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Effects of Benzo(e)pyrene on Reactive Oxygen/Nitrogen Species and Inflammatory Cytokines Induction in Human RPE Cells and Attenuation by Mitochondrial-involved Mechanism

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Abstract

Purpose: To identify inhibitors that could effectively lower reactive oxygen/nitrogen species (ROS/RNS), complement and inflammatory cytokine levels induced by Benzo(e)pyrene [B(e)p], an element of cigarette smoke, in human retinal pigment epithelial cells (ARPE-19) in vitro.

Methods: ARPE-19 cells were treated for 24 hours with 200 μM, 100 μM, and 50 μM B(e)p or DMSO (dimethyl sulfoxide)-equivalent concentrations. Some cultures were pre-treated with ROS/RNS inhibitors (NG nitro-L-arginine, inhibits nitric oxide synthase; Apocynin, inhibits NADPH oxidase; Rotenone, inhibits mitochondrial complex I; Antimycin A, inhibits mitochondria complex III) and ROS/RNS levels were measured with a fluorescent H₂DCFDA assay. Multiplex bead arrays were used to measure levels of Interleukin-6 (IL-6), Interleukin-8 (IL-8), Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), Transforming Growth Factor alpha (TGF-α) and Vascular Endothelial Growth Factor (VEGF). IL-6 levels were also measured by an enzyme-linked immunosorbent assay. Real-time qPCR analyses were performed with primers for C3 (component 3), CFH (inhibits complement activation), CD59 (inhibitor of the complement membrane attack complex (MAC)) and CD55/DAF (accelerates decay of target complement target proteins).

Results: The ARPE-19 cultures treated with B(e)p showed significantly increased ROS/RNS levels ($P < 0.001$), which were then partially reversed by 6 μM Antimycin A (19%, $P = 0.03$), but not affected by the other ROS/RNS inhibitors. The B(e)p treated...
INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness and low vision in individuals over 55 years of age in the industrialized world. AMD can be classified into two types: wet or neovascular and dry or atrophic. In wet AMD, choroidal neovascular membrane (CNV) develops, which causes hemorrhage, swelling, and macular scarring resulting in severe visual loss. Dry AMD can result in atrophy of photoreceptors and retinal pigment epithelium (RPE) that can also decrease central vision over time. The combined prevalence of neovascular AMD and geographic atrophy has been estimated to be 1.4% in the United States, thus affecting 1.75 million individuals presently with a 50% expected increase by the year 2020. Although the clinical features of AMD have been well documented for more than a century, the detailed pathogenesis of AMD is not fully understood. However, cigarette smoking is one of the most consistent risk factors for AMD in several worldwide population based studies. In 1993, the Beaver Dam Eye Study showed that cigarette smoking increases the risk of wet AMD. Epidemiologic studies have confirmed that smoking is a risk factor in both forms of AMD.

A family of toxic compounds, the polycyclic aromatic hydrocarbons (PAH), are found in cigarette smoke, and they form DNA changes causing cellular proliferation. One of the most investigated PAH is Benzo(a)Pyrene [B(a)p] which has been shown to cause damage to bovine RPE cells and various other cell lines. The concentration of B(a)p in cigarette smoke is 9 ng/cigarette. More recently, another PAH compound, B(e)p which has a concentration of 5–40 ng/cigarette, was shown to decrease cell viabilities of 5–40 ng/cigarette. Furthermore, the damaging effects of B(e)p can be partially reversed by pre-treatment with memantine, resveratrol and genistein. B(e)p with its structure of five fused aromatic rings (PAH) with a high molecular weight can be found in substances besides cigarette smoke including automobile exhaust, charcoal-broiled foods, and incomplete combustion of organic materials.

Oxidative stress plays an important role in AMD as evident by the AREDS trial. There is mounting biochemical evidence of the role of oxidative stress in drusen formation, which is considered a hallmark of AMD. The superoxide radicals (O2−) are generated through two sources inside the cell, mitochondrial respiration (through complexes and pathways) or via the cell membrane bound NAD (P) H oxidase [Figure 1]. The superoxide radical is converted to hydrogen peroxide (H2O2) by superoxide dismutases (SOD) and the H2O2 can be converted to O2 and H2O through enzymes such as glutathione synthase and catalase. Increased reactive oxygen/nitrogen species (ROS/RNS) levels are known to cause apoptosis through release of mitochondrial cytochrome C into the cytosol by opening the mitochondrial permeability transition pore. In addition, elevated ROS/RNS levels can contribute greatly to inflammation and oxidative damage by damaging proteins, lipids and DNA. If mitochondrial DNA (mtDNA) is damaged, then oxidative phosphorylation is diminished and additional ROS/RNS is generated. However, it is not clear how smoking...
components might induce inflammation and oxidative damage in human RPE cells.

