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TERT regulates telomere-related senescence and apoptosis through DNA damage response in male germ cells exposed to BPDE *in vitro* and to B[*a*]P *in vivo*☆

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ABSTRACT

Increasing evidence shows that impaired telomere function is associated with male infertility, and various environmental factors are believed to play a pivotal role in telomerase deficiency and telomere shortening. Benzo[*a*]pyrene (B[*a*]P), a ubiquitous pollutant of polycyclic aromatic hydrocarbons (PAHs), can act as a reproductive toxicant; however, the adverse effect of B[*a*]P on telomeres in male reproductive cells has never been studied, and the related mechanisms remain unclear. In this study, we explored the effects of benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE), the active metabolite of B[*a*]P, on telomere dysfunction in mouse spermatocyte-derived cells (GC-2) and also the potential role of telomerase in BPDE-induced spermatogenic cell damage. The results showed that BPDE induced cell viability inhibition, senescence, and apoptosis in GC-2 cells in a dose-dependent manner. Shortened telomeres, telomere-associated DNA damage, reduced telomerase activity, and TERT expression were also observed in BPDE-treated cells, accompanied with the activation of DNA damage response pathway (ATM/Chk1/p53/p21). Moreover, by establishing the TERT knockdown and re-expression cell models, we found that TERT regulated telomere length and the expression of DNA damage response-related proteins to influence senescence and apoptosis in GC-2 cells. These *in vitro* findings were further confirmed *in vivo* in the testicular cells of rats orally administrated with B[*a*]P for 7 days. B[*a*]P treatment resulted in histological lesions, apoptosis, and senescence in the testes of rats, which were accompanied by shortened telomeres, reduced levels of TERT protein, and increased expression of DNA damage response-related proteins. In conclusion, it can be concluded that TERT-mediated telomere dysfunction contributes to B[*a*]P- and BPDE-induced senescence and apoptosis through DNA damage response in male reproductive cells.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are well-documented mutagens and carcinogens. They are generated during the incomplete combustion of organic materials and distributed widely not only in the certain occupational settings but also in the general environment. Thus, PAHs can inevitably enter the human body through inhalation, ingestion, and dermal contact absorption, and their contamination has raised a lot of public health concerns. In addition to the mutagenic and carcinogenic activities, exposure to PAHs has been shown to be associated with reproductive toxicity. Specifically, epidemiological researches suggest that exposure to PAHs correlates with reduced semen quality and a higher risk of infertility (Han et al., 2011; Hsu et al., 2006; Xia et al., 2009). Therefore, understanding the mechanism underlying the toxic effects of PAHs on male reproduction will provide insights into developing strategies to avoid male infertility.

Benzo[*a*]pyrene (B[*a*]P), the most extensively studied high-molecular-weight PAHs, is metabolized by cytochrome P450-mediated metabolism to form active metabolites including the carcinogenic metabolite benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) after entering the biological system. Previous studies in animal models have clearly demonstrated that direct

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exposure to B[*a*]P has a toxic effect on reproduction. Specifically, B[*a*]P exposure was reported to be related to the decreased spermatozoa quality (Mohamed el et al., 2010), increased BPDE-DNA adduct formation, and apoptosis in sperm cells (Revel et al., 2001). Moreover, BPDE could covalently bind to DNA to activate the DNA damage checkpoint and/or to induce cell death including apoptosis (Barkley and Santocanale, 2013; Said et al., 1995; Xiao et al., 2007). Cell death is the key response of cells exposed to B[*a*]P (Jiang et al., 2011), and the induction of DNA damage has recently been implicated in B[*a*]P-induced cell death (Holme et al., 2007; Lin et al., 2008b). In general, DNA damage caused by environmental or endogenous genotoxic agents represents a serious survival challenge for cells. In male germ cells, however, the cellular events for B[*a*]P-induced reproductive toxicity are largely unknown. More specifically, the underlying molecular mechanisms that DNA damage mediates cell death triggered by B[*a*]P remain to be elucidated.

