An Environmental Quinoid Polycyclic Aromatic Hydrocarbon, Acenaphthenequinone, Modulates Cyclooxygenase-2 Expression through Reactive Oxygen Species Generation and Nuclear Factor Kappa B Activation in A549 Cells

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Received July 26, 2006; accepted October 24, 2006

Diesel exhaust particles (DEPs) contain oxygen-containing polycyclic aromatic hydrocarbons (PAHs) called quinoid PAHs. Some quinoid PAHs generate free radicals as they undergo enzymatic and nonenzymatic redox cycling with their corresponding semiquinone radicals. Reactive oxygen species (ROS) produced by these reactions can cause severe oxidative stress connected with inflammatory processing. Although humans and animals are continuously exposed to these chemicals in the environment, little is known about which quinoid PAHs are active. In this study, we estimated the intracellular ROS production and nuclear factor kappa B (NF-κB) translocation in A549 cells exposed to isomers of quinoid PAHs having two to four rings. We found that both acenaphthenequinone (AcQ) and 9,10-phenanthrenequinone (PQ) enhanced ROS generation and that AcQ translocated NF-κB from the cytosol to the nucleus. However, PQ, which has been reported to induce apoptosis, did not influence NF-κB activation. In addition, AcQ induced cyclooxygenase-2 (COX-2) expression which is a key enzyme in the inflammatory processing involved in the activation of NF-κB. Upregulation of NF-κB and COX-2 expression by AcQ treatment was suppressed by the antioxidant N-acetylcysteine (NAC). These results provide that AcQ might play an important role in human lung inflammatory diseases as an air pollutant.

Key Words: quinoid PAHs; ROS; NF-κB; COX-2.

Diesel exhaust particles (DEPs) are one of the major urban air pollutants. DEPs are easily deposited in the airways and alveoli through respiration, thereby inducing oxidative stress and inflammation. Recently, adverse human health effects, including airway and cardiovascular diseases, particularly in persons with preexisting lung diseases caused by DEPs have been reported (Atkinson et al., 2001; Auger et al., 2006). DEPs are mainly composed of carbon nuclei which adsorb a vast number of organic compounds such as polycyclic aromatic hydrocarbons (PAHs), nitro PAHs, quinones, heterocyclics, aldehydes, and aliphatic hydrocarbons (Bai et al., 2001; Draper, 1986). Although many studies have clarified molecular signaling pathways of PAHs in several human diseases, quinoid PAHs have received relatively little attention.

Reactive oxygen species (ROS), such as superoxide anions, hydroxyl radicals, and hydrogen peroxides, are highly reactive and modify intracellular molecules. Numerous oxidants generated by stimuli inside and outside the cells upset the redox balance and subject the cells to oxidative stress conditions (Bai et al., 2005). Moreover, accumulation of ROS causes severe oxidative stress within cells through the formation of oxidized cellular macromolecules, including lipids, proteins, and DNA (Kikuno et al., 2006). Oxidation of these molecules is known to be associated with damage to cells, and this damage induces various biological responses such as inflammation and apoptosis. Quinones and PAHs, which are contained in particulate matter (PM) and DEP (Cho et al., 2004), produce ROS through redox cycling directly or indirectly (Kumagai et al., 2002; Nel et al., 2001; Shima et al., 2006). Moreover, quinones induce oxidative stress by the generation of ROS and reduction of cellular glutathione (GSH) level (Bolton et al., 2000). These facts lead to a number of signaling pathways, such as the mitogen-activated protein kinase pathways and nuclear factor kappa B (NF-κB) signal pathways.

