang et al., 2011; Yang et al., 2011), which further supports our observation and suggests that BaP-induced mitochondrial events exists also in normal human bronchial epithelial cells.

Quantities of studies have demonstrated the alteration cellular mitochondrial DNA (mtDNA) and mitochondrial dysfunction might play important roles in malignant tumor developing (Bellance et al., 2009). Mitochondrial impairment is not observed systematically in every cancer cell, as is high lactate production, suggesting a multifactorial origin of cancer cells' metabolic reprogramming. NRF-1 is a

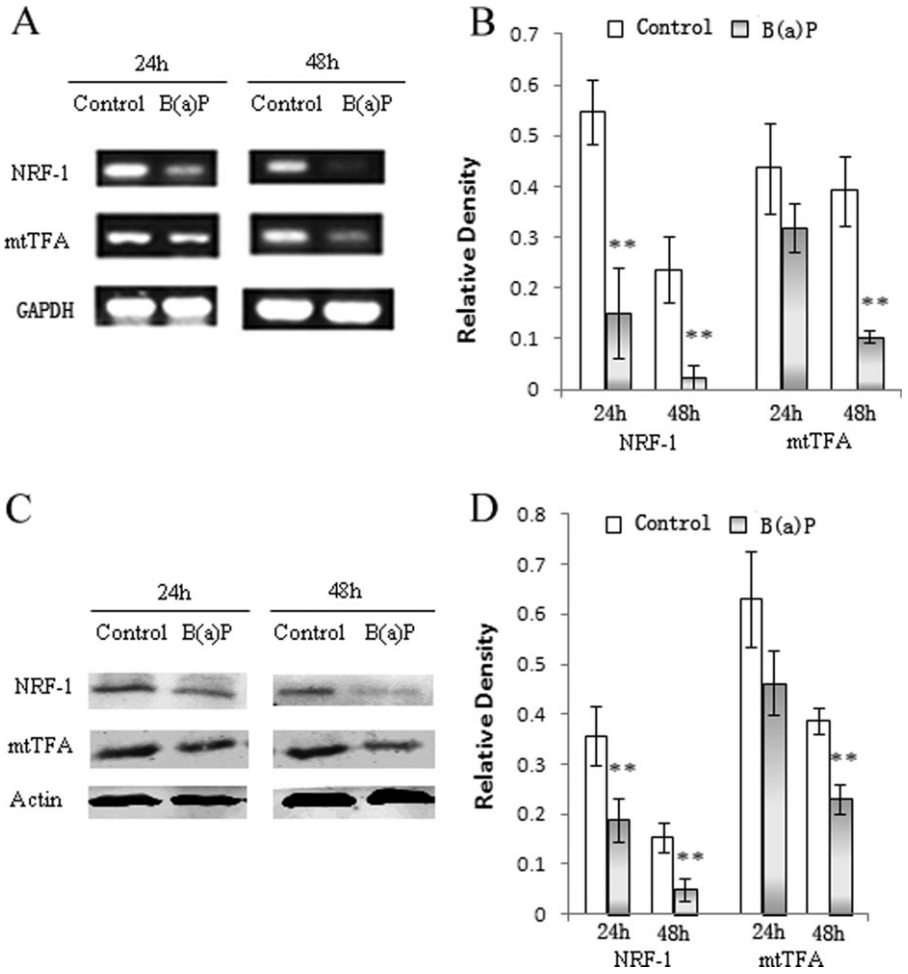

Fig. 4. Effect of BaP on NRF-1 and mtTFA level. (A, B) RT-PCR was performed to know the change of NRF-1 and mtTFA mRNA levels. The fluorescent intensity of each band was measured with Quantity One, and the transcription levels of NRF-1 and mtTFA mRNA relative to that obtained for GAPDH. The *y*-axis is meant to represent the levels of NRF-1 and mtTFA mRNA relative to those present in control cells. (C, D) Western blot analysis was performed to know the change of NRF-1 and mtTFA protein levels. The fluorescent intensity of each band was measured with Quantity One, and the protein levels of NRF-1 and mtTFA mRNA relative to that obtained for β-actin. The *y*-axis is meant to represent the levels of NRF-1 and mtTFA protein relative to those present in control cells. The result is representative of three independent experiments with similar result. $^{**}P < 0.01$ versus control group.

