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Inhibition of osteoclast differentiation by polycyclic aryl hydrocarbons is dependent on cell density and RANKL concentration

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Abstract

We investigated the effect of representative polycyclic aryl hydrocarbons (PAHs), benzo[*a*]pyrene (BaP), and 7,12-dimethylbenz-[*a*]anthracene (DMBA) on osteoclast differentiation and function by using dispersed cancellous bone derived rabbit osteoclasts and the RAW264.7 cells. These cells differentiate into osteoclasts when exposed to receptor activator of NF-κB ligand (RANKL). The rabbit osteoclasts were exposed to 10^{-6} to 10^{-9} M BaP or DMBA and the tartrate-resistant acid phosphatase (TRAP)-positive cells were counted. The effect of PAHs on osteoclast differentiation in dispersed rabbit osteoclast-containing stromal cell populations was cell density dependent, suggesting that the cell density of stromal cells, osteoclast precursors, and/or mature osteoclasts are factors regulating the effect of PAHs. To investigate the direct effect of BaP on osteoclast differentiation, RAW264.7 cells were exposed to 10^{-5} to 10^{-6} M BaP. Treatment of RAW264.7 cells cultured with 25 ng/ml soluble RANKL and 10^{-5} M BaP for 5 days decreased osteoclast differentiation, TRAP activity levels, and resorption of bone-like substrata. The inhibition was prevented by 10^{-6} to 10^{-7} M resveratrol, an aryl hydrocarbon receptor (AhR) antagonist, and by higher concentrations of RANKL. To investigate the ability of RANKL to reverse BaP-mediated inhibition, gene expression was determined by RT-PCR. Cytochrome P450 1B1 (CYP1B1) mRNA, one of the genes activated by BaP, was present only in the groups exposed to BaP; the levels of CYP1B1 mRNA decreased in the presence of increasing concentrations of RANKL. These results suggest that the inhibitory effects of PAHs on osteoclastogenesis are direct and likely involve interaction of the RANKL and PAH signaling pathways.

Keywords: Osteoclast; RANKL; Benzo[*a*]pyrene; CYP1B1; TRAP

1. Introduction

Cigarette smoking is a risk factor for periodontal disease [1], dental implant failure [2], bone healing [3–6], and osteoporosis [7–9]. However, the mechanisms underlying the effects of components in cigarette smoke on bone remodeling are not known. Polycyclic aryl hydrocarbons (PAHs) are environmental pollutants present in industrial combustion products, exhaust fumes, furnace gases, as well as in cigarette smoke [10]. They have been implicated in immunotoxicity, developmental and reproductive toxicity, disruption of endocrine pathways, and carcinogenesis [10,11]. Benzo[*a*]pyrene (BaP) and 7,12-dimethylbenz-[*a*]anthracene (DMBA) are two typical PAHs, that are present in cigarette smoke at high concentrations (40–100 ng/cigarette) [9].

PAHs induce many of their toxic effects via the aryl hydrocarbon receptor (AhR), a cytosolic transcription factor [10–12]. This receptor is maintained in the cytosol in a complex with the 90 kDa heat shock protein, Hsp90, the X-associated protein 2 (XAP2), and p23. Upon ligand binding, these molecules dissociate from the complex and the ligand–receptor complex translocates into the nucleus. In the nucleus, it binds to aryl hydrocarbon receptor nuclear translocator (ARNT), and the formed complex recognizes dioxin response element (DRE) sequences resulting in the upregulated expression of numerous genes,

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such as those for phase I and phase II detoxification enzymes [11,12]. PAHs are not the only substances that are capable of binding the AhR. Resveratrol and α-naphthoflavone (naturally occurring ligands) are AhR antagonists [13,14], while several molecules, such as 7-ketocholesterol [15], 2-(1′H-indole-3′-carbonyl)-triazole-4-carboxylic acid methyl ester [16], indirubin, and indigo represent endogenous ligands that can activate AhR [14].

Bone as a tissue is constantly being remodeled. Two major cell types involved in bone remodeling are osteoblasts, which form bone and osteoclasts, which resorb bone. Osteoblasts are derived from cells of mesenchymal lineage, while osteoclasts are derived from hematopoietic precursors of the monocyte/macrophage lineage, which fuse to form multinucleated cells [17]. The main features of osteoclasts include the ability to resorb bone and other mineralized tissues, to form actin rings, to express calcitonin receptor, integrin $\alpha_v\beta_3$, and tartrate-resistant acid phosphatase (TRAP) [18,19]. Osteoblasts and osteoclasts interact; osteoblasts (and stromal cells) produce a cytokine necessary for osteoclast differentiation and activation, called receptor activator of nuclear factor κB ligand (RANKL). RANKL is available in both membrane bound and soluble form and is essential for osteoclast differentiation, activation, and survival [20]. It is a member of TNF superfamily and binds to its receptor, RANK, on the surface of osteoclasts [21]. Upon binding, a signal transduction cascade is initiated via TNF receptor associated factors (TRAFs 1, 2, 3, 5, 6) and involving several transcription factors such as NF-κB, nuclear factor of activated T cells (NFAT), and AP-1 [22,23].