Mainly, NF-κB consists of two major subunits, p50 (NF-κB1) and p65 (RelA), and represents a family of transcription factors that participate in regulating the immune response, cell survival, inflammation, and cancer (Ghosh and Karin, 2002; Mann and Oakley, 2005). In cells, NF-κB exists in an inactive cytoplasmic form but enters the nucleus in response to various stimuli including oxidants and certain environmental stresses.
(Huang et al., 2002; Stancovski and Baltimore, 1997). On activation, NF-κB regulates the expression of almost 400 different genes, which include enzymes (e.g., cyclooxygenase-2 [COX-2] and iNOS), cytokines (such as TNF, IL-1, IL-6, IL-8, and chemokines), adhesion molecules, cell cycle regulatory molecules, and angiogenic factors (Ahn and Aggarwal, 2005). For example, DEP was shown to contribute to the inflammatory response by increasing the activation of NF-κB in airway epithelial cells (Bonvallot et al., 2001) and DEP-induced transactivation of NF-κB is mediated by PI3K/Akt signaling pathway (Ma et al., 2004). These findings revealed that under certain conditions, DEP induces inflammatory processing by the activation of NF-κB.

Prostaglandins are synthesized through the activities of two isoforms of COX, designated COX-1 and COX-2 (Fitzpatrick, 2004). COX-1 is constitutively present in most cells, whereas COX-2 is not detected in most normal tissue, but is rapidly induced by lipid peroxides, oxidant stress, NF-κB, cytokines (TNF-α, IL-1β, IL-6), and transforming growth factor β1, all of which are considered to be proinflammatory stimuli (Yu et al., 2006). Thus, the transcription of COX-2 may be modulated by NF-κB. In fact, the COX-2 gene contains numerous cis-acting promoter elements, including NF-κB sites (Chen et al., 2005). NF-κB plays a critical role in mediating COX-2 expression (Chung et al., 2002; Jung et al., 2006; Zha et al., 2004). In addition, recent studies showed that enhanced expression of COX-2 might be due to increased production of ROS, and antioxidants significantly decreased COX-2 expression by attenuated prostaglandins production (Hayek et al., 1997; Wu et al., 1998).

In the present study, we hypothesized that redox-sensitive transcription factor NF-κB and its dependent enzyme COX-2 induction might involve environmental quinoid PAHs to human lung diseases. We tested six PAHs having two to four rings and all of their quinoid derivatives, all of which are normally inducers of ROS and NF-κB activity and thus a role in human lung diseases through COX-2 expression.

MATERIALS AND METHODS

Cells and culture conditions. A549, human lung carcinoma, cells were obtained from Riken Gene Bank (Tsukuba, Japan). Cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) Media supplemented with 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO) in a humidified 5% CO₂ at 37°C containing 100 IU/ml penicillin, 100 mg/ml streptomycin (Wako Pure Chemical, Osaka, Japan), and 2.5 mg/l amphotericin B (Sigma-Aldrich). For all experiments, cells were used at the exponential phase and plated in 100-mm culture dishes, and cultures at 70–80% confluence were used for the exposures. Cells were allowed to adhere to the dish overnight, and then the culture medium was replaced with fresh DMEM (serum free or 1%) with or without test compounds as indicated.

Chemicals and reagents. AcQ, PQ, and chrysene (Chr) were purchased from Sigma-Aldrich. Acenaphthene (ANT), anthracene, anthraquinone, 1,2-benzanthraquinone, and 1,4-chrysenequinone (CQ) were purchased from Wako Pure Chemical. Naphthalene was purchased from Kanto Chemical (Tokyo, Japan). 1,2-Naphthoquinone and phenanthrene (PA) were purchased from Tokyo Chemical (Tokyo, Japan). 1,4-Anthraquinone and phenanthrene-1,4-quinone were purchased from Chiron AS (Trondheim, Norway). Benz[a]anthracene was purchased from Nakalai Tesque (Kyoto, Japan). The stock solutions of these test compounds were maintained in dimethylsulfoxide (DMSO) solutions and were added directly to the cell culture medium as 1000× stocks to give the desired final concentration. N-acetylcysteine (NAC), ROS scavenger, was purchased from Sigma-Aldrich. BAY 11-7085, NF-κB inhibitor, was purchased from Wako Pure Chemical.