68-kDa protein containing an N-terminal nuclear localization signal and C-terminal transcriptional activation domain that is ubiquitously expressed (Gugneja et al., 1996). NRF-1 plays a critical role in integrating nuclear–mitochondrial interactions by initiating transcription of nuclear-encoded mtDNA-specific transcription factors including mtTFA, TFB1M, and TFB2M (Scarpulla, 2006). Regulation of mitochondrial biogenesis involves the coordinated actions of both mtDNA and nuclear-encoded gene products including NRF-1, NRF-2, mtTFA, and PGC-1α (Kelly and Scarpulla, 2004). NRF-1 stimulates transcription of the mtTFA gene by binding to an NRF-1 response element in the promoter (Scarpulla, 2006). Subsequently, mtTFA increases the transcription of mtDNA-encoded gene target. mtTFA is essential for mtDNA replication (Larsson et al., 1998). Chemical-induced NRF-1 expression results expression of mtTFA, which subsequently entered mitochondrial to increase the expression of mtDNA-encoded genes, mitochondrial biogenesis, and oxidative phosphorylation (Mattingly et al., 2008). Downregulation of mtTFA decreased the expression of mtDNA-encoded genes, mitochondrial biogenesis, and oxidative phosphorylation (Kang et al., 2007). To verify whether transcriptional or post-transcriptional regulation of NRF-1 was affected in BaP-induced mitochondrial event. We investigated the change of NRF-1 mRNA and protein at different time after BaP treatment

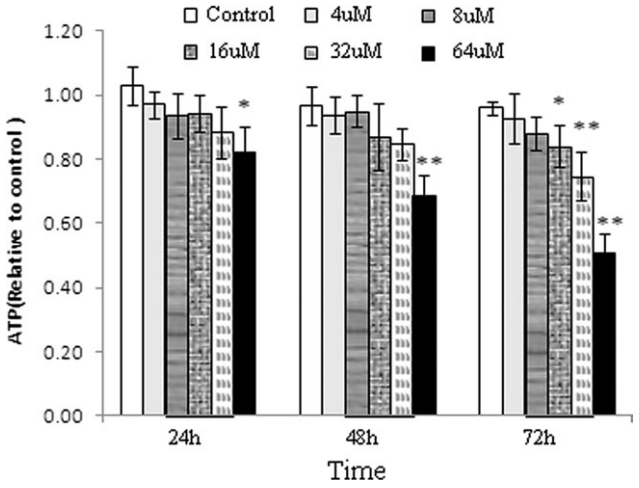

Fig. 5. Effect of BaP on ATP level. ATP levels were detected using Cell-Titer-Glo® Luminescent Cell Viability Assay. The results were expressed as ratio of ATP to control. The *y*-axis is meant to represent the levels of ATP treated with BaP relative to those present in control cells. The result is representative of three independent experiments with similar result. $^{*}P < 0.05$,$^{**}P < 0.01$ versus control group.

with RT-PCR and Western blot analysis, respectively. The results indicated downregulation of mRNA and protein of NRF-1 at 24 and 48 h after BaP treatment, suggesting that transcription and protein of NRF-1 were affected by BaP-induced mitochondrial event. NRF-1 subsequently promotes transcription of mitochondrial transcription factor mtTFA and then mtTFA targets mtDNA-encoded genes (Klinge, 2008). We investigated mtTFA transcription and protein levels induced by BaP. The results showed that there were no changes of transcription and protein levels of mtTFA at 24 h after 16 μM BaP treatment, the significantly downregulation of transcription and protein levels of mtTFA were detected at 48 h after 16 μM BaP treatment, suggesting that mtTFA expression decreasing lagged behind downregulation of NRF-1. Moreover, transcriptional levels of NRF-1 and mtTFA decreased about 90% and 74% respectively, protein levels of NRF-1 and mtTFA decreased about 78% and 40% respectively, suggesting mtTFA gene level decrease might be regulated by NRF-1 downregulation. This is similar to NRF-1 subsequently promotes transcription of mtTFA induced by estrogens-induced mitochondrial biogenesis (Klinge, 2008).

In all, a series of mitochondrial events were observed after BaP treatment, also NRF-1 and one of its target proteins, mtTFA were detected downregulation during these mitochondrial events.

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