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Evidence of selective activation of aryl hydrocarbon receptor nongenomic calcium signaling by pyrene

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ABSTRACT

In its classical genomic mode of action, the aryl hydrocarbon receptor (AhR) acts as a ligand activated transcription factor regulating expression of target genes such as CYP1A1 and CYP1B1. Some ligands may also trigger more rapid nongenomic responses through AhR, including calcium signaling (Ca^{2+}). In the present study we observed that pyrene induced a relatively rapid increase in intracellular Ca^{2+}-concentrations ($[Ca^{2+}]_i$) in human microvascular endothelial cells (HMEC-1) and human embryonic kidney cells (HEK293) that was attenuated by AhR-inhibitor treatment and/or transient AhR knockdown by RNAi. *In silico* molecular docking based on homology models, suggested that pyrene is not able to bind to the human AhR in the agonist conformation. Instead, pyrene docked in the antagonist conformation of the AhR PAS-B binding pocket, although the interaction differed from antagonists such as GNF-351 and CH223191. Accordingly, pyrene did not induce CYP1A1 or CYP1B1, but suppressed CYP1-expression by benzo[*a*]pyrene (B[*a*]P) in HMEC-1 cells, confirming that pyrene act as an antagonist of AhR-induced gene expression. Use of pharmacological inhibitors and Ca^{2+}-free medium indicated that the pyrene-induced AhR nongenomic $[Ca^{2+}]_i$ increase was initiated by Ca^{2+}-release from intracellular stores followed by a later phase of extracellular Ca^{2+}-influx, consistent with store operated calcium entry (SOCE). These effects was accompanied by an AhR-dependent reduction in ordered membrane lipid domains, as determined by di-4-ANEPPDHQ staining. Addition of cholesterol inhibited both the pyrene-induced $[Ca^{2+}]_i$-increase and alterations in membrane lipid order. In conclusion, we propose that pyrene binds to AhR, act as an antagonist of the canonical genomic AhR/Arnt/CYP1-pathway, reduces ordered membrane lipid domains, and activates AhR nongenomic Ca^{2+}-signaling from intracellular stores.

1. Introduction

The aryl hydrocarbon receptor (AhR), which is a basic helix-loop-helix PAS transcription factor, plays a central role in regulating toxicity from PAHs. In its classical mode of action, ligand-activated AhR translocates to the nucleus and dimerizes with the AhR nuclear translocator (Arnt). The AhR-Arnt complex then binds to so-called dioxin or xenobiotic response elements (DREs or XREs) in the promotor region of target genes. The prototypical genes activated by AhR are the cytochrome P450 enzymes CYP1A1 and CYP1B1, but AhR also regulates a number of other genes including many central proinflammatory mediators [1,2]. Notably, AhR-signaling is highly ligand specific and not restricted to the classical mode of action [3,4]. Nonclassical AhR-signaling involves crosstalk with a number of other transcription factors and signaling molecules independently of Arnt activation [2–4]. In addition, it has been suggested that many of the toxic effects of the AhR ligand 2,3,7,8-tetrachlorodibenzodioxin (TCDD) could be due to non-genomic AhR-signaling where the receptor rather functions as a

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signaling molecule in the cytosol [1]. As such, the emerging pattern of AhR signaling is strikingly similar to the signaling from a number of nuclear receptors, including estrogen receptor (ER), which also involves both genomic and nongenomic modes of action [5].

As for ER-signaling, AhR nongenomic responses appear to involve both activation of protein kinases as well as mediation of calcium (Ca^{2+}) responses within min after exposure, hence preceding the genomic activation of target genes [1,3,5]. These early cytosolic responses of the AhR are far less understood than the genomic mode of action and have predominately been investigated for TCDD. It is therefore unclear whether genomic and nongenomic signaling is triggered simultaneously through interaction with the same motifs within the AhR-PAS-B binding pocket, or whether the rank order of ligand potencies could differ for induction of genomic and nongenomic signaling.

Polycyclic aromatic hydrocarbons (PAHs) constitute a central group of AhR ligands originating from incomplete combustion of organic materials. The main toxicological concern has historically been carcinogenic effects, and CYP1A1/CYP1B1is central in the metabolic activation and subsequent mutagenic effects of several PAHs [6]. In risk assessment the toxicity of PAHs has been converted into toxicity equivalency factors (TEFs) based on estimates of relative carcinogenicity compared to the known human carcinogen benzo[*a*]pyrene (B[*a*]P) [7]. Pyrene is among the most abundant PAHs in outdoor air occurring at concentrations that are orders of magnitudes higher than B[*a*]P [8], but is not classifiable as carcinogen to humans (IARC group 3 compound), and has a very low TEF value of 0.001 [7]. However, pyrene exposure has been associated with asthma in children [9] and cardiovascular disease [10]. Studies in zebrafish embryos suggest that pyrene may induce peripheral vascular defects, cardiotoxicity and neuronal cell death [11–13]. Moreover, pyrene and its derivatives, but not B[*a*]P or β-naphthoflavone, potentiate pro-inflammatory responses induced by a toll-like receptor-3 ligand [14]. This suggests that pyrene triggers some effects that are distinctly different from B[*a*]P.

