Aryl Hydrocarbon Receptor- and Calcium-dependent Induction of the Chemokine CCL1 by the Environmental Contaminant Benzo[a]pyrene*

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Polycyclic aromatic hydrocarbons (PAHs) are widely distributed immunotoxic environmental contaminants well known to regulate expression of pro-inflammatory cytokines such as interleukine-1β and tumor necrosis factor-α. In the present study, we demonstrated that the chemokine CCL1, notably involved in cardiovascular diseases and inflammatory or allergic processes, constitutes a new molecular target for PAHs. Indeed, exposure to PAHs such as benzo[a]pyrene (BP) markedly increased mRNA expression and secretion of CCL1 in primary human macrophage cultures. Moreover, intranasal administration of BP to mice enhanced mRNA levels of TCA3, the mouse orthologue of CCL1, in lung. CCL1 induction in cultured human macrophages was fully prevented by targeting the aryl hydrocarbon receptor (AhR) through chemical inhibition or small interfering RNA-mediated down-modulation of its expression. In addition, BP and the potent AhR agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin were found to enhance activity of a CCL1 promoter sequence containing a consensus xenobiotic-responsive element known to specifically interact with AhR. Moreover, 2,3,7,8-tetrachlorodibenzo-p-dioxin triggered AhR binding to this CCL1 promoter element as revealed by chromatin immunoprecipitation experiments and electrophoretic mobility shift assays. In an attempt to further characterize the mechanism of CCL1 induction, we demonstrated that BP was able to induce an early and transient increase of intracellular calcium concentration in human macrophages. Inhibition of this calcium increase, using the calcium chelator 1,2-bis(o-aminophenoxy)ethane-N,N',N''-tetraacetic acid tetra(acetoxymethyl) ester or the calcium store-operated channel inhibitor 2-aminoethoxydiphenyl borate, fully blocked CCL1 up-regulation. Taken together, these results bring the first demonstration that PAHs induce expression of the chemokine CCL1 in an AhR- and calcium-dependent manner.

3 The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; AhR, aryl hydrocarbon receptor; CYP, cytochrome P450; BP, benzo[a]pyrene; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; BeP, benzo[e]pyrene; 2-APB, 2-aminoethoxydiphenylborate; XRE, xenobiotic-responsive elements; ChIP, chromatin immunoprecipitation assays; 3′ M4NF, 3′-methoxy-4′-nitroflavone; Fura-2-AM, Fura-2-acetoxymethylester; CCL1, CC- chemokine ligand 1; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N',N''-tetraacetic acid tetra(acetoxymethyl) ester; RT, reverse transcription; RT-qPCR, reverse transcription-real-time quantitative-PCR; ELISA, enzyme-linked immunosorbent assay; siRNA, small interference RNA; SIaHR, AhR siRNA; SIaHRmut, AhR-mutated siRNA.

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sions of IL-1β, IL-8, or tumor necrosis factor-α occur in cells exposed to PAHs or to other potent AhR agonists such as TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), which may contribute to the adverse inflammatory or immunotoxic effects due to PAH exposure (14–17). To identify new cytokines regulated by PAHs, we have recently analyzed the transcriptome of BP-treated human macrophages using microarrays. Data from these experiments suggest that CCL1 (CC-chemokine ligand 1, also named I-309), a chemokine triggering Th2 immune response and strongly implicated in cardiovascular diseases, asthma, and allergic inflammation (18–20), may be up-regulated by AhR agonists.

In the present study, we demonstrate for the first time that BP markedly induces CCL1 production in human macrophages by an AhR-dependent mechanism. Moreover, BP was shown to trigger an early and transient increase of intracellular calcium concentration, which seems essential to CCL1 up-regulation by this environmental contaminant.

### EXPERIMENTAL PROCEDURES

**Reagents**—PAHs, α-naphthoflavone (αNF), resveratrol, and 2-aminoethoxydiphenylborate (2-APB) were purchased from Sigma-Aldrich. TCDD was obtained from Cambridge Isotope Laboratories (Cambridge, MA), and 1,2-bis(α-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA-AM) was obtained from Calbiochem. Pluronic and Fura-2 acetoxy-methylester (Fura-2-AM) were provided from Molecular Probes (Eugene, OR), and Ficol was obtained from Amersham Biosciences. Granulocyte macrophage-colony stimulating factor (specific activity, 1.2 × 10^6 units/mg) was purchased from Schering Plough (Lyon, France). 3’-Methoxy-4’-nitroflavone (3’M4’NF) was a kind gift from Dr. T. Gasiewicz (Dep’t of Environmental Medicine, University of Rochester Medical Center, Rochester, NY). Polyclonal rabbit antibody anti-AhR was obtained from Biomol Research Laboratories (Plymouth, PA), and nonspecific IgG peroxidase-conjugated donkey anti-rabbit was from Amersham Biosciences. All other reagents were commercial products of the highest purity available. Chemicals were commonly used as stock solutions in Me2SO. The final concentration of solvent did not exceed 0.2% (v/v); control cultures received the same dose of solvent as for treated counterparts.

