Science of the Total Environment xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Benzo[*a*]pyrene impedes self-renewal and differentiation of mesenchymal stem cells and influences fracture healing

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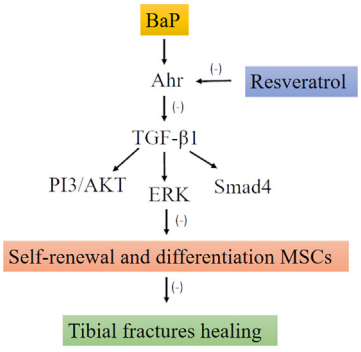
HIGHLIGHTS

- The toxicity of BaP to mesenchymal stem cells was firstly investigated.
- Ahr plays a key role in BaP's negative effect by inhibiting TGF-β1/SMAD4 and TGF-β1/ERK/AKT signaling pathways.
- Resveratrol rescues BaP's negative effect in vivo and in vitro studies.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 5 December 2016
Received in revised form 17 February 2017
Accepted 18 February 2017
Available online xxxx

Editor: Henner Hollert

Keywords:
Benzo[*a*]pyrene
Mesenchymal stem cells
Fracture healing
Aryl hydrocarbon receptor
Resveratrol

ABSTRACT

Mesenchymal stem cells (MSCs) are implicated in the bone-forming process during fracture repair. Benzo[*a*]pyrene (BaP)—a cigarette smoke component and powerful motivator of the aryl hydrocarbon receptor (Ahr)—unfavorably influences bone condition and osteoblast differentiation. The first thing we noticed decreases self-renewal and differentiation of human bone marrow mesenchymal stem (hBM-MSCs) from smokers and activates Ahr signaling in MSCs by up-regulating the Ahr target gene cytochrome P450 (CYP) 1B1 expression. In vitro studies, we employed C3H10T1/2 and bone marrow mesenchymal stem cells (BM-MSCs) with BaP and discovered that BaP impaired innate properties of MSCs. Further investigation into MSCs showed that exposure to BaP activated Ahr signaling and inhibited TGF-β1/SMAD4 and TGF-β1/ERK/AKT signaling pathways. Corresponding with the outcomes, tibial fracture calluses produced by BaP-administered rats appeared to delay healing. This effect of BaP was abrogated by resveratrol, a natural Ahr antagonist, in vitro and in vivo. These data demonstrated that Ahr may play a key role in BaP–impaired innate properties by inhibiting SMAD-dependent signaling pathways TGF-β1/SMAD4 and SMAD-independent TGF-β1/ERK/AKT signaling pathways. Furthermore, resveratrol inhibited MSCs from adverse effects caused by BaP.

1. Introduction

Smoking is the main source of avoidable morbidity and death across the globe in the 21st century (Wipfli and Samet, 2009). The World Health Organization approximates 6 million patients perish annually due to smoke-associated conditions. Several meta-analyses have discovered that there is up to a 40% growth in the total risk of hip fracture and 85% inflation in the chance of hips breaking in people who smoke (Kanis et al., 2005; Ward and Klesges, 2001). Cigarettes contain >1000 chemicals, of which at least 150 are established toxins. Cigarette smoke causes a wide range of pathologies including carcinogenesis, immune dysfunction, and developmental/reproductive abnormalities.

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http://dx.doi.org/10.1016/j.scitotenv.2017.02.152
0048-9697/

Most of these toxic effects are mediated by Ahr (aryl hydrocarbon receptor), a ligand-activated transcription factor. Constitutive activation of Ahr via genetic manipulation causes development of cancers, inflammation and immune abnormality in mice. Recent investigation disclosed that cigarette smoke contains high levels of agonists for Ahr and strongly activates Ahr-mediated signaling pathway (Kitamura and Kasai, 2007). Once a xenobiotic ligand is recognized by Ahr, the Ahr–ligand complex translocates into the nucleus and forms a heterodimer with its partner coactivator, Ahr nuclear translocator (Arnt). The AhR/Arnt heterodimer then binds to its recognition DNA sequence, the xenobiotic responsive element (XRE) [also called the dioxin responsive element (DRE)] (Mimura and Fujii-Kuriyama, 2003). Ahr ligands with the highest affinity are halogenated and polycyclic aromatic hydrocarbons (PAHs) (Denison et al., 2002). Binding of the Ahr–ligand complex to XRE leads to transcriptional induction of dioxin-responsive genes such CYP1B1.

Studies have demonstrated that cigarette and smog contain large amounts of benzo[*a*]pyrene (BaP), a typical polycyclic aryl hydrocarbon. The chemical binds to the aryl hydrocarbon receptor (Ahr), a transcription factor extracted from many sources, including osteoblasts and osteoclasts (Nebert et al., 2004). Further reports have verified that BaP's toxic effects induce bone depletion via the Ahr and prompt CYP1 enzymes. In vivo and in vitro evaluations have shown that BaP interrupts bone development in mammals and induces the depletion of bone accumulation and bone solidity, potentially via an enlargement in bone changes in rats (Lee et al., 2002). BaP exposure inhibits skeletal development of *Sebastiscus marmorata* embryos (He et al., 2011), but no reports have discussed the manifestation of BaP subjection in mesenchymal stem cells (MSCs), particularly in fracture healing.