Several groups have investigated the effect of PAHs on bone remodeling in vivo and in vitro. In vitro, Gierthy et al. [24] demonstrated that TCDD inhibits bone nodule formation, alkaline phosphatase activity, and osteocalcin expression in rat calvaria-derived osteoblasts, while Singh et al. [8] reported that TCDD inhibited osteogenesis probably through inhibition of osteodifferentiation. In vivo studies have demonstrated that PAHs dose-dependently inhibit bone growth, modelling, and mechanical strength in rats [25], cause a loss of bone mass and bone strength in ovariectomized rats [9], decrease osteoblast proliferation and differentiation, and delay ossification in the first cervical and lumbar vertebrae and in the metacarpals and metatarsals of mouse fetuses [26].

Most of the studies published so far have focused on the effects of PAHs on bone formation. Studies on the effects of PAHs on osteoclasts are limited to one study by Naruse et al. [27], who demonstrated that 3-methylcholanthrene (3-MC), another aryl hydrocarbon, inhibited osteoclast formation indirectly by suppressing RANKL mRNA production by stromal cells.

Here, we show a direct effect of BaP, one of the aryl hydrocarbons in cigarette smoke, on osteoclast differentiation and function and further examine the mechanism of its effects.

2. Materials and methods

2.1. Chemicals

BaP, DMBA, resveratrol, DMSO, and TRAP staining kit were purchased from Sigma. Silver nitrate, hydroquinone, and sodium thiosulphate were purchased from Fisher Scientific. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin, streptomycin, gentamycin, and fungizone were purchased from Gibco.

2.2. Dispersed rabbit osteoclasts

Osteoclasts were isolated from the long bones of newborn New Zealand white rabbits as described previously [28]. The cells were resuspended in 6 ml of α-MEM containing 10% FBS, 100 μg/ml penicillin G, 0.5 μg/ml gentamycin, 0.3 μg/ml fungizone, and plated in six-well plates at 100 μl/well. They were allowed to attach for 24 h, then washed three times to remove floating cells, after which medium containing 10^{-6} to 10^{-9} M BaP, 10^{-7} to 10^{-9} M DMBA, and/or DMSO/EtOH (vehicle) was added. The cells were incubated for a further 48 h, fixed and stained for TRAP activity as a marker of osteoclast differentiation using the Sigma TRAP staining kit and the number of TRAP positive cells was counted.

2.3. Osteoclast differentiation from the RAW264.7 cell line

The mouse macrophage cell line RAW264.7 was obtained from the American Type Culture Collection (ATCC). These cells, when cultured in the presence of RANKL, differentiate into osteoclasts [20]. The cells were plated at a density of 2500 cells/well in 96-well plates or 16-well Osteologic™ slides (BD Biosciences) in DMEM containing 10% FBS, 100 μg/ml penicillin and streptomycin. The medium also contained 0–200 ng/ml recombinant glutathione S-transferase-soluble RANKL (GST-sRANKL), 0.1% DMSO (vehicle), 10^{-5} to 10^{-9} M BaP, or 10^{-6} to 10^{-7} M resveratrol, depending on the experimental group. All cultures were incubated at 37 °C in humidified air containing 5% CO_2. The cells were incubated for 5 days with media changes on days 1 and 3. To measure TRAP activity, the cells were lysed with 0.1% Triton X-100 in citrate buffer (pH 4.8) and the enzyme activity was determined using *p*-nitrophenyl phosphate as a substrate in the presence of tartaric acid buffer (adapted from Shui et al. [29] and Sigma protocol). Total protein levels were measured using the BCA protein assay kit (Pierce). In order to assess osteoclastic activity, resorption of a bone-like mineral substratum (Osteologic™ slides) was measured. After culture, Osteologic™ slides were bleached to remove cells and then stained using the von Kossa stain to visualize the remaining mineral substrate. For staining, the slides were incubated with fresh 3% (w/v) sodium nitrate for 20 min in

Table 1
List of primers for RT-PCR

Name	Forward primer (5′–3″)	Reverse primer (5′–3″)	T (°C)	Cycle no.	Reference
GAPDH	TGCCAGCCTCGTCCCGTAGAC	CCTCACCCCATTTGATGTTAG	60	22	[45]
CYP1A1	CAGATGATAAGGTCATCACGA	TTGGGGATATAGAAGCCATTC	54	35	[46]
CYP1B1	GGCGTTCGGTCACTACTCTG	AGGTTGGGCTGGTCACTCAT	60	35	[46]
TRAP	ACACAGTGATGCTGTGTGGCAACTC	CCAGAGGCTTCCACATATATGATGG	60	30	[41]

the dark, washed five times with dH_2O, developed in fresh 0.5% hydroquinone for 3 min, washed again five times with dH_2O, and fixed with 2% sodium thiosulphate for 5 min. The slides were then washed three times with dH_2O and air-dried. Resorbed areas showed as clear areas against the contrasting brown to black background. The area of resorption was measured by image analysis using ImagePro™ software.