Intracellular ROS activity. Cells were inoculated at a density of 1 × 10⁴ cells per well in a Falcon 96-well plate and were allowed to adhere overnight. Cells were then incubated in serum-free DMEM-containing chemicals and 10µM 2′,7′-dichlorofluorescin diacetate (DCFDA, Sigma-Aldrich) at 37°C. The change in fluorescence was measured using a Fluoroskan Ascent FL (Thermo Electron, Waltham, MA) at excitation and emission wavelengths of 485 and 530 nm, respectively. A fluorometric assay was performed to determine the relative levels of ROS, such as superoxide radical, hydroxyl radical, and hydrogen peroxide. This assay measures the oxidative conversion of stable, nonfluorescent DCFDA to the highly fluorescent 2′,7′-dichlorofluorescein in the presence of esterases and ROS, especially hydrogen peroxide (Lee et al., 2004).

Protein preparation and Western blot analysis. Cells were harvested and washed twice in phosphate-buffered saline (PBS) at 4°C. Total cell lysates were lysed in lysis buffer (20mM Hepes-NaOH [pH 7.5], 60mM β-glycerol phosphate, 20mM NaF, 150mM NaCl, 5mM EGTA, 1mM Na₂-EDTA, 0.5% Tween-20, 0.5% NP-40, 0.5% sodium deoxycholate, 1mM DTT, 2 µg/ml leupeptin). The supernatant was collected, and protein concentrations were then determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL).

Luciferase reporter gene assay. Cells were harvested and washed with PBS. The cells were suspended in 5 ml of transfection medium of FBS-free OPTI-MEM I medium (GIBCO, Grand Island, NY) containing 20 µg of luciferase reporter vector and 50 µl of LipofectAMINE (Invitrogen, Carlsbad, CA) and then transfected for 30 min at 37°C. The reporter vectors transfected to cells were NF-κB promoter-driven luciferase expressing plasmid pTAL-NF-κB. Then, 25 ml of assay medium was added to the cell suspension and the cells were plated on 48-well plates at a cell density of 5 × 10⁴ cells (300 µl of diluted cell suspension solution) per well. After 18 h, the cells were washed with a fresh assay FBS-free medium were treated with DMSO and each test compound. The final DMSO concentration in the assay medium was adjusted to 0.1% (vol/vol). After the treatment, the cells were harvested in 50 µl of PicaGene cell lysis buffer LUC (Toyo Ink, Tokyo, Japan). Luciferase activity in the cell lysate was assayed using a PicaGene luciferase kit (Toyo Ink).
Reverse transcriptase-polymerase chain reaction. Total RNA was isolated using TRIzol reagent (Invitrogen), as per the manufacturer’s instructions. Two micrograms of total RNA were used to generate cDNA template for RT-PCR. The first strand synthesis was performed using a random hexamers (Promega, Madison, WI) and, Superscript II reverse transcriptase (Invitrogen). The first strand cDNA products were further diluted and used as PCR template. The PCR reaction in a total volume of 25 μl containing 0.25 units of Taq polymerase, 0.25mM dNTPs, PCR buffer (PE Applied Biosystems, Foster City, CA), and 50 ng of the relevant oligonucleotide primers. Separate PCRs for COX-2 amplification were performed using the following primers: COX-2, sense 5′-CTGGCTAGACAGCGTAAACT, antisense 5′-GATACTTTCGT-TACGTGGG (product size 632 bp). PCR conditions allowing to be in the exponential phase of PCR were: number of cycles (n) = 26, annealing temperature (AT) = 49°C for COX-2. In this study, GAPDH was used as the expression control. PCR reaction products were separated on a 1.2% agarose gel and visualized under ultraviolet light after ethidium bromide staining.

Enzyme immunoassay for prostaglandin E2. Cells were transferred to a 48-well plate and were allowed to adhere overnight. Cells were incubated for 6 h in 1% FBS DMEM in the presence of 1–15μM AcQ, and the level of prostaglandin E2 (PGE2) present in the media was analyzed using an enzyme immunoassay kit (R&D Systems, Minneapolis, MN), and measurements were made according to the manufacturer’s instructions.

Statistics. The results are presented as means ± SE. Statistical significance of differences between the untreated control and the treated groups were determined using one-way ANOVA. A value of p < 0.05 was considered to be significant.