The critical events in mature bone development are the enlistment, proliferation, and differentiation of MSCs alongside endochondral and intramembranous bone development in the afflicted areas (Bruder et al., 1994). In endochondral ossification, mesenchymal cells first separate into chondrocytes (de Crombrugghe et al., 2001), while in intramembranous bone development, mesenchymal cells separate exclusively into osteoblasts(de Crombrugghe et al., 2001); therefore, both endochondral and intramembranous bone configuration are contingent upon osteoblast differentiation from MSCs. Bone marrow-derived mesenchymal stem cells (BM-MSCs) are multipotent, self-renewing, and have a great multiplying measure. Studies have shown that the production of converting growth factor-β1 (TGF-β1) within the bone-marrow microenvironment has a principal part in controlling and stimulating the differentiation of BM-MSCs during the repair of fractures in vivo (Einhorn, 1998; Lind et al., 1993) and in vitro (Claes et al., 1998; Long et al., 1995). Much of the evidence of the cross-talk of Ahr with TGF-β was reviewed recently (Puga et al., 2005). $Ahr^{(-/-)}$ fibroblasts secreted significantly more TGF-β1 into the culture medium than $Ahr^{(+/+)}$ fibroblasts, and showed increased levels of activated Smad4 and TGF-β1 mRNA (Qian et al., 1996). These results demonstrate novel evidence relevant to the mechanisms of Ahr-mediated TGF-β1 regulation. BaP has been found to induce toxicologic effects in other systems by inhibiting TGF-β1 signaling pathways (Kamaraj et al., 2010). The RNA expression profiles support the fact that the Ahr represses TGF-β gene expression and affects the gene expression of several TGF-β-modulating and -processing genes (Guo et al., 2004).

Based on this, we examined human MSCs from smokers and non-smokers to investigate whether BaP has an influence on self-renewal, differentiation, and gene expression accounts. We then employed C3H10T1/2 and BM-MSCs exposed to BaP to verify the above conclusions and to evaluate BaP's mechanism(s) of action. Our outcomes revealed that BaP inhibits the biological characteristics of MSCs commitment in an Ahr-dependent manner. Thus, we further examined the TGF-β1 signaling pathways extracellular regulated protein kinases (ERK) and Similar to Mothers Against Decapentaplegic homolog 4 (SMAD4). The results revealed decreased TGF-β1, phosphorylation of ERK, and SMAD4. In addition, the outcomes of tibial fracture evaluations in rats given BaP and the Ahr antagonist, resveratrol, showed that BaP delayed the fracture repair process and can be rescued by resveratrol.

Taken together, our results suggested BaP activated Ahr to decrease TGF-β1 signaling pathways that influence the bone-fixing procedure to a limited extent by inhibiting MSCs capable of self-renewal and inhibited the proliferation rate.

2. Materials and methods

2.1. Animal groups

Male Sprague-Dawley rats were purchased from the Animal Facility of Chongqing Medical University, China [Certificate No: SCXK (YU) 20070001]. Twenty-four male rats from eight to ten weeks old were randomly divided into four groups (6 rats/group) as follows: gavaged orally with corn oil delivery vehicle control (Group 1) or BaP (Sigma Aldrich, St. Louis, MO) given in corn oil at 120 mg/kg/day (Groups 2), animals gavaged orally with 400 mg/kg/day of resveratrol (Sigma Aldrich, St. Louis, MO) in corn oil (Group-3), rats given in corn oil with 120 mg/kg/day of BaP and 400 mg/kg/day of resveratrol (Group-4). Treatment was continued for 6 d. The rats were then performed tibial fractures. After tibial fracture surgery, treatment was continued up to 21 days.

2.2. Fracture surgeries

Tibial fractures, a 6 mm long incision was made in the skin on the anterior side of the tibia. A sterile 0.7 mm pin was inserted into the tibial marrow cavity, temporarily withdrawn to facilitate transection of the tibia with a scalpel at mid-shaft, and then reinserted. The incision was closed with 3 USP 5-0 sutures.

2.3. Histology

After detachment of the surrounding tissue, for tibiae, were fixed in 10% neutral-buffered formalin, decalcified in HCl/EDTA, and embedded in paraffin. Followed by 3 mm sections were cut and staining with hematoxylin and eosin.

2.4. Primary mesenchymal stem cells and cell line

Under a protocol approved by the Ethical Committee of Chongqing Medical University with written informed consent from each patient, we obtained Human bone marrow mesenchymal stem (hBM-MSCs) from twelve non-smoking healthy donors and twelve smoking healthy donors who provided informed consent. The cells were prepared from bone marrow aspirates using standard protocols, as detailed prior (Strong et al., 2014). Briefly, bone marrow aspirates were taken from the iliac crest of adult donors. Cells were isolated using a density gradient and cultured in complete culture media (CCM), composed of α-MEM (Gibco, Carlsbad, CA, USA), 20% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/mL penicillin/100 μg/m strepto-mycin (GIBCO), and 2 mM L-glutamine (GIBCO). When the cultures reached 70% confluency, the cells were harvested with 0.25% trypsin/1 mM EDTA, resuspended at 1×10^6 cells/mL in α-MEM with 5% dimethyl sulfoxide (DMSO) and 30% FBS, frozen in 1 mL aliquots overnight at −80 °C, and stored in liquid nitrogen for no >6 months before thawing. Donor demographic information is available in Supplemental material, Table S1.

Bone marrow cells were isolated from Sixty rats (aged 3 weeks, weighing 50–60 g) Both femurs and tibiae by flushing the femurs with α-MEM supplemented with 10% fetal bovine saline and antibiotic-antimycotic, using a 19-gauge needle. Isolated bone marrow cells were seeded onto 10-mL tissue culture dishes, and cultured with α-MEM supplemented with 10% FBS. When the cells were 70%–80% confluent, they were harvested with 0.05% trypsin-EDTA, replated at 2×10^4 cells/cm^2, and cultured for 5 days. BM-MSCs between the 3 and 6 passage were used for the experiments.

Please cite this article as: Zhou, Y., et al., Benzo[*a*]pyrene impedes self-renewal and differentiation of mesenchymal stem cells and influences fracture healing, Sci Total Environ (2017), http://dx.doi.org/10.1016/j.scitotenv.2017.02.152