We prepare our own GST-sRANKL through a bacterial expression system [30]. The biological activity of the GST-sRANKL so obtained varies from lot to lot; therefore, each new lot of sRANKL was titrated in order to be able to add the same concentration of biologically active sRANKL in all experiments. On average, the biological activity of commercially available sRANKL is somewhat higher than the biological activity of our GST-sRANKL.

2.4. RT-PCR

RAW264.7 cells were plated in 25 cm^2 tissue culture flasks at 5×10^5 cells/flask, and incubated in the presence of 10^{-5} M BaP and/or 0.1% DMSO, 0–200 ng/ml RANKL for 20 h. At the end of the incubation period, total RNA was extracted using TRIzol reagent (Invitrogen). Total RNA (3 μg) was treated with DNase I (Invitrogen) at 1 unit of DNase I/μg RNA according to the manufacturer's protocol and then reverse transcribed using Revert Aid H^- First Strand Kit (MBI Fermentas). PCR reactions were performed in 50 μl volumes using HotStarTaq polymerase (Qiagen). The initial activation was performed at 95 °C for 15 min, followed by 45 s at 94 °C for denaturation, 1 min at a set temperature (see Table 1) for annealing, 1 min at 72 °C for extension, and 10 min at 72 °C for a final extension. The cycle number, the annealing temperatures, and the oligonucleotides sequences for the PCR are summarized in Table 1. PCR products were separated on a 1.5% (v/v) agarose gel and visualized using GeneSnap software.

3. Results

3.1. Cell density affects the action of BaP and DMBA in dispersed rabbit osteoclasts

To examine the effect of BaP on dispersed osteoclasts isolated from rabbit long bones, a dose–response study was performed. The cells were grown in the presence of 10^{-7} to 10^{-9} M BaP, 10^{-7} to 10^{-9} M DMBA, and/or vehicle for 48 h. Due to the variability in cell yields in different isolations, there was a high degree of variability in initial cell density between different experiments. To normalize the results obtained, the osteoclast number in experimental groups was expressed as a percent of the osteoclast number in vehicle control groups (Fig. 1). At high osteoclast densities (and stromal cell densities), all concentrations of BaP and DMBA decreased osteoclast numbers by

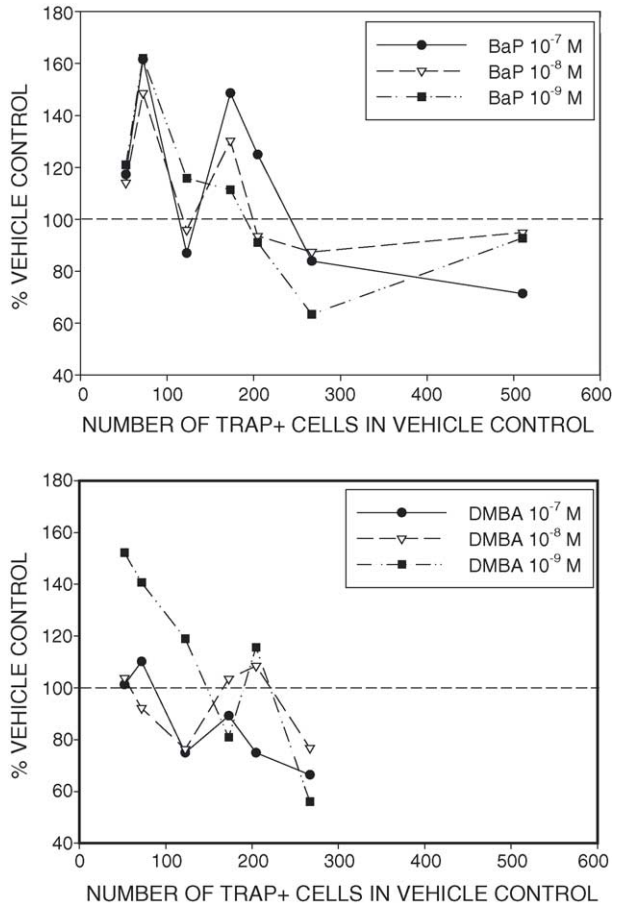

Fig. 1. In a mixed stromal cell population, BaP and DMBA decrease the number of TRAP-positive cells at high cell density, and increase the number of TRAP-positive cells at cell densities lower than 100 and between 150 and 200. Rabbit derived osteoclasts were plated at 100 μl/well (four separate experiments, seven different cell densities) and incubated with BaP and DMBA for 48 h. The cells were then fixed, TRAP stained, and the number of multinucleated cells was determined. The X-axis represents the average number of TRAP-positive cells in the vehicle control group alone for each experiment ($n = 3$). The Y-axis represents the number of TRAP-positive cells in the presence of BaP and DMBA, expressed as percent of vehicle control.