In addition to age and smoking, genetic factors play a major role in susceptibility to AMD. Recently, a strong genetic association has been described between AMD and Complement Factor H (CFH), which is a major plasma protein (155KDa) composed of 1213 amino acids.[24] that is known to inhibit the alternative complement pathway.[25] The pathological relevance of the genetic studies is supported by the demonstration that drusen contain complement proteins, including CFH,[25-28] complement components C3,[29,30] C2, CFB,[31,32] C5, Cq1, the membrane attack complex (MAC), and C5b-9.[33]

Increased serum levels of C3a have been reported in AMD patients.[34] In addition, T-cell derived cytokines cause upregulation of complement pathway components including C3, CD46, CD35, CD59 and others.[35]

CFH inhibits the C3b function, limits wasteful consumption of complement components and prevents non-specific damage to host tissues.[36] After CFH binds to C3b, the complex interacts with complement receptors and complement factor B to promote opsonization and phagocytosis. This regulation occurs in both the fluid phase and cellular surfaces.[37] Recent studies show that RPE cells produce CFH[38] and also pro-inflammatory cytokines such as Interleukin-6 (IL-6) and Interleukin-8 (IL-8).[39] However, the response of CFH or cytokine levels from RPE cells after exposure to B(e)p have not been investigated. Therefore, the present study was designed to examine the effects of B(e)p on ROS/RNS production, pro-inflammatory cytokines and complement components.

**METHODS**

**Cell Culture**

ARPE-19 cells were obtained from ATCC (Manassas, VA). Cell passage numbers from 16–28 were used for these experiments. Cells were grown according to previously established methods.[11] Cells were plated in 24 well plates (Becton Dickinson Labware, Franklin Lakes, NJ) for ROS/RNS (10^4 cells per well) assays. For the protein assay, 10^4 cells were plated in 60 x 15 mm well plates. The enzyme linked immunosorbent assay (ELISA) was performed in 96 well plates with 10^4 cells per well. When ARPE-19 cells became confluent, they were incubated for 24 hours in serum-free medium to make them relatively non-proliferating. This simulates the natural human RPE cells, which remain in a non-proliferating phase and which are not exposed to the circulation because of the outer blood-retinal barrier. The cells were exposed to varying concentrations of B(e)p.

**Exposure to B(e)p**

B(e)p was obtained from Sigma-Aldrich, St. Louis, MO, as a commercial powder. Concentrations of 200μM, 100μM and 50μM B(e)p were used for the experiments. The stock solution of 100mM B(e)p was prepared by dissolving the B(e)p in dimethyl sulfoxide (DMSO). The working concentration of B(e)p was prepared by diluting the stock solution in the culture media. Previously, we showed that B(e)p treatment for 24 hours on ARPE-19 cells was cytotoxic,[41] and hence this time point was chosen for the inhibition studies. The equivalent amounts of DMSO served as control cultures.

**Pretreatment with Inhibitors**

All inhibitors were obtained from Sigma-Aldrich (St. Louis, MO). Cells were pre-treated for 4 hours with one of the following enzyme inhibitors: 2, 4, or 6 μM of Antimycin A (inhibitor of mitochondrial complex III); 30 or 60 μM of Apocynin (inhibitor of NADPH oxidase); 100, 200 or 400 μM of NG nitro-L-arginine (L-NNA; inhibitor of nitric oxide synthase) or 2 or 4 μM of Rotenone (inhibitor of mitochondrial complex I). After inhibitor pretreatment, cells were incubated another 24 hours with 200μM B(e)p or DMSO-equivalent exposure (control) plus the inhibitor. The control cells were untreated cultures, the B(e)p treated cultures and DMSO-equivalent treated cultures. Experiments were repeated at three separate times in duplicates.

**Detection of ROS/RNS**

ROS/RNS production was measured with the fluorescent dye 2′,7′-dichlorodihydro fluorescein diacetate assay (H2DCFDA; Invitrogen) which detects H2O2, hydroxyl radicals, and peroxynitrite anions. Briefly, the ARPE-19 cells were plated in 24-well plates. The cells were washed with sterile phosphate buffered saline (PBS), and incubated with 500 μl of 10 μM H2DCFDA for 15 minutes at 37°C and again washed with PBS. The H2DCFDA (10 μM) was prepared by adding 2 μl of 5 mM (H2DCFDA) stock/ml in PBS. The 5 mM H2DCFDA stock solution was prepared fresh by mixing 0.001 g of H2DCFDA in of DMSO. ROS/RNS production was measured with the fluorescence imager (excitation 488 nm, emission 520 nm, FMBIO III; Hitachi, Yokohama, Japan).