RESULTS

Enhancing Effect of PAHs and Quinoid PAHs on ROS Generation

We selected six PAHs, having two, three, and four aromatic rings and eight of their quinoid derivatives, all of which are present in the environment. Of these compounds, only benz[a]anthracene is reported to be carcinogenic impact according to WHO (1998). Among quinoid PAHs, a few reports have shown that PQ has a positive effect on apoptosis via ROS generation (Sugimoto et al., 2005). However, it is not known whether other quinoid PAHs can generate ROS. Therefore, we first examined the effects of eight quinoid PAHs and their parent PAHs on intracellular ROS generation in A549 cells. Five compounds (ANT, AcQ, PQ, Chr, and CQ) significantly increased the ROS level (Table 1), whereas the others did not. Exposure of cells to the parent PAHs (ANT and PA) increased cellular ROS levels in a concentration-dependent manner, while exposure of cells to the parent PAHs (ANT and PA) increased ROS levels either slightly or not at all (Fig. 1). Exposure of cells to Chr and CQ did not significantly increase the ROS level.

Effect of NF-κB is Enhanced by AcQ

To examine the effect of AcQ and PQ on NF-κB activation, we transiently transfected A549 cells. After 18 h incubation, cells were treated with 5μM of AcQ and PQ for 1 h. Treatment of A549 cells with 5μM of AcQ markedly enhanced NF-κB luciferase activity (Fig. 2A). AcQ also increased both p65 and p50 protein levels in the nuclear extracts. Interestingly, PQ had little effect on NF-κB luciferase activity or protein levels compared to AcQ. It is important to note that, at a given concentration, AcQ resulted in greater NF-κB than PQ (Fig. 2A). Consequently, AcQ was used in the following experiments.

AcQ significantly stimulated NF-κB luciferase activity in a concentration-dependent manner (Fig. 2B). To confirm the

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>ROS (% of control)</th>
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<tbody>
<tr>
<td>None</td>
<td></td>
<td>100.00 ± 3.14</td>
</tr>
<tr>
<td>Naphthalene</td>
<td></td>
<td>93.31 ± 1.50</td>
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<tr>
<td>1,2-Naphthoquinone</td>
<td></td>
<td>65.98 ± 0.03***</td>
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<td>Acenaphthene</td>
<td></td>
<td>120.48 ± 0.74***</td>
</tr>
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<td>AcQ</td>
<td></td>
<td>147.64 ± 0.63***</td>
</tr>
<tr>
<td>Anthracene</td>
<td></td>
<td>96.83 ± 2.11</td>
</tr>
<tr>
<td>Anthraquinone</td>
<td></td>
<td>89.85 ± 0.77*</td>
</tr>
<tr>
<td>1,4-Anthraquinone</td>
<td></td>
<td>86.44 ± 3.54*</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td></td>
<td>100.86 ± 0.17</td>
</tr>
<tr>
<td>PQ</td>
<td></td>
<td>156.68 ± 1.16***</td>
</tr>
<tr>
<td>Phenanthrene-1,4-quinone</td>
<td></td>
<td>92.95 ± 1.22</td>
</tr>
<tr>
<td>Benz[a]anthracene</td>
<td></td>
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</tr>
<tr>
<td>Benz[a]anthracene-7,12-quinone</td>
<td></td>
<td>96.02 ± 3.17</td>
</tr>
<tr>
<td>Chr</td>
<td></td>
<td>122.24 ± 0.54***</td>
</tr>
<tr>
<td>CQ</td>
<td></td>
<td>128.24 ± 1.60***</td>
</tr>
</tbody>
</table>

Note. ROS was detected using DCFDA after treatment of test compounds (5μM) for 1 h. Data are shown as the percent increase compared to untreated cells and are presented as mean ± SE of triplicate measurements. Statistical significance: *p < 0.05 and ***p < 0.001 versus untreated control.
translocation of NF-κB, we examined the Western blot analysis using primary antibodies for the p65 and p50 subunits of NF-κB in cytosolic and nuclear fractions. Treatment of cells with AcQ dramatically decreased the amounts of p65 and p50 in the cytosol but increased their levels in the nucleus. These data clearly show that AcQ causes nuclear accumulation of NF-κB which is a sign of proinflammatory behavior.