**Cell Culture**—Primary human macrophages were obtained from blood monocytes as previously described (21). Briefly, mononuclear cells, isolated from blood buffy coats (kindly provided by the Etablissement Français du Sang, Rennes, France) through ficoll gradient centrifugation, were initially seeded at the density of 2 × 10^6 cells per cm^2 into plastic culture plates for 2 h. Non-adherent cells were then removed, and adherent monocytes were further cultured at 37 °C under 5% CO2 for 6 days in RPMI 1640 medium supplemented with 10% decomplemented fetal bovine serum (Invitrogen), 2 μg/ml genistein, 100 units/ml penicillin, 10 μg/ml streptomycin, and 400 units/ml granulocyte macrophage-colony stimulating factor. Such a protocol allows the obtainment of pure macrophage cultures with <1% of contaminating cells. Macrophages were then routinely cultured in the medium described above.

Human endothelial EAhy-926 cells (22) (generously provided by Dr. C. J. Edgell, University of North Carolina, Chapel Hill) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 10 μg/ml streptomycin, and 2 μg/ml genistein.

**Animal Treatment**—Male adult C57Bl/6 mice (10 weeks old, weighing 25 g) were treated by intranasal instillation of 500 μg of BP dissolved in tricaprylin (50 μl/mouse) under etomidate anesthesia. Control mice received tricaprylin only. Twenty-four hours after BP treatment, mice were quickly anesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg) and underwent removal of lungs, which were stored at −80 °C until analysis.

**RNA Isolation and Analysis**—Total RNAs were extracted from cells or lungs using the TRIzol method (Invitrogen). They were then subjected to semiquantitative reverse transcription (RT)-PCR or to reverse transcription-real-time quantitative-PCR (RT-qPCR) analyses as previously described (23, 24). For RT-PCR assays, cDNA aliquots were amplified using Master mix (Promega) and 0.1 μM of forward and reverse gene primers listed in Table 1. Each PCR cycle consisted of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. β-Actin primers were used as an internal control. PCR products were finally visualized using ethidium bromide-staining agarose gels. RT-qPCR assays were performed using the fluorescent dye SYBR Green methodology and an ABI Prism 7000 detector (Applied Biosystems, Foster City, CA).
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City, CA). Gene-specific primers (Table 1) were designed with the Primer3 software (available at frodo.wi.mit.edu/cgi-bin/primer3_www.cgi). The specificity of each gene amplification was checked up to the end of qPCR reactions through the analysis of dissociation curves of the PCR products. Amplification curves were read with ABI Prism 7000 SDS software using the comparative cycle threshold method. Relative quantification of the steady-state target mRNA levels was performed after normalization of the total amount of cDNA tested to an 18S RNA endogenous reference.

Determination of Cytokine Levels—Levels of CCL1 and IL-1β in the supernatants of macrophage cultures were quantified using Duoset ELISA development system kits obtained from R&D systems (Abington, UK). Briefly, 96-well plates, initially coated with 1 μg/ml mouse anti-human CCL1 or IL-1β antibody overnight at room temperature, were incubated for 2 h with macrophage culture supernatants or recombinant human CCL1 or IL-1β standard. After washes, plates were processed according to the manufacturer's instructions.

Reporter Gene Constructs and Luciferase Assays—The 2975-bp region of the CCL1 gene promoter (GenBankTM under accession number AC002482), spanning -3038 to -63, was obtained through PCR amplification and cloned into the firefly luciferase reporter vector pGL3 basic (Promega); the resulting plasmid construct was designed as pCCL1-FL(-2975). The pCCL1-FL.Mut(-2975) plasmid corresponds to the pCCL1-FL(-2975), with three mutations located at positions -312, -311, and -310 on the XRE consensus site located at -312 (consensus XRE-312: GCCTG; mutated XRE-312: GCCCA). The pCYP1A1-FL(-1639) construct containing a 1639-bp region (from -1566 to +73) of the human CYP1A1 gene upstream of the firefly luciferase reporter gene had been previously described (25) and was a kind gift from Dr. R. Barouki (INSERM U490, Paris, France).

EAhy-926 cells cultured in 24-well plates were transiently co-transfected with one of the firefly luciferase promoter-containing plasmids described above and the Renilla luciferase-encoding pRL-TK vector (Promega), used as an internal control, according to the LipofectamineTM 2000 method (Invitrogen). Briefly, 400 μl of transfection medium (OptiMEM) containing 200 ng of luciferase reporter plasmid was added per well of confluent EAhy-926 cells along with 20 ng of the pRL-TK plasmid and 0.4 μl of LipofectamineTM 2000. After a 24-h period, cells were exposed to PAHs for another 24-h period. Dual luciferase assays (firefly and Renilla) were then performed with a Promega kit according to the manufacturer's instructions. pGL3 basic (a promoterless pGL3-luciferase construct) and pGL3 SV40 (a pGL3-luciferase construct with the SV40 promoter) vectors were used in parallel as negative and positive firefly luciferase controls, respectively.