10–40% compared to vehicle controls after 48 h incubation. In contrast, at osteoclast densities below 100 TRAP-positive cells per well and at osteoclast densities of around 150–200 TRAP-positive cells per well, BaP and DMBA treatment generally increased osteoclast numbers compared to vehicle controls. As well, a consistent "dip" was seen between 100 and 150 TRAP-positive cells. The explanation for this pattern is not clear. However, it is reproducible and suggests that density of certain cell types in the population could be responsible for this phenomenon. Thus, the cell density of stromal cells, osteoclast precursors, and/or osteoclasts appears to determine the effects of BaP and DMBA. To clarify this issue, we investigated whether PAHs have a direct effect on osteoclasts by performing experiments with the mouse macrophage cell line, RAW264.7. These cells, when cultured for 5 days in the presence of sRANKL (25–200 ng/ml), differentiate into osteoclasts [20].

3.2. BaP inhibits osteoclastogenesis at 25 and 50 ng/ml but not at 100 ng/ml sRANKL in RAW264.7 cells, while the AhR antagonist resveratrol reverses the BaP-mediated inhibition

To investigate the direct effect of BaP on osteoclast function, the resorption of a bone-like substratum was assessed. BaP (10^{-5} and 10^{-6} M) inhibited osteoclast-mediated resorption of Osteologic^{TM} slides in the presence of 25 ng/ml sRANKL (Fig. 2A). To test possible involvement of the AhR in BaP-mediated effects on osteoclast cell function, the cells were cultured in the presence or absence of the AhR antagonist resveratrol. Resveratrol (10^{-6} M) reversed the BaP-induced inhibition of resorption in the presence of 25 ng/ml RANKL (Fig. 2B). To determine if the inhibition of osteoclastic resorption was due to an inhibition of osteoclast function and/or a decrease of osteoclast formation, we measured TRAP activity in cultures grown on a plastic substratum (Fig. 3A). Inhibition of TRAP activity was observed at 10^{-5} M BaP in the presence of 25 or 50 ng/ml sRANKL; however, no effect on TRAP activity was observed at 10^{-5} to 10^{-10} M BaP in the presence of 100 ng/ml sRANKL (Fig. 3A); results for 10^{-7} to 10^{-10} (no effect) not shown). In order to evaluate the effect of BaP on cell proliferation, total protein was measured (Fig. 3B). Since RAW cells do not produce extracellular matrix, total protein levels could be used to estimate cell proliferation. There was no difference in total amount of protein between the groups cultured with 25–100 ng/ml sRANKL ($n = 4$ experiments), suggesting that 10^{-5} to 10^{-6} M BaP did not affect cell proliferation in the presence of sRANKL. There was, however, a significant difference in total amount of protein between vehicle control and 10^{-5} M BaP in the absence of sRANKL. To further test the possible involvement of the AhR, experiments were conducted with resveratrol, an AhR antagonist. Resveratrol (10^{-6} to 10^{-7} M) reversed BaP-mediated inhibition of TRAP activity in the presence of 25 and 50 ng/ml RANKL (Fig. 4).