Effects of NAC on AcQ-Induced Responses

Preincubation of A549 cells with the oxidant NAC significantly decreased the increase of ROS level produced by 15 μM AcQ (Fig. 3A). To clarify whether ROS is involved in AcQ-induced NF-κB activation, we preincubated the A549 cells with 4 mM NAC for 1 h and then exposed 1–15 μM AcQ for 1 h. NAC also inhibited the AcQ-dependent NF-κB activation by reducing the levels of p65 and p50 (Fig. 3B top panel) and repressed the AcQ-stimulated NF-κB luciferase activity (bottom panel). These data demonstrate that NAC, a strong antioxidant, blocked NF-κB activity induced by AcQ.

Induction of COX-2 Expression by AcQ is Mediated ROS and NF-κB

To examine the effect of AcQ on COX-2 expression for 6 h poststimulation in A549 cells, we exposed the cells to 1–15 μM AcQ. Production of PGE2, a major COX product in A549 cells, was significantly increased by AcQ in a concentration-dependent manner (Fig. 4A). Consistent with this observation, AcQ exposures led to induction of COX-2 protein level. Exposure of cells to AcQ, especially at a concentration of 15 μM increased both the COX-2 protein level and the PGE2 expression (Fig. 4B). However, the increases of COX-2 protein and PGE2 were significantly suppressed by the addition of 4 mM NAC or 10 μM BAY 11-7085, a NF-κB inhibitor. Interestingly, PQ, an even better oxidant, had no effect on COX-2 mRNA and protein levels compared to AcQ. At a given concentration, AcQ resulted in greater COX-2 than PQ (Fig. 4C). These results strongly indicate that only AcQ-induced COX-2 expression is involved in the generation of ROS and activation of NF-κB.

DISCUSSION

PAHs and numerous structurally related PAH derivatives, which are widespread as environmental contaminants, are byproducts of incomplete combustion of organic materials (Hayakawa et al., 2006). Consequently, the continuous exposure and inhalation of these chemicals threaten human health gradually and cause several chronic diseases, including inflammation, allergy, and cancer (Durant et al., 1996; Knize et al., 1999; Takizawa et al., 2003; Wogan et al., 2004). In addition, some PAHs species are chemically oxidized to quinones by cytochrome P450 enzymes, which generate ROS. These quinones are able to participate in an establishing futile redox cycle during which cytotoxic ROS is accumulated (Bolton et al., 2000; Shima et al., 2006). This redox cycling between quinones and semiquinone radicals is responsible for the cytotoxic properties of the parent PAHs and leads to lipid peroxidation, DNA oxidation, and DNA strand breaks (Bai et al., 2005). Through this mechanism, quinones contribute to cellular oxidative hazardous effects including acute cytotoxicity, immunotoxicity, and carcinogenesis. Compared to other environmental pollutants, however, the cellular toxic effects of quinones have received little attention.

Among the compounds tested, AcQ and PQ have much stronger abilities to enhance the ROS level than their parent PAHs. Moreover, AcQ and PQ were able to change the intracellular GSH level in a concentration-dependent manner (data not shown). Interestingly, we found that 1,4-naphthoquinone but not CQ can considerably decrease the ROS level in a concentration-dependent manner. To explain this, additional
studies may be required to determine the exact molecular pathway. PQ was recently shown to produce singlet oxygen and hydroxy radicals and to cause oxidative damage leading cells to apoptosis (Kumagai et al., 2002; Sugimoto et al., 2005). However, few reports have examined the adverse effects of other quinoid PAHs on the redox balance. Especially, little is known about how exposure to quinoid PAHs affects NF-κB activation in human cells. Our results show that one of the quinoid PAHs, AcQ, increases ROS generation which results in NF-κB activation in A549 cells.