Design and Transfection of AhR-targeted Specific siRNA—Small interfering RNA (siRNA) oligonucleotide sequences, designed as recommended (26) with two 2′-deoxymethylene overhands on each strand, were: AhR siRNA (SiAhR), 5′-AAG-UCCGGUCUCUAUGCCGCTT-3′, and control AhR-mutated siRNA (SiAhRmut), 5′-AACUCGGUCUCUAUGCCGCTT-3′. The absence of matching of these sequences with that of any other human gene was checked by using the NCBI standard nucleotide-nucleotide BLAST program. Transfection of siRNAs was performed using the HiPerFect Transfection kit (Qiagen). Macrophages, cultured in 24-well plates, were incubated with the transfection mix containing 5 nM siRNA and 4.5 μl of HiPerFect reagent in 500 μl of culture medium, in agreement with the manufacturer’s instructions. After a 40-h incubation, the transfection medium was removed, and cells were thereafter exposed to BP for an additional 8-h period.

Chromatin Immunoprecipitation (ChIP) Assays—ChIP assays were performed essentially as previously described (27, 28). Briefly, macrophages either untreated or exposed to BP or TCDD for 30 min, were cross-linked with 1% formaldehyde in phosphate-buffered saline at room temperature for 10 min. After washing and suspension in cell lysis buffer, chromatin was digested using the BamH1 restriction enzyme at 37 °C overnight. Protein-DNA complexes were then immunoprecipitated using rabbit polyclonal antibody against AhR or nonspecific IgG anti-rabbit and protein G-Sepharose as described by Corre et al. (29). The recovered DNA was then subjected to PCR using primers listed in Table 1 and covering XRE sequences found in CCL1 and CYP1A1 promoter (positive control) (30) or an XRE-free sequence of the tyrosinase gene (negative control) (29).

Electrophoretic Mobility Shift Assays—Nuclear extracts were isolated from cultured macrophages using the BD TM Transfctor extraction kit (BD Biosciences). Binding of nuclear extracts to [γ-32P]ATP (Amersham Biosciences) end-labeled double-stranded oligonucleotides was then analyzed with the Promega gel shift assay system (Promega). Two double-stranded oligonucleotides, corresponding to (−326 to −294) region of the CCL1 promoter were used: the wild-type CCL1-312-WT (5′-ATCTTGAGAAAGTGCGTGGGAACTGTCCTGGT-3′) (underlined characters represent the core XRE) and the mutated CCL1-312-Mut (5′-ATCTTGAACAGTGCCAAAGGAACCTGTCCTGGT-3′). DNA-protein complexes were separated by electrophoresis on a polyacrylamide gel and detected by autoradiography.

Intracellular Calcium Measurements—Variations in intracellular concentrations of calcium were analyzed by microspectrofluorometry using the Ca2+ -sensitive probe Fura-2-AM as previously reported (13). Briefly, macrophages were incubated at 37 °C in cell suspension buffer (134.8 mM NaCl; 4.7 mM KCl; 1.2 mM K2HPO4; 1 mM MgCl2; 1 mM CaCl2; 10 mM glucose; 10 mM HEPES; pH 7.4) supplemented with 1.5 μM Fura-2-AM and 0.006% pluronic acid. Following probe loading, cells, placed in a continuously perfused recording chamber mounted on the stage of an epifluorescence microscope (Nikon Diaphot), were irradiated alternately with 340 and 380 nm light, and fluorescence from the trapped dye was measured at 510 nm. The ratio of fluorescence intensities recorded after excitation at 340 nm (F340) and at 380 nm (F380) was used to estimate intracellular concentrations ([Ca2+]i). The monochromator and the photometers, which allow emission and detection of fluorescence from 3 to 5 cells in the field of view, were part of a DeltaRAM system from Photon Technology International (PTI, Birmingham, UK), which also provided software systems to acquire and process data.

Statistical Analysis—Results are presented as mean ± S.D. Significant differences were routinely evaluated with the non-
parametric Wilcoxon test except for the variations of intracellular calcium concentration for which the Mann-Whitney U test was used. The level of significance was p < 0.05.