**Screening for Cytokines Levels**

The expressions of cytokines from the supernatant of B(e)p treated and DMSO-equivalent treated ARPE-19 cultures were measured using the multiplex bead array format (Luminex, Austin, TX). The high sensitivity multiplex assay kit was used to obtain simultaneous quantitative determination of the human cytokines. The array was analyzed as per manufacturer’s instructions. The increased levels of IL-6 levels in the supernatant of ARPE-19 cell cultures were verified with the quantitative sandwich enzyme linked immunoassay technique (ELISA;
**Isolation of RNA and Amplification of cDNA**

Cells from untreated and B(e)p treated cultures were pelleted, and RNA isolated using the RNeasy Mini-Extraction kit (Qiagen) following the manufacturer’s protocol. The RNA was quantified using a NanoDrop 1000 (ThermoScientific). For Q-PCR analyses, 100 ng of individual RNA samples were reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen).

**Real-time Quantitative PCR (RT-qPCR) Analyses**

RT-qPCR was performed using 4 different primers (QuantiTect Primer Assay, Qiagen) for genes associated with the complement pathway (C3, CFH, CD59, CD55/DAF) [Table 1]. The Q-PCR using the Quantifast SYBR Green PCR Kit (Qiagen) and a Bio-Rad iCycler iQ5 detection system was performed using the cDNA samples from the untreated and B(e)p treated cultures. TBP gene was used as a housekeeper. The analyses were performed in triplicate and the experiments were repeated twice. Statistical analyses of gene expression levels were performed to measure difference between haplogroups using Prism, Version 5.0 (GraphPad Software Inc.).

**Statistical Analysis**

Data from all experiments was analyzed by Student’s t Test (GraphPad Prism, version 3.0; GraphPad Software Inc., San Diego, CA) and presented as Mean ± Standard Error of Mean (SEM). Statistically significant values were denoted as (*), (**) and (***) on the figures for P values <0.05, <0.01 and <0.001 respectively.

**RESULTS**

Inhibitors were tested to determine whether they could block the ROS/RNS levels that were induced by 200 μM B(e)p [Figure 1]. DMSO-treated cultures were standardized to 100%. Antimycin A 6 μM reduced the ROS/RNS levels to 260.7 ± 23.01% (P = 0.03), from the B(e)p induced ROS/RNS level of 311.1 ± 22.55%. The 2 μM and 4 μM Antimycin A did not block the ROS/RNS levels. In addition, the other inhibitors, Apocynin, L-NNA and Rotenone, did not reverse B(e)p induced ROS/RNS levels [Figure 2a-d].

Supernatants of the ARPE-19 cell cultures treated with 50, 100 and 200 μM B(e)p or DMSO-equivalent controls were analyzed with the multiplex bead array, which scanned for IL-6, IL-8, GM-CSF, TGF-α and VEGF proteins. These analyses showed significantly higher levels of IL-6 and GM-CSF in the B(e)p treated cultures as compared to the DMSO-equivalent treated cultures [Figure 3]. After B(e)p treatment, there was a 33% increase of IL-6 levels (133.0 ± 8.1 versus 99.9 ± 0.06, P = 0.016) and 28.7% higher GM-CSF levels (128.7 ± 0.33 versus 99.9 ± 0.06, P = 0.0001). There were no significant changes between the B(e)p treated and DMSO-equivalent control cultures for the levels for IL-8 (101.7 ± 3.9), TGF-α (110.0 ± 8.08) and VEGF (85.3 ± 6.68). As IL-6 is a well-recognized pro-inflammatory cytokine, we wanted to further analyze this response at different B(e)p concentrations. Therefore, ARPE-19 cells were exposed