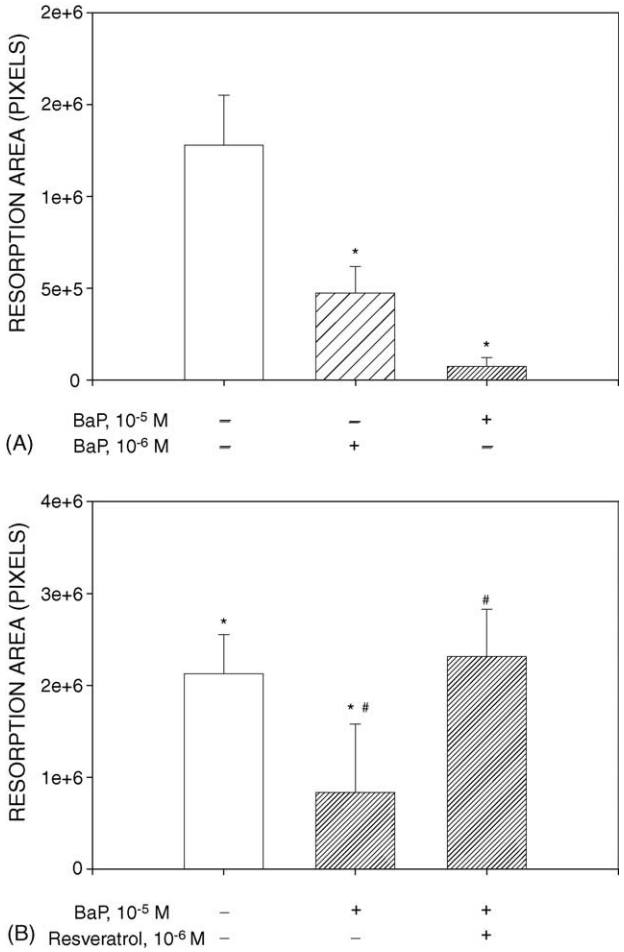

Fig. 2. BaP inhibits osteoclastic resorption and resveratrol reverses the inhibition. The RAW264.7 cells were plated at 2500 cells/well in 16-well Osteologic^{TM} slides ($n = 4$), incubated in the presence of (A) 10^{-5} and 10^{-6} M BaP and 25 ng/ml sRANKL, and (B) 10^{-5} M BaP, 10^{-6} M resveratrol and 25 ng/ml sRANKL for 5 days with media changes on days 1 and 3. The slides were stained using von Kossa staining. The area of resorption was determined by image analysis. The results are presented as mean ± standard deviation. $^{*}p < 0.05$ as compared to vehicle control; $^{\#}p < 0.05$ compared to BaP 10^{-5} M + 10^{-6} M resveratrol.

3.3. sRANKL inhibits BaP-mediated CYP1B1 mRNA expression

The observation that either high concentrations of RANKL or the AhR antagonist resveratrol could reverse BaP inhibition suggested that there might be interacting signaling pathways between RANKL and PAHs. In order to determine the mechanisms underlying the putative interaction between RANKL and PAHs, RT-PCR of genes regulated by PAHs and RANKL was performed. PAHs induce a number of phase I and phase II detoxification enzymes, including cytochrome P450. Cytochrome P450 1A1 (CYP1A1) is the most studied isoform in relation to the effects of aryl hydrocarbons, while CYP1B1 is believed to be responsible for BaP metabolism in many cells and

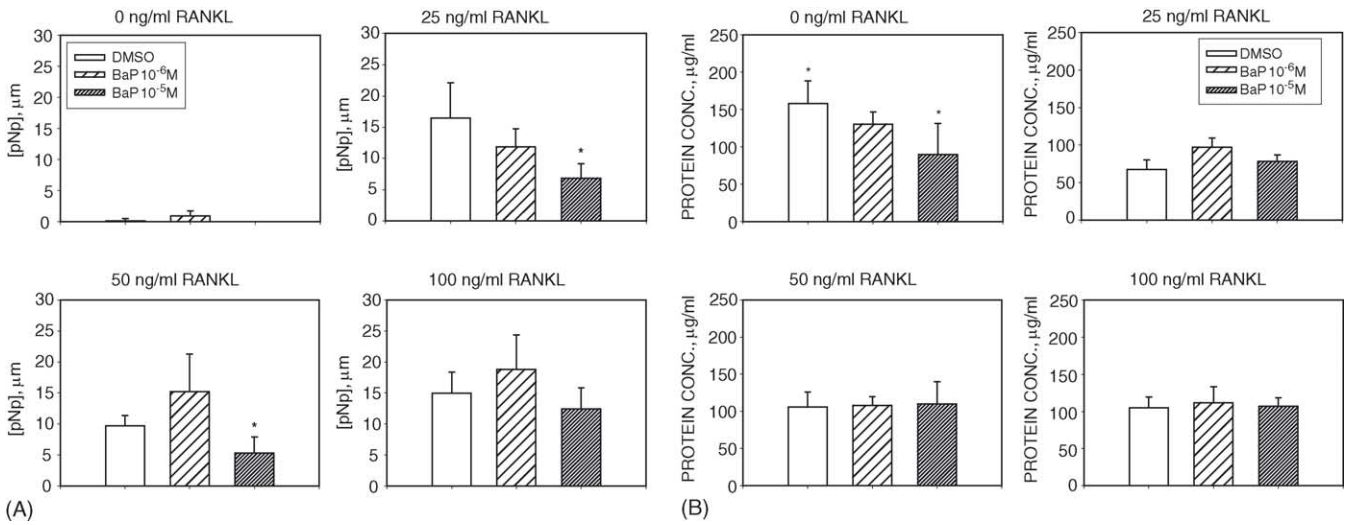

Fig. 3. (A) Treatment with 10^{-5} M BaP decreases TRAP activity levels at 25 and 50 ng/ml, but not at 100 ng/ml sRANKL. RAW264.7 cells were plated at 2500 cells/well in 96-well plates ($n = 8$) and cultured in the presence of 10^{-5} or 10^{-6} M BaP and 25, 50, and 100 ng/ml sRANKL for 5 days with media change on days 1 and 3. On day 5, the cells were lysed and TRAP activity of the lysates was determined as described in Section 2. The experiments were repeated at least three times. *p*Np = *p*-nitrophenol. (B) BaP has no effect on total protein levels in the presence of sRANKL. The RAW264.7 cells were plated at 2500 cells/well in 96-well plates ($n = 6$), incubated in the presence of 10^{-5} M BaP and 25, 50, and 100 ng/ml sRANKL for 5 days with media changes on days 1 and 3. The cells were lysed and the total protein levels of the lysates were determined using a BCA protein kit (Pierce). The results are presented as mean ± standard deviation. $^{*}p < 0.05$ as determined by Student's *t*-test. The experiments were repeated four times.

tissues [31,32]. Therefore, the expression of CYP1A1 and CYP1B1 was examined in RAW264.7 cultures exposed to 10^{-5} M BaP and either 25 or 200 ng/ml RANKL for 20 h. mRNA extracted from mouse liver exposed to BaP was used as a positive control for the RT-PCR.