The reported lack of effect on CYP1A1/CYP1B1expression in human cells suggest that pyrene may have limited ability to bind and activate AhR [15–17]. In contrast, it appears that pyrene induce CYP-expression in fish and rats, although with considerable lower potency than B[*a*]P [18–20], and it has been suggested that developmental toxicity of pyrene in zebrafish is mediated through AhR-dependent mechanisms due to qualitative similarities with effects from TCDD [11]. Studies from our lab have shown that the nitro-derivative 1-nitropyrene is capable of inducing CYP1A1 expression in rat Hepa1c1c7 cells, but fails to do so in human bronchial epithelial BEAS-2B cells [21,22]. This could be due to differences in ligand selectivity and gene regulation between the human AhR and AhR from other species [23–25]. One possibility is that pyrene and pyrene derivatives could bind the human AhR, but fail to induce the classical genomic signaling.

Previous studies from our consortium have shown that pyrene induces a relatively rapid increase in $[Ca^{2+}]_i$ in human microvascular endothelial HMEC-1 cells, almost twice the magnitude of calcium responses induced by B[*a*]P and other PAHs investigated [16]. In the present study we have explored the mechanism of pyrene-induced increase in $[Ca^{2+}]_i$ in HMEC-1 cells. Our results suggest that pyrene is a AhR ligand that may selectively activate nongenomic signaling, triggering increase in $[Ca^{2+}]_i$ through store-operated calcium entry (SOCE) in parallel with alteration in membrane organization in HMEC-1 cells. This strongly supports the notion that pyrene may have unique toxicological effects, and shows that AhR nongenomic signaling may be triggered independently of the classical genomic pathway.

2. Materials and methods

2.1. Chemicals

Benzo[a]pyrene (B[a]P), pyrene, 2-methyl-2H-pyrazole-3-carboxylic acid (2-methyl-4-o-tolylazo-phenyl)-amide (CH223191), cholesterol, hydrocortisone and EGTA were purchased from Sigma-Aldrich (St. Louis, MO, USA; now Merck). TRIzol® reagent, pluronic acid and Fura-2 acetoxymethylester (Fura-2-AM) were provided by InVitrogen (Carlsbad, CA, USA). 1-[2-(4-Methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl-1H-imidazole hydrochloride (SKF 96365) and N-[4-[3,5-Bis(trifluoromethyl)-1H-pyrazol-1-yl]phenyl]-4-methyl-1,2,3-thiadiazole-5-carboxamide (BtP2) was obtained from TOCRIS (Bristol, UK). 2-Aminoethoxydiphenylborate (2-APB) was purchased from Merck Millipore (Burlington, MA, USA. Di-4ANEPP-DHQ, MCDB 131 medium, and L-glutamine (200 mM) were purchased from Thermo Fischer Scientific (Carlsbad, CA, USA). Fetal bovine serum (FBS) by Biochrom AG (Berlin, Germany), and endothelial growth factor by Nerliens Meszansky (Oslo, Norway). Penicillin and streptomycin were from Lonza (Walkersville, MD, USA). All real-time RT-PCR reagents and TaqMan probes/primers were purchased from Applied Biosystems (Foster City, CA, USA). All other reagents were commercial products of the highest purity available.

2.2. Cell culture

Human endothelial HMEC-1 cells, obtained from ATCC through LGC Standards (Wesel, Germany) were routinely maintained in MCDB 131 medium containing epidermal growth factor (10 ng/mL), hydrocortisone (1 μg/mL), penicillin (50 unit/mL), and streptomycin (50 μg/mL) and supplemented with 10% FBS, according to the providers' instructions. Experiments were performed on HMEC-1 cells at passage numbers 22–32. Human embryonic kidney HEK293 cells were maintained in Dulbecco's modified Eagle's medium containing penicillin (50 unit/mL), and streptomycin (50 μg/mL) and supplemented with 10% FBS. The HEK293 cells was a gift from the late Hervé Paris, INSERM, Toulouse, France. The passage number of and origin of these cells, that was used in a limited number of experiments are not known. HEK293 cells permanently expressing β1-/β2ADR were obtained by β1-/β2ADR cDNA transfection using Lipofectamine (Invitrogen) as described elsewhere [26]. Chemicals were commonly prepared as stock solution in dimethyl sulfoxide (DMSO). The final concentration of solvent did not exceed 0.2% (v/v); control cultures received similar concentration of DMSO.

Cells used for Ca^{2+} measurements or fluorescence measurements of membrane order were grown on glass coverslips to 50–60% confluency and serum starved for a minimum of 12 h prior to exposure. The glass coverslips were sterilized in ethanol of increasing concentrations from 70 to 99%, and then coated with serum proteins using pure FBS prior to cell culture. Cells used for q-PCR were cultivated in 6-well plates, grown to near confluence and serum starved for a minimum of 12 h prior to exposure.

2.3. Calcium measurements

HMEC-1 were grown on glass lamellas to 50–60% confluency and serum starved for a minimum of 12 h prior to exposure. Before exposure cells were mounted in exposure chambers containing 1 mL cell suspension buffer. Calcium cell suspension buffer contained: 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. Cells were washed two times with the buffer before loading with Fura-2AM for 30 min. Inhibitors were added during this loading period, and after the loading buffer had been washed off. Calcium-free cell suspension buffer containing the extracellular calcium chelator ethylene glycol tetra acetic acid (EGTA) was used after loading in experiments on extracellular Ca^{2+} dependency. Variations in intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$ were analysed in HMEC-1 cells exposed to all four DEP-OE, by micro-spectrofluorometry using the Ca^{2+} sensitive probe Fura-2AM, as previously reported [27]. Briefly, cells were incubated at 37 °C in cell suspension buffer supplemented with 1.5 μM Fura-2AM and 0.006% pluronic acid.