| Table 1. Q-PCR analyses for gene expression of complement genes after treatment with B(e)p versus DMSO-equivalent |
| Symbol | Gene Name | GenBank accession no | Function | p | ∆ΔCT/Fold^a |
| C3 | Complement Component 3 | NM_000064 | Central in activation of complement system; required for both classical and alternative pathways | 0.59 | 0.195±0.350/1.14 |
| CFH | Complement Factor H | NM_000186 | Essential in regulation of complement activation | 0.44 | 0.133±0.166/1.10 |
| CD59 | Molecule, Complement Regulatory Protein | NM_000611 NM_203329 NM_203331 NM_001127223 NM_001127225 NM_001127226 NM_001127227 | Cell surface glycoprotein that regulates complement-mediated cell lysis, inhibits complement membrane attack complex and is involved in lymphocyte signal transduction | 0.75 | -0.0483±0.146/0.97 |
| CD55/DAF | Decay accelerating factor for complement | NM_000574 NM_001114543 NM_001114544 NM_00111475 | Involved in the regulation of complement cascade by accelerating the decay of complement proteins and disrupting the cascade | 0.52 | -0.172±0.260/0.89 |

^aN=3 with six values for each sample. Positive values indicate up regulation of the gene. Negative values indicate down regulation of the gene. Fold=2^-∆∆CT. DMSO, dimethyl Sulfoxide; L-NNA, NG nitro-L-arginine; ROS/RNS, reactive oxygen/nitrogen species

R & D Systems, Minneapolis, MN). The ELISA assay was performed as per manufacturer’s instructions. The substrate solution color intensity was measured using a Molecular Devices Spectra Max Plus 384 spectrophotometer.
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**DISCUSSION**

Retinal and choroidal tissues have some of the highest oxygen consumption rates in the body.\(^{39}\) This large oxygen gradient, along with high levels of polyunsaturated fatty acids in photoreceptors and measuring ROS/RNS in the B(e)p treated RPE cell repeated exposure to light, make a highly oxidative milieu.\(^{40}\) The retinal micro-environment is highly sensitive to these events, which ultimately elevates the levels of oxidative stress, a major pathological factor in AMD.

Smoking is one of the environmental factors proven in several studies to contribute to AMD.\(^{43}\) A number of toxic compounds exist in cigarette smoke and one of the well-known agents is B(e)p belonging to a group of polycyclic aromatic hydrocarbons. In our previous study, ARPE-19 cells showed a concentration-dependent decrease in cell viability after exposure to B(e)p.\(^{11}\) In addition, B(e)p promoted apoptosis through activation of multiple caspases, which could be inhibited by exposure to memantine, resveratrol and genistein.\(^{13}\)

ROS and RNS are powerful oxidizing agents produced in different compartments of the cell. Figure 1 provides a simplified schematic presentation of different pathways for ROS/RNS production and their fate. Within the retinal cells, the mitochondria are the major sites of ROS production\(^{41}\) with superoxides being generated at complex III (inhibited by Antimycin A)\(^{42,43}\) and complex I (inhibited by Rotenone)\(^{44}\) of the electron transport chain (ETC). A non-mitochondrial source of superoxide formation is the membrane bound NADPH oxidase (inhibited by Apocynin).\(^{45-47}\) Superoxide is an unstable molecule that is rapidly converted to \(\text{H}_2\text{O}_2\) by superoxide dismutase (SOD). If the \(\text{H}_2\text{O}_2\) molecule is processed through the Fenton reaction, the destructive hydroxyl radical will be formed. Alternatively, the superoxide can react with nitric oxide, which is produced by nitric oxide synthase (inhibited by L-NNA) to form peroxynitrite, another powerful oxidizing agent. By knowing the exact site of action for each of the inhibitors and measuring ROS/RNS in the B(e)p treated RPE cell...
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In the present study, L-NNA was evaluated at three different dosages (400, 200 and 100 μM) and found to have no effect in blocking the B(e)p induced ROS/RNS production, which indicates that B(e)p does not act via the nitric oxide pathway. One possible limitation of our study is that the assay measures multiple types of ROS/RNS. However, the inhibitors used are very specific and if the pathways were involved, we would expect a decline in the ROS/RNS values after inhibitor treatment. On the other hand, different components of ROS/RNS can readily convert to each other and we are going to deal the oxidative stress of B(e)p (as a whole) on RPE cells. Then, our method of ROS/RNS measurement would be useful to find a way to attenuate total destructive effects of B(e)p as a whole. By the way, our study showed that only one of the inhibitors (Antimycin A), which only acts on mitochondrial complex III, attenuates the oxidizing action of B(e)p and provides guidance to focus on the mitochondrial respiratory chain.

