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Combustion Particle-Induced Changes in Calcium Homeostasis: A Contributing Factor to Vascular Disease?

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Abstract
Air pollution is the leading environmental risk factor for disease and premature death in the world. This is mainly due to exposure to urban air particle matter (PM), in particular, fine and ultrafine combustion-derived particles (CDP) from traffic-related air pollution. PM and CDP, including particles from diesel exhaust (DEP), and cigarette smoke have been linked to various cardiovascular diseases (CVDs) including atherosclerosis, but the underlying cellular mechanisms remain unclear. Moreover, CDP typically consist of carbon cores with a complex mixture of organic chemicals such as polycyclic aromatic hydrocarbons (PAHs) adhered. The relative contribution of the carbon core and adhered soluble components to cardiovascular effects of CDP is still a matter of discussion. In the present review, we summarize evidence showing that CDP affects intracellular calcium regulation, and argue that CDP-induced impairment of normal calcium control may be a critical cellular event through which CDP exposure contributes to development or exacerbation of cardiovascular disease. Furthermore, we highlight in vitro research suggesting that adhered organic chemicals such as PAHs may be key drivers of these responses. CDP, extractable organic material from CDP (CDP-EOM), and PAHs may increase intracellular calcium levels by interacting with calcium channels like transient receptor potential (TRP) channels, and receptors such as G protein-coupled receptors (GPCR; e.g., beta-adrenergic receptors [βAR] and protease-activated receptor 2 [PAR-2]) and the aryl hydrocarbon receptor (AhR). Clarifying a possible role of calcium signaling and mechanisms involved may increase our understanding of how air pollution contributes to CVD.

Keywords Diesel exhaust particles · Polycyclic aromatic hydrocarbons · Endothelial dysfunction · Aryl hydrocarbon receptor · Calcium signaling

Abbreviations

ADRs	Adrenergic receptors
AhR	Aryl hydrocarbon receptor
ARNT	AhR nuclear translocator
B[*a*]P	Benzo[*a*]pyrene
CVD	Cardiovascular diseases
CDP	Combustion-derived particles
CYP	Cytochrome P450
$[Ca^{2+}]_i$	Cytosolic concentration of calcium
DEP	Diesel exhaust particles
DEP-EOM	Extractable organic material of DEP
GPCRs	G protein-coupled receptors
MMPs	Matrix metalloproteinases
NF-κB	Nuclear factor-κB
1-NP	1-nitropyrene
OC	Organic chemicals
oxLDL	Oxidized low-density lipoproteins
PM	Particular matter
PAHs	Polycyclic aromatic hydrocarbons
PAR-2	Protease-activated receptor 2
ROS	Reactive oxygen species
ROCE	Receptor-operated calcium entry
RTKs	Receptor tyrosine kinases

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TRP	Transient receptor potential
SOCE	Store-operated calcium entry
XREs	Xenobiotic response elements

Introduction

Air pollution is a major environmental contributor to adverse health effects, carrying responsibility for about one in every nine deaths globally [1]. This is mainly due to increased risk of cardiovascular disease (CVD). Exposure to particulate matter (PM), in particular fine ($PM_{2.5} < 2.5$ μm) and ultrafine (UFP or $PM_{0.1} < 0.1$ μm) PM, originating from combustion of organic material including diesel fuel, has been linked to development and