BPDE is well documented to directly react with DNA to form DNA adducts, which ultimately results in DNA damage. Moreover, telomeric DNA is a rich source of guanine bases, and this structural characteristic indicates that telomeres are more susceptible to internal/external factors. Telomeres are nucleoprotein complexes that consist of noncoding TTAGGG double-stranded repeats and bound to specialized telomeric-interacting proteins (Lu et al., 2013). The function of telomeres is mainly to distinguish chromosomal ends from DNA breaks, consequently protecting chromosomes from fusions and degradation and maintaining the genomic integrity (Ju and Lenhard Rudolph, 2008). Telomere loss occurs during each round of cell division, which prevents cell proliferation infinitely by inducing differentiation, replicative senescence, or apoptosis (Allsopp et al., 1992). To counteract telomere loss, telomerase is required. Telomerase is a ribonucleoprotein that includes the telomerase reverse transcriptase (TERT) and the telomerase RNA (TERC). Telomerase inhibition by targeting TERT has been shown to induce the gradual dysfunction and shortening of telomeres, DNA damage, and telomere fusion, while increasing apoptosis (Sahin et al., 2011; Zhang et al., 1999). The ectopic expression of TERT activates telomerase activity and promotes telomere elongation and cell proliferation, thereby implicating TERT as rate-limiting factor for telomerase activity that plays a key role in telomerase function (Counter et al., 1998). Recent work indicated that dysfunctional telomeres could be recognized as DNA damage to initiate a DNA damage response (DDR) that leads to the activation of ataxia-telangiectasia mutated (ATM); ataxia telangiectasia-, Rad3-related (ATR), and the downstream kinases Chk1 and Chk2; and phosphorylated p53. Phosphorylated p53 regulates diverse cellular processes such as cellular senescence and/or apoptosis in a transactivation-dependent or transactivation-independent manner (Deng et al., 2008). Studies have revealed that DNA damage could induce cellular senescence through p53/p21-dependent pathway (Ninomiya et al., 2014). However, the potential roles of TERT in governing telomere dysfunction and senescence or apoptosis are largely unknown in the setting of BPDE-induced germ cell damage.

Our previous epidemiological investigation has found that urinary levels of PAH metabolites were associated with decreased human sperm telomere length, and *in vivo* results obtained in a rat model also demonstrated the adverse effects of B[*a*]P in sperm telomere length and telomerase expression of male germ cells (Ling et al., 2016). These results suggest that PAH compounds may affect the telomere maintenance in spermatogenesis. Given these possible connections between telomere dysfunction, DDR, and cellular senescence or apoptosis, we herein evaluate whether telomere dysfunction mediates spermatogenic cell injury through the activation of DDR in an *in vitro* model of BPDE-exposed GC-2 cells and in an *in vivo* model of B[*a*]P-exposed rats. Specifically, TERT is a major determinant of telomerase activity, which has supplied a tool to directly investigate the role of telomerase activity. Thus, we used the TERT knockdown and re-expression cell models to explore the potential role of telomerase in BPDE-induced male reproductive toxicity.

2. Materials and methods

2.1. Chemicals and antibodies

BPDE was purchased from Midwest Research Institute (Kansas City, MO, USA). B[*a*]P and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Commercially available antibodies against TERT (ab183015), TRF1 (ab1423), TRF2 (ab13579), RAP1 (ab175329), ATM (ab78), Chk1 (ab47574), p-ATM (ab36810), p-Chk1 (ab47318), and γ-H2AX (ab2893) were obtained from Abcam (Cambridge, MA, USA). The p53 (#2524), p-p53 (#12571), and β-Gal (#2372) antibodies were purchased from Cell Signaling Technology (Boston, MA, USA). The p21 antibody (sc-397) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The β-actin antibody was from Beyotime Institute of Biotechnology (Shanghai, China).

2.2. Cell culture and treatment

The mouse spermatocyte-derived GC-2 spd (ts) cells (GC-2 cells) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in DMEM medium (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (Gibco) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were treated with BPDE (50, 100, and 200 nM) dissolved in DMSO. Control cells received DMSO (0.05% final concentration) alone. All cell culture experiments were replicated at least three times.

2.3. Construction of TERT knockdown and re-expression cells

GC-2 cells with stable knockdown of TERT were established with short hairpin RNA (shRNA) targeting TERT gene, subsequently infected with lentiviral vectors for TERT re-expression. Briefly, GC-2 cells were separately transfected with the pSGU6-GFP vector carrying TERT-shRNA or the negative control shRNA using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). After 48 h of incubation, the cells were harvested and protein was isolated to analyze TERT knockdown efficiency by Western blotting. shRNA was synthesized by Sangon Biotechnology Company (Shanghai, China). The shRNA sequences were 5′-GGAAGAACCTCACATTCTTTC-3′ (TERT) and 5′-GTTCTCCGAACGTGTCACGT-3′ (NC, negative control). Stably transfected cells were selected by neomycin (G418) (Calbiochem, San Diego, CA, USA) at a concentration of 0.4 mg/ml for 14 days, and then positive clones were mixed for subsequent experiments (Liu et al., 2014b).

The lentiviral vector pLV-EGFP-TERT and the control vector pLV-EGFP were constructed by Saiye Biotechnology Company (Guangzhou, China). For the transient lentivirus expression experiment, stable TERT knockdown cells were seeded and cultured in medium overnight at 37 °C. Cells were transfected with pLV-EGFP-TERT or negative control vector at a multiplicity of infection (MOI) of 10. Fluorescence microscopy was used to detect the expression of GFP, and the re-expression efficiency was evaluated by Western blotting. The stable TERT knockdown cells and cells transfected with the TERT re-expression vector were treated with 200 nM BPDE for an additional 72 h and then harvested for use in the subsequent experiments.