Our RT-qPCR data provides in vitro evidence that B(e)p did not change the expression levels for any of the complement pathway components. In general, CFH plays a critical protective role in host cells against inflammation and diseases. Both increased age and smoking decrease plasma levels of CFH[58,59] and CFH deficiency is associated with uncontrolled complement activation along with cellular and tissue damage. Dysregulation of the complement pathway plays an important role in the development of AMD.[42] It has been shown that RPE cells are a local source of CFH[60] and in vitro studies have shown that mouse and human RPE cells produce significant amounts of CFH protein. Although it has been shown that RPE synthesis of CFH is suppressed by pro-inflammatory proteins and oxidized photoreceptor rod outer segment,[42] in our study the ARPE-19 cultures showed increased IL-6 levels after B(e)p treatment but no change in CFH expression. This is in contrast with another study which showed extensive increase in gene expression of C3, CFH and CD59 in RPE cells 24 hours after exposure to B(a)p (another member of the PAH molecules).[61] Marazita et al recently applied cigarette smoke concentrate and hydrogen peroxide to ARPE-19 to make them senescent. Those senescent ARPE-19 cells showed upregulated IL-6, IL-8, VEGF and simultaneously downregulated CFH expression.[62] In our study we could not find any significant changes in VEGF. The difference between study results may be due to the presence of other components of the cigarette smoke or hypoxia, which could have additive effects to the B(e)p exposure. Our study has been done at the gene level on complement factors, but more studies at the protein level could clarify the amount of each complement factor in this situation and could better explain those changes after B(e)p treatment.

IL-6 is a potent pro-inflammatory cytokine that binds to its receptor and the complex then interacts with gp130 on the cell surface. This leads to dimerization of gp130 which initiates IL-6-mediated signaling in target cells[63] through the activation of signal transducer and activator of transcription (STAT) proteins.

Our findings show that B(e)p increases oxidative stress and pro-inflammatory cytokine levels, thus suggesting the damage caused by B(e)p in ARPE-19 cells is similar to known pathogenic mechanisms for AMD. Previously, Kim et al[51] used 0.2 μM Rotenone on H2O2 stressed ARPE-19 to increase the ROS/RNS production. In our study, Rotenone and Apocynin did not diminish the ROS/RNS production in B(e)p treated cells, suggesting that it does not act through mitochondrial complex I or NAPDH oxidase. The ROS/RNS assay used in this study also measures the combined levels of H2O2, hydroxy and peroxynitrite. The latter is generated by combination of superoxide and nitric oxide, which can reportedly cause vascular endothelial toxicity.[52-54] Nitric oxide, which is formed by nitric oxide synthase activity, can induce retinal angiogenesis.[55,56] The nitric oxide synthase inhibitor, L-NNA, prevents the formation of nitric oxide and subsequently peroxynitrite formation.[57] In the present study, L-NNA was evaluated at three different dosages (400, 200 and 100 μM) and found to have no effect in blocking the B(e)p induced ROS/RNS production.
of transcription 3 (STAT3), a known transcription factor that induces inflammation and extracellular signal-regulated kinase and mitogen-activated protein kinase (ERK-MAP kinase) cascade, which mainly promotes cell proliferation. Increased serum levels of IL-6 are related to AMD progression. Formation of CNV by laser treatment caused IL-6 expression in the RPE-choroid complex. Significant suppression of CNV happened due to antibody-based blockade of IL-6 receptor or genetic ablation of IL-6. The present study demonstrated that after 24 hours of treatment with B(e)p, the ARPE-19 cells showed significant increase of IL-6 levels. These findings suggest that the cigarette smoke element B(e)p could elevate IL-6 levels and possibly contribute to the inflammatory environment found in retinal diseases such as AMD. Further animal studies will be necessary to verify our tissue culture findings.

In ARPE-19 cultures, B(e)p induced a 28% increase in GM-CSF levels (P = 0.0001). GM-CSF is a glycoprotein that has been localized to the retina and functions to stimulate differentiation of hematopoietic progenitor cells into tissue-resident dendritic cells, macrophages and granulocytes. Recent studies suggest neuro-protective properties of GM-CSF in cerebral neurons and retinal cells. While GM-CSF has been associated with retinal ganglion cells, this is the first study to show that it is produced by the ARPE-19 cells. The significance of higher levels of GM-CSF after B(e)p treatment is speculative and requires further investigation.

In summary, we found that smoking component B(e)p generates ROS/RNS most probably through mitochondrial complex III and induces inflammatory processes through IL-6 and GM-CSF, which makes them targets for drug therapy to diminish the levels of oxidative stress and inflammation in human RPE cells.

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Conflicts of Interest
There are no conflicts of interest.

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