Both CYP1A1 and CYP1B1 were expressed in the mouse liver exposed to BaP. There was no constitutive expression of mRNA for CYP1A1 in the osteoclast cultures (Fig. 5A). The expression of CYP1B1 mRNA, a major cytochrome P450 isoform in macrophages [31,33], was considerably increased upon stimulation with 10^{-5} M BaP when compared to vehicle-treated control (Fig. 5A and B). This stimulation was not affected by 25 ng/ml RANKL, but was significantly inhibited by 200 ng/ml RANKL (Fig. 5A and B). Induction of CYP1B1 by BaP indicated that the AhR mechanism is functional in the RAW264.7 cells.

To determine the effect of BaP on early osteoclast-specific gene expression, TRAP mRNA expression (Fig. 5A and B) was assessed. As expected, TRAP mRNA expression between the groups with or without sRANKL was different. However, when quantified, the effect of 10^{-5} M BaP on TRAP mRNA expression in the presence of 25 ng/ml sRANKL was not statistically significant (Fig. 5A and B).

4. Discussion

We have shown here that BaP directly inhibits osteoclast formation from RAW264.7 cells and also presented evidence indicating an interaction between BaP and sRANKL signaling. These results apparently contradict the conclusions of Naruse et al. [27] that aryl hydrocarbons do not have a direct effect on osteoclasts. Nevertheless, we believe our results are consistent with the findings of Naruse et al. and the different conclusions can be explained by our observation that sRANKL can reverse BaP-mediated effects.

We first evaluated the effects of BaP and DMBA on dispersed rabbit osteoclasts, and found that at high cell density, BaP and DMBA decreased the number of TRAP-positive cells, while at low cell density, an increase in TRAP-positive cells was observed (Fig. 1). The results obtained at high cell density are consistent with the findings of Naruse et al. [27], who used osteogenic stromal ST2 cells and mouse spleen cells to generate osteoclasts, and demonstrated that 3-methylcholanthrene (3-MC), another representative PAH, decreased osteoclast formation through inhibition of RANKL mRNA expression in stromal cells. Our results support their hypothesis that in a co-culture system, PAH-induced effects on osteoclastogenesis are, in part, mediated through the stromal cell population. However, at specific cell densities, we found an increase in osteoclast numbers compared to the vehicle control. To explain our results, we hypothesized that various types of stromal cells present in the dispersed osteoclast preparation may have masked a direct and possibly stimulatory effect of PAHs on osteoclasts in the high-density cultures. To test this hypothesis, we investigated the direct effect of BaP on osteoclast differentiation and function using the RAW cell line model and found that there was no effect on TRAP activity in response to 10^{-6} to 10^{-10} M BaP in the presence of 100 ng/ml sRANKL. This is also consistent with the findings reported by Naruse et al. [27]. They demonstrated that there was no effect of 3-MC on the formation of TRAP-positive cells when the RAW cells were cultured in