RESULTS

Effect of BP on CCL1 Expression and Production in Human Macrophages and on TCA3 Expression in Mouse Lung—CCL1 chemokine expression was investigated in primary human macrophages exposed to various BP concentrations (from 0.1 to 20 μM) for 24 h (Fig. 1A). As for CYP1A1, used here as positive control of treatment, the BP effect on CCL1 mRNA levels was dose-dependent: CCL1 mRNA up-regulation was detected after 0.1 μM BP, reaching a maximum after 10 μM BP. Such data, obtained with semiquantitative RT-PCR assays, were confirmed using RT-qPCR analyses, which revealed a 3.9 ± 2.2-, 12.6 ± 6.6-, and 27.9 ± 9.9-fold induction of CCL1 mRNA levels in response to 0.1, 1, and 10 μM BP treatment, respectively (n = 4). CCL1 secretion in supernatants of macrophagic cultures was concomitantly enhanced by BP in a dose-dependent manner as assessed by ELISA, reaching a value of 12.8 ± 3.4 ng/ml in the supernatant of cultures exposed to 10 μM BP (Fig. 1A). Kinetic analysis of CCL1 gene induction by BP showed a maximum rate of mRNA level at 12–24 h, as for CYP1A1 up-regulation and a maximum of CCL1 secretion from 24-h exposure (Fig. 1B). Using shorter BP treatment time, CCL1 mRNA induction was detected as soon as 90-min exposure to BP (data not shown).

Alveolar macrophages are the main cellular source of CCL1 in the lung (31). To determine whether in vivo BP treatment could also be effective on CCL1 levels, the expression of TCA3, the mouse CCL1 orthologue gene (32), was determined using RT-qPCR assays. For this purpose, mice were treated by intranasal instillation of BP. As shown in Fig. 1C, BP exposure increased TCA3 mRNA levels in lung tissue (mean induction of 3.9-fold). Induction of lung CYP1A1 used as a validity control of mice BP treatment was concomitantly observed (data not shown) (33).

Lack of Contribution of IL-1β to BP-mediated CCL1 Up-regulation—IL-1β has been previously identified as a PAH target (34), and it was consequently found to be up-regulated at both mRNA and protein level in BP-exposed macrophagic cultures (Fig. 2A). Because CCL1 secretion has been shown to be triggered by IL-1β (35), it was therefore tempting to speculate that up-regulation of CCL1 expression in BP-treated macrophages might have been a consequence of primary induction of IL-1β. To test this hypothesis, we evaluated the effect of IL1-RA, an antagonist of IL-1 receptor (36), on BP-mediated induction of CCL1. IL1-RA, used at doses (50 and 100 ng/ml) that fully abolished IL-1β-mediated CCL1 mRNA up-regulation (Fig. 2B), failed to counteract CCL1 induction in BP-exposed macrophages (Fig. 2C), making a direct contribution of IL-1β to CCL1 regulation by BP very unlikely.

Effects of Ahr Agonsits and/or Antagonists and of Ahr Knockdown on CCL1 Up-regulation—We analyzed the effects of hydrocarbons such as benzo[e]pyrene (BeP) and TCDD, known as very weak and potent activators of Ahr, respectively (37, 38), on CCL1 expression levels. As shown in Fig. 3A, 10 μM BeP failed to significantly increase CCL1 mRNA level and CCL1 secretion in macrophagic cultures; by contrast, 10 nM TCDD
markedly enhanced CCL1 expression at both mRNA and protein levels, similarly to BP. We next investigated the effects of 3′M′4′NF, a potent antagonist of AhR (39, 40), on BP-triggered induction of CCL1. As shown in Fig. 3B, 3′M′4′NF, used either at 1 or 5 μM, markedly counteracted the up-regulation of CCL1 mRNA and secretion in BP-exposed macrophage cell cultures. It concomitantly blocked induction of the AhR-regulated gene CYP1A1 at mRNA levels, fully confirming that 3′M′4′NF was active under our experimental conditions. Similar inhibition of BP-mediated up-regulation of CCL1 expression was obtained using resveratrol or αNF as AhR antagonists instead of 3′M′4′NF (data not shown).

Besides pharmacological AhR inhibition, we analyzed whether AhR knockdown, using specific AhR-targeted siRNAs, may impair CCL1 induction in response to PAHs. Transfection of SiAhR allowed a strong reduction of AhR mRNA and AhR protein levels in primary macrophages (data not shown) and, as expected, CYP1A1 induction by BP was also prevented by transfection of SiAhR (Fig. 3C). Similarly, AhR knocking down markedly counteracted the inducing effects of BP toward CCL1 mRNA levels in macrophagic cultures when compared with transfection of control siRNA (SiAhRmut) (Fig. 3C).

Effects of AhR Agonists and/or Antagonists on CCL1 Promoter Activity—We next studied the effects of BP, BeP, TCDD, and/or 3′M′4′NF on CCL1 promoter activity. For these experiments, we performed transient transfection assays in EAhy-926 cells with the pCCL1-FL(−2975) reporter vector. This construct contained 2975 bp of the CCL1 gene 5′-flanking region. As shown in Fig. 4A, potent AhR agonists as BP and TCDD significantly enhanced luciferase activity of pCCL1-FL(−2975), unlike the very weak AhR activator BeP. Similar effects were observed with the pCYP1A1-FL(−1639) plasmid, a CYP1A1 promoter construct used here as positive control. Moreover, the AhR antagonist 3′M′4′NF was able to abrogate the increase of CCL1 and CYP1A1 promoter activity due to TCDD (Fig. 4B).