Oxidative stress is described generally as a condition under which increased production of free radicals, ROS (including singlet oxygen and reactive lipid peroxidation products, such as reactive aldehydes and peroxides), and oxidant-related reactions result in damage to cells. Within this definition of oxidative stress, emphasis is placed on the damages of the cellular...
structure and function (Yu and Chung, 2006). Accumulation of ROS by oxidant stress can affect gene transcription through its effect on redox-sensitive transcription factor NF-κB. Upon activation by several oxidants, the p65/p50 dimer translocates into the nucleus where it activates mainly proinflammatory genes (Chung et al., 2002; Lee et al., 2004). PAHs and/or other environmental pollutants are known to generate ROS and transactivate NF-κB (Burdick et al., 2003; Cho et al., 2005; Li et al., 2004; Shishodia et al., 2003). The major goal of our study was to determine whether quinoid PAHs activate NF-κB translocation and have a role in the proinflammatory process. We found that AcQ had a marked effect on NF-κB activation and the activity was stronger than that of PQ (Fig. 2). The remarkable difference between AcQ and PQ may be due to their different effects on cells, such as causing cellular apoptosis or an inflammatory process. Furthermore, pretreatment of cells with NAC prevented the ROS generation enhanced by AcQ and abrogated AcQ-induced NF-κB activation (Fig. 3). These results support the hypothesis that the enhancement of ROS by AcQ plays a role in the NF-κB activation pathway than PQ.

Our results further indicated that AcQ-induced oxidative stress and AcQ-enhanced NF-κB activation lead to the constitutive expression of proinflammatory genes such as COX-2. Unlike COX-1, which is constitutively expressed, COX-2, which is entirely related to the inflammatory process, is an isoform induced by various stimuli (Chen et al., 2000; Lee et al., 2005; Mitchell et al., 1994; Patel et al., 2005). However, the relationship between quinoid PAHs and inflammatory processing remains unclear. Here, we showed that incubation of A549 cells with AcQ induced PGE2 production and COX-2 expression in a concentration-dependent manner after 6 h exposure (Fig. 4). We also found evidence that the induction of expression of COX-2 in A549 cells by AcQ was a direct response of ROS generation and NF-κB activation. Similarly, recent papers showed that suppression of ROS generation inhibits NF-κB–modulated COX-2 expression (Barbieri et al., 2004; Lu and Wahl, 2005; Yang et al., 2005). Moreover, AcQ contributed to change another NF-κB–regulated enzyme, iNOS expression via the ROS generation and NF-κB activation. Similarly, recent papers showed that suppression of ROS generation inhibits NF-κB–modulated COX-2 expression (Barbieri et al., 2004; Lu and Wahl, 2005; Yang et al., 2005). Moreover, AcQ contributed to change another NF-κB–regulated enzyme, iNOS expression via the ROS generation and NF-κB activation (data not shown). Thus, we speculate that AcQ is able to affect proinflammatory processing by these NF-κB signal cascades. Our finding supports the hypothesis that a potential quinoid PAHs, AcQ, contribute to COX-2 expression through the ROS generation and NF-κB activation other than quinoid PAHs tested. However, the mechanism by which process can generate ROS by AcQ is still unclear. Further studies are needed to elucidate the exact source of ROS production by AcQ.

In summary, our results demonstrate that some quinoid PAHs, especially AcQ and PQ, increase oxidative stress as represented by an increase of intracellular ROS and depletion of GSH. Although both compounds increased oxidative stress, AcQ more strongly up-modulated NF-κB activity than did PQ. In addition, AcQ increased the expression of the proinflammatory protein COX-2 via NF-κB activation. However, PQ had no effect on the COX-2 mRNA and protein level. The reason why PQ is not effective on COX-2 expression may be that the toxic effect of PQ is leading to apoptosis. This indicates that AcQ is a potential quinoid PAH that might play a role in inflammatory processing in human pulmonary lung disease.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci. oxfordjournals.org/.
ACKNOWLEDGMENTS

This work was supported in part by the 21st Century center of excellence program and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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