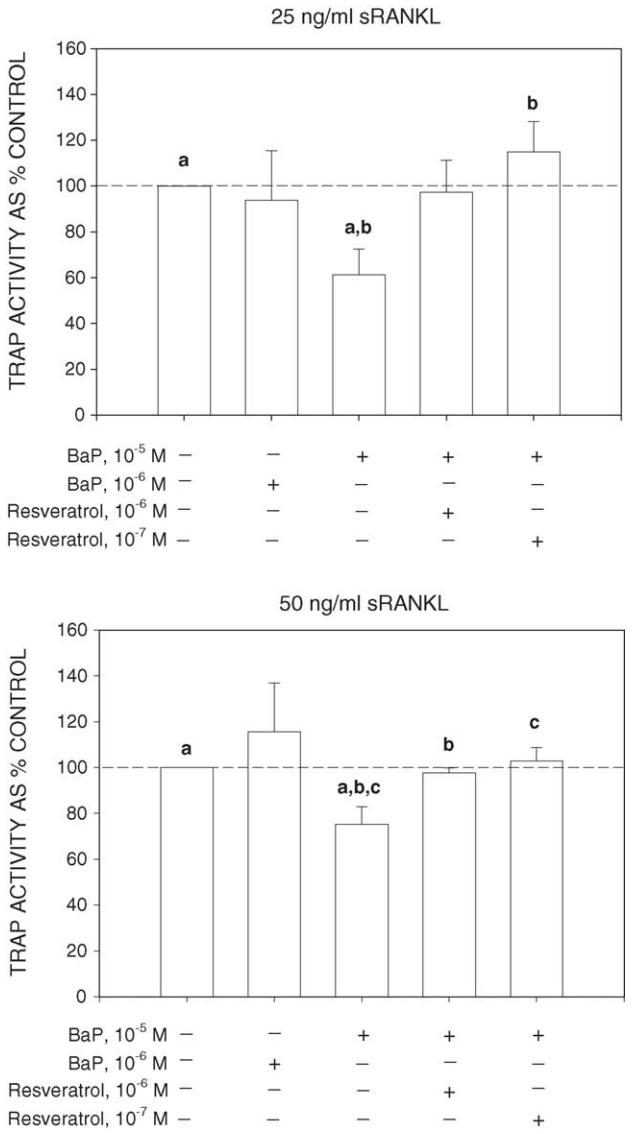

Fig. 4. BaP (10^{-5} M) inhibits TRAP activity levels at 25 and 50 ng/ml sRANKL. RAW264.7 cells were cultured for 5 days in the presence of various concentrations of sRANKL, with or without BaP and resveratrol. The data are presented as percent of vehicle control (DMSO) combining data from six different experiments (mean ± standard deviation). (a, b, c) Statistical significance between two bars is indicated by identical letters, $p < 0.05$ as determined by SPSS v.12.0 statistical software using an independent sample *t*-test (equal variances not assumed).

the presence of 100 ng/ml of a commercially available human soluble RANKL (PeproTech EC Ltd.). However, we observed an inhibition of TRAP activity and formation of TRAP-positive cells when 10^{-5} M BaP was used in the presence of 25–50 ng/ml sRANKL. We suspect that the reason why this group did not observe inhibition of osteoclastogenesis was that they did not perform the experiments at lower sRANKL concentrations.

Even though 10^{-5} M BaP is a concentration that is higher than that attained in vivo under experimental conditions, this concentration of BaP has been used to study the effects of PAHs on macrophages [34,35]. This group found that 10^{-5} and 10^{-6} M BaP had no effect on cell viability using human monocytes, but later demonstrated that these concentrations caused caspase- and mitochondrion-related apoptosis [36]. We found that 10^{-5} M BaP decreased protein values (used as a parameter for cell number) in the absence of sRANKL, but had no effect in the presence of sRANKL (Fig. 3B), suggesting that sRANKL prevented the BaP-induced inhibition of cell proliferation.

Anti-apoptotic and pro-survival properties of sRANKL have been demonstrated by several groups [37,38]. In the model used here, sRANKL appeared to reverse BaP-mediated inhibitory effects not only on total protein levels but also on osteoclast formation.

The observation in our study that both the AhR antagonist resveratrol and RANKL could reverse BaP-induced effects suggested a BaP–RANKL signaling interaction. Aryl hydrocarbon–TNF-α interaction, through AhR–NF-κB competition has been demonstrated previously using 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a halogenated aryl hydrocarbon and a potent AhR agonist [12,39,40]. Ke et al. [40] showed that increasing concentrations of TNF-α and LPS decreased TCDD-induced gene expression. RANKL and TNF-α both belong to the TNF superfamily and both act via NF-κB in order to initiate gene transcription, while BaP and TCDD initiate their gene transcription through the AhR. We hypothesized that in osteoclasts, there is a similar AhR–NF-κB competition. To investigate this hypothesis, we examined BaP-induced and sRANKL-induced gene expression.

BaP induces a range of phase I and phase II detoxification enzymes, including different isoforms of cytochrome P450, such as CYP1A1 and CYP1B1. The levels of CYP1B1 mRNA were significantly upregulated in all groups exposed to BaP (Fig. 5A), significantly decreased in 200 ng/ml sRANKL groups and marginally decreased in 25 ng/ml sRANKL group (Fig. 5B), demonstrating the ability of sRANKL to reverse BaP-mediated gene expression. These results are consistent with our hypothesis that there is competition between the PAH and RANKL signaling pathways.

We also found that 10^{-5} M BaP did not suppress TRAP mRNA expression in the presence of 25 ng/ml sRANKL. This observation appears not to correspond to the TRAP activity results (Fig. 3A). However, TRAP mRNA expression was measured after only 20 h of incubation, while TRAP activity assays were performed on day 5, and it has been reported previously that most osteoclast-related genes are not upregulated until day 2 of the culture in the RAW cell model system [41] and days 3–6 in other models [42].