Direct AhR Interaction with CCL1 Promoter in BP- or TCDD-treated Macrophages—Analysis of the CCL1 promoter sequence indicated the presence of two AhR-related consensus XRE located at −2803 (XRE-2803) and −312 (XRE-312) (Fig. 5A), in agreement with a previous report (41). To investigate whether AhR interacts in vivo with these consensus XREs, we performed a ChIP assay using either

**FIGURE 3. Effects of AhR agonists (A) and antagonist (B) and of AhR knockdown (C) on CCL1 up-regulation.** A, macrophages were either untreated (Ct) or exposed to 10 μM BP, 10 μM BeP, or 10 nM TCDD for 12 h. Levels of CCL1 and β-actin mRNAs were then determined by RT-PCR (lower panel), whereas secreted CCL1 levels in culture supernatants were determined by ELISA (upper panel). The data shown are representative (RT-PCR) or are the means ± S.D. (ELISA) of three independent experiments. CCL1 values in culture supernatants are expressed as the percentage of the control values found in untreated cultures, arbitrarily set at 100%. *, p < 0.05 when compared with untreated macrophages. B, primary macrophages, either untreated (Ct) or exposed to 10 μM BP, were pretreated during 1 h and co-treated by the AhR antagonist 3′M′4′NF (1 or 5 μM) for 12 h. Levels of CCL1, CYP1A1, and β-actin mRNAs and of secreted CCL1 were then determined and expressed as described above. The data shown are from three independent experiments. *, p < 0.05 when compared with macrophages not exposed to BP. C, macrophages were transfected with siRNAs directed against AhR (SiAhRmut) or with control ineffective mutated SiAhR (SiAhRmut) and were either untreated (Ct) or exposed to 2 μM BP for 8 h. Expression of CCL1, CYP1A1, and β-actin mRNAs was then determined by RT-PCR (n = 3).

**FIGURE 4. Effects of AhR agonists and antagonist on CCL1 promoter activity.** EAhy-926 cells were co-transfected with the pRL-TK vector that codes for Renilla luciferase and the pCCL1-FL(−2975) or pCYP1A1-FL(−1639) firefly luciferase-constructs. Cells were then either untreated (Ct) or exposed to 10 μM BP, 10 μM BeP, or 10 nM TCDD (A) or co-exposed to both TCDD and the AhR antagonist 3′M′4′NF (5 μM) (B) for 24 h. Dual luciferase assays (firefly and Renilla) were next performed, and the data, expressed as means ± S.D. of four independent experiments, correspond to activities of firefly luciferase constructs normalized to that of pRL-TK Renilla luciferase-construct. *, p < 0.05, when compared with untreated cells.
untreated or BP- or TCDD-treated macrophages. Analysis of anti-AhR antibody-precipitated DNA using primers spanning the two XRE motifs found in CCL1 promoter revealed a specific PCR product only for the XRE-312 motif (Fig. 5B). A strong signal was also obtained for the CYP1A1 promoter sequence (30), used here as a positive control. By contrast, no PCR band was observed when using nonspecific IgG instead of the anti-AhR antibody for DNA immunoprecipitation or with primers spanning an XRE-free sequence of an unrelated gene: the tyrosinase (29) (Fig. 5B). The efficiency of primers to amplify DNA was assessed by using cell extract (input) instead of immunoprecipitated DNA complex for PCR assays (Fig. 5B).

To further investigate the ability of the XRE-312 sequence to bind AhR, we carried out electrophoretic mobility shift assays with a 32-bp CCL1 promoter sequence spanning the XRE-312 as a probe. As shown in Fig. 5C, a complex (bound DNA) was formed when nuclear extracts from control untreated macrophages were added to radiolabeled XRE-containing CCL1–312-WT oligonucleotide (lane 2); the intensity of this complex was increased when nuclear extracts from TCDD-treated cells were used (lane 3). The formation of this complex was, however, abolished in the presence of an excess of unlabeled CCL1–312-WT probe (lane 4) or by using a mutated XRE-containing CCL1–312-Mut probe instead of the wild-type probe (lanes 5 and 6). Addition of anti-AhR antibody, in supershift assay (lanes 7–9) was able to induce a “supershift” of the CCL1–312-WT-protein complex formed with nuclear extracts prepared from TCDD-treated cells (lane 8), indicating further that AhR was present in the complex; an excess of unlabeled probe led to disappearance of the supershifted band (lane 9).

We next investigated whether mutating the XRE-312 sequence could affect promoter activity in response to AhR agonists. For this purpose, we performed transient transfection assays in EAhy-926 cells with the pCCL1-FL (−2975) containing the wild-type XRE-312 and with the pCCL1-FL.Mut (−2975) containing a mutated XRE-312. As shown in Fig. 5D, TCDD treatment failed to induce activity of pCCL1-FL.Mut (−2975), whereas it enhanced that of pCCL1-FL (−2975) as reported above.