In summary, we have shown that the inhibitory effects of PAHs on osteoclasts are both direct (possibly via AhR–NF-κB competition) and indirect (via the stromal cells). Further investigation is required to understand the mechanism of PAH-mediated effects on osteoclast formation, whether it is similar to the one described for TCDD–TNF-α interaction in hepatoma cells, and whether it involves a competition for coactivator complexes, such as p300/CBP and SRC-1. There

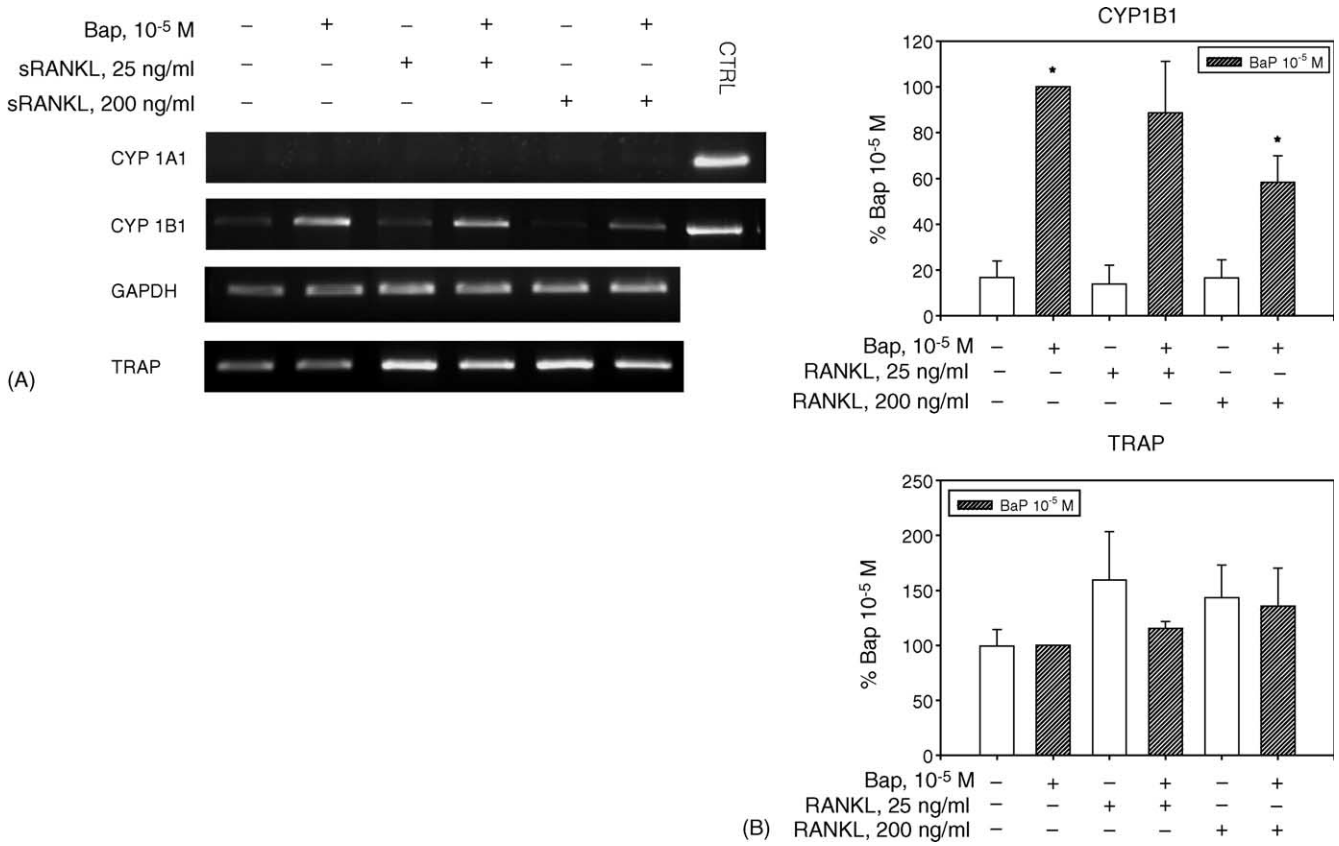

Fig. 5. (A) sRANKL inhibits BaP-mediated CYP1B1 mRNA expression. Total RNA was isolated from RAW264.7 cells incubated in the presence of 10^{-5} M BaP, 25 and 200 ng/ml sRANKL, and/or vehicle for 20 h, using the TRIzol method. RT-PCR was performed with the primers listed in Table 1 and PCR products were separated on a 1.5% agarose gel. (B) Quantification of the RT-PCR results shown in panel (A). The density of the bands was analyzed by GeneTools software; the values for TRAP and CYP1B1 were normalized to GAPDH value for each sample. The results are presented as mean ± standard deviation of three experiments. $^{*}p < 0.05$ compared to 10^{-5} M BaP as determined by Student's *t*-test.

are also reports that demonstrate an existence of an AhR-independent mechanism of CYP1B1 upregulation in the bone marrow cells by the PAHs [43,44], raising the possibility of a different mechanism of signal transduction.

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