Role of [Ca2+], in BP-mediated Up-regulation of CCL1 Expression

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FIGURE 5. Direct AhR interaction with CCL1 promoter in BP- or TCDD-treated macrophages. A, schematic representation of CCL1 promoter. XRE motifs that putatively bind AhR are shown in black, and their position is indicated relatively to the transcription initiation site; arrows indicate the position of primers used in ChIP assays. B, chromatin immunoprecipitation assays were performed on human macrophages either untreated (Ct) or exposed to 2 μM BP or 10 nM TCDD for 30 min, using anti-AhR antibody or nonspecific IgG. Recovered DNA was subjected to PCR using primers specific for CCL1 and centered on the two consensus XRE motifs. Specific primers for XRE-containing human CYP1A1 promoter and sequence-XRE-free human tyrosinase were used in parallel as positive and negative controls, respectively. The data shown are representative of three independent experiments. C, AhR binding to XRE-312 of CCL1 promoter. DNA binding assays were performed using nuclear extracts prepared from macrophages either untreated (control) or exposed to 10 nM TCDD for 30 min. Radiolabeled oligonucleotides used as probes were 32-bp CCL1 promoter sequences spanning the XRE-312; CCL1–312-WT contained the wild-type XRE, whereas CCL1–312-Mut contained a mutated XRE. “Competitor” corresponds to unlabeled CCL1–312-WT. For supershift experiments, DNA binding reactions were incubated for a further 45 min on ice with a polyclonal anti-AhR antibody. Retarded complexes (Bound DNA) and supershifted bands (Supershift) are indicated by black arrows. The data shown are representative of two independent experiments. D, effect of XRE-312 mutation on CCL1 promoter activity. EAhy-926 cells were co-transfected with the pRL-TK vector that codes for Renilla luciferase and with the pCCL1-FL (−2975) (containing the wild-type XRE-312) or with the pCCL1-FL.Mut (−2975) (containing a mutated XRE-312) firefly luciferase constructs. Cells were then either untreated (Ct) or exposed to 10 nM TCDD for 24 h. Dual luciferase assays (firefly and Renilla) were performed, and the data, expressed as means ± S.D. of four independent experiments, correspond to activities of firefly luciferase constructs normalized to that of pRL-TK Renilla luciferase construct. *, p < 0.05, when compared with untreated cells.
Using microspectrofluorometry and the Ca\(^{2+}\)-sensitive probe Fura-2-AM, we demonstrated that BP was also capable of eliciting a transient increase of [Ca\(^{2+}\)], in primary human macrophages. As shown in Fig. 6A (inset), continuous recording of [Ca\(^{2+}\)], indicated that the [Ca\(^{2+}\)], increase began at 5.92 ± 3.02 min after addition of 1 \(\mu\)M BP, to reach a maximal level after 39.9 ± 8.14 min. This maximal level of [Ca\(^{2+}\)], increase, expressed as the ratio of Fura-2 fluorescence in macrophages exposed to 1 \(\mu\)M BP relative to that detected in untreated counterparts, was 1.94 ± 0.29 (Fig. 5A). To fully confirm that these [Ca\(^{2+}\)], changes were transient, macrophages were incubated with 1 \(\mu\)M BP for 15, 40, 50, 60, 240, and 480 min. As shown in Fig. 6A, the BP-induced increase of [Ca\(^{2+}\)], was significant only at 15 and 40 min of treatment with a return to the steady-state level thereafter. Interestingly, treatment by a higher concentration of BP (10 \(\mu\)M) or by TCDD (10 nm) resulted in both cases in an increase of [Ca\(^{2+}\)], of similar magnitude in macrophages (Fig. 6B). The increase of [Ca\(^{2+}\)], occurring in macrophages in response to BP was fully abolished when cells were placed in a Ca\(^{2+}\)-depleted buffer supplemented with the Ca\(^{2+}\)-chelating agent, EGTA (without extracellular calcium (Wo), Fig. 6C), thus indicating it required extracellular Ca\(^{2+}\). Moreover, the [Ca\(^{2+}\)], increase in BP-treated macrophages was also counteracted by co-treatment with 50 \(\mu\)M 2-APB, a cell-permeable inhibitor of inositol 1,4,5-trisphosphate-induced Ca\(^{2+}\) release and of store-operated calcium channels (Fig. 6D), thus pointing to a role for both inositol 1,4,5-trisphosphate and store-operated channels in AhR agonists-triggered cellular Ca\(^{2+}\) movements, as recently suggested (13).

To determine whether the [Ca\(^{2+}\)], increase observed in BP-treated macrophages might play a role in CCL1 up-regulation, we first analyzed the effects of BAPTA-AM, a chelating agent of extracellular calcium. As shown in Fig. 7A, BAPTA, used under its cell-permeable form BAPTA-AM, fully blocked BP-mediated induction of CCL1 mRNA and secretion levels. It similarly inhibited BP-triggered CYP1A1 mRNA induction. By contrast, it did not modify basal CCL1 expression in untreated macrophages. 2-APB, which inhibited transient [Ca\(^{2+}\)], increase due to BP as reported above, also markedly decreased BP-triggered up-regulation of CCL1 mRNA and secretion (Fig. 7B). This inhibitory effect, however, required pretreatment of cells with 2-APB before initial addition of BP. Indeed, this inhibitory effect was attenuated when addition of 2-APB was performed 30 min after onset of BP treatment and fully disappeared when addition of 2-APB was further delayed (60 or 120 min after that of BP) (Fig. 6B), i.e., when 2-APB was added after BP-induced transient increase of [Ca\(^{2+}\)], has occurred. The same results were observed with respect to CYP1A1 induction in response to BP.

**DISCUSSION**

Our results demonstrate that the chemokine CCL1, which has been implicated in asthma, allergic inflammation, cardiovascular diseases, and vascular wall biology (18–20), constitutes a new molecular target of PAHs. Indeed, treatment of human macrophagic cells by such compounds, like BP, was found to enhance levels of both CCL1 mRNAs and secretion. This treatment also led to the increase of CCL1 promoter activity. Moreover, in vivo BP treatment of mice induced mRNA levels of TCA3, the mouse orthologue of CCL1. By contrast, BP failed to alter expression of other

**FIGURE 6. Effect of BP on [Ca\(^{2+}\)], in human macrophages.** A, macrophages, either untreated (Ct) or exposed to 1 \(\mu\)M BP, were incubated with the Ca\(^{2+}\)-sensitive probe Fura-2-AM and were submitted alternatively to 340 and 380 nm excitation wavelengths; the fluorescence from the trapped dye was measured at 510 nm. The F340/F380 ratio, i.e., ratio of fluorescence intensities after excitation at 340 nm and 380 nm, respectively, was used to estimate [Ca\(^{2+}\)]. The curve represents F340/F380 ratio variations in BP-exposed cells normalized with the ratio detected in untreated counterparts, at 15, 40, 50, 60, 240, and 480 min after the addition of BP; data are means ± S.D. of three independent experiments. *, \(p < 0.05\), when compared with untreated cells. The inset corresponds to the continuous recording of normalized [Ca\(^{2+}\)], changes over the initial 40-min period of BP treatment (the recordings shown are representative of three independent experiments). B, macrophages were maintained under control conditions (Ct) or treated with 1 or 10 \(\mu\)M BP or 10 nm TCDD for 40 min. [Ca\(^{2+}\)], changes were then determined as described above. Results are expressed as the F340/F380 ratio normalized to that detected in untreated control cells and are the means ± S.D. of three different experiments. *, \(p < 0.05\), when compared with untreated cells. C, macrophages, maintained either in the presence of extracellular calcium (W) or without (i.e., in the presence of 10 nm EGTA, Wo), were either untreated (Ct) or treated by 10 \(\mu\)M BP for 40 min. [Ca\(^{2+}\)], alterations, expressed as the F340/F380 ratio normalized to that detected in untreated control cells, were then determined as described above; data are given as the means ± S.D. of three different experiments. *, \(p < 0.05\), when compared with BP-untreated cells. D, macrophages were either co-treated or not with 10 \(\mu\)M BP and/or 50 \(\mu\)M 2-APB for 40 min; when 2-APB was used, a 1-h pretreatment was applied prior to co-treatment with BP. [Ca\(^{2+}\)], alterations, expressed as the F340/F380 ratio normalized to that detected in untreated control cells, were then determined as described above; data are given as the means ± S.D. of three different experiments. *, \(p < 0.05\), when compared with BP-untreated cells.
CC-chemokines such as CCL5 or CCL22 in primary human macrophages (data not shown). This demonstrates that the effects observed were rather specific for CCL1 and did not correspond to a general modulation of CC-chemokine expression by this environmental chemical.

It is noteworthy that PAHs can up-regulate various cytokines/chemokines in addition to CCL1, such as IL-1β, IL-2, and tumor necrosis factor-α (14–17). The up-regulation of IL-1β, which takes place in BP-treated primary macrophages, may deserve special attention owing to the known inducing role of IL-1β toward CCL1 expression (35). However, IL-1RA, an IL-1β receptor antagonist, failed to alter BP-mediated induction of CCL1, whereas it fully abolished IL-1β-triggered CCL1 up-regulation. Moreover, co-treatment of macrophages with BP and the protein synthesis inhibitor cycloheximide did not alter CCL1 mRNA induction (data not shown), thus demonstrating that de novo protein synthesis was not required for BP-mediated up-regulation of CCL1. Taken together, these data likely rule out a role for IL-1β in CCL1 induction by BP and rather suggest a direct CCL1 regulation by this environmental contaminant. Such a conclusion is fully supported by ChIP assays demonstrating the direct binding of AhR to CCL1 promoter in BP-exposed human macrophages.

AhR, well known to contribute in a major way to regulation of PAH-responsive genes, plays a crucial role in CCL1 induction by aryl hydrocarbons. Such conclusion is supported by the following points: (i) TCDD, a very potent agonist of AhR, markedly enhanced CCL1 expression in primary macrophages, whereas BeP, which poorly interacts with AhR, did not significantly alter CCL1 levels; (ii) the use of AhR antagonists such as 3′M4′NF, resveratrol, or αNF, fully countered BP-mediated up-regulation of CCL1; (iii) in vitro knockdown of AhR expression in primary macrophages using siRNAs prevented CCL1 induction by BP; (iv) activity of CCL1 promoter, which contains XRE sequences known to interact with AhR, was inducible by BP and TCDD in an 3′M4′NF-inhibitable manner; and (v) ChIP assay, electrophoretic mobility shift assays, and functional promoter mutation studies provided direct evidence that AhR interacts in vivo and in vitro with the XRE located at −312 (XRE-312) on the CCL1 promoter. It is noteworthy that the sequence of CCL1 promoter containing the XRE located at −2803 (XRE-2803), failed to bind AhR, suggesting that it is not functional and therefore likely does not play a major role in CCL1 up-regulation in response to aryl hydrocarbons. This conclusion is fully supported by the fact that TCDD similarly induced the activity of both pCCL1-FL(−2975), containing XRE-2803 and XRE-312, and pCCL1-FL(−1915) promoter construct, spanning −1978 to −63 and lacking XRE-2803, but retaining XRE-312 (data not shown). Moreover, mutating XRE-312 was sufficient to fully prevent TCDD-mediated increase of CCL1 promoter activity. This lack of contribution of XRE-2803 may be due to the identity of nucleotides adjacent to the core XRE sequence, which have been shown to influence the efficiency of AhR binding (42, 43). Interestingly, PAH-unresponsive genes, including CCL5 or CCL22, have been described to contain XREs in their promoter regions (41), suggesting that these XRE were also not functional. Besides CCL1, regulation of other cytokines, such as IL-2, in response to AhR agonists has been hypothesized to be linked to XRE sequences (44). By contrast, post-transcriptional mechanisms may contribute to TCDD regulation of IL-1β (45), whereas induction of tumor necrosis fac-
tor-α expression by PAHs occurs independently of AhR/XRE sequences (14). Taken together, these data favor the idea that cytokines/chemokines, which constitute an important emerging group of aryl hydrocarbon targets, may be in fact affected by these environmental contaminants through different ways.

A previous report (46) demonstrated the relation between alterations in intracellular Ca²⁺ ([Ca²⁺]i) and immune dysregulation due to aryl hydrocarbon exposure. In an attempt to determine whether Ca²⁺, also known as a gene-expression regulator, is involved in CCL1 induction by aryl hydrocarbons, we looked for an effect of these compounds on intracellular Ca²⁺ level in human macrophages. These cells exposed to BP exhibited an early and transient increase of [Ca²⁺]i, which however, did not occur in Ca²⁺-depleted buffer and in 2-APB-treated cells. This indicates that BP-mediated [Ca²⁺]i change in macrophages are most likely due to inositol 1,4,5-trisphosphate- and store-operated calcium channel-dependent mechanisms. It is noteworthy, however, that activation of the ryanodine receptor by BP metabolites has also been incriminated in PAH-mediated [Ca²⁺]i, increase, especially in lymphocytes (6, 47). This suggests that pathways of [Ca²⁺]i, mobilization by AhR agonists may differ according to the cell type. Counteracting BP-related alterations in intracellular Ca²⁺ due to aryl hydrocarbon exposure. In an attempt to determine whether Ca²⁺ induction triggered by aryl hydrocarbons. The significance of this finding is supported by the early and transient increase in murine form of CCL1, which however, is not established carcinogenic effects of PAHs (62). It is also noteworthy that CCL1 was induced in primary human macrophages in response to BP doses as low as 0.1–1 μM (25–250 ng/g), which are close to those found in some foods (up to 50 ng/g) (63), in cigarette smoke (20–40 ng/cigarette) (64), or in contaminated soil, suggesting that humans are likely exposed to PAH concentrations triggering CCL1 release. In addition, CCL1 concentrations presently obtained in BP-treated macrophage cultures, reaching the value of 12.8 ± 3.4 ng/ml, were in the range of those required for triggering chemotaxis of cells expressing CCR8 (CCL1-receptor) (65). Moreover, CCL1 secretion induction occurred after a relative short treatment with BP (6–12 h), indicating that acute and transient exposure to PAHs is sufficient to enhance CCL1 secretion. Finally, induction of the murine form of CCL1, i.e. TC3, after intranasal administration of BP to mice, brings evidence that CCL1 up-regulation may be achieved after in vivo exposure and may therefore contribute to the in vivo toxic effects of PAHs.

In summary, the chemokine CCL1 was identified as a new molecular target of PAHs. Owing to the various roles played by this chemokine, especially in cardiovascular pathology or allergic and inflammatory diseases, it is tempting to speculate that its up-regulation by PAHs may participate to some of the deleterious effects of these environmental contaminants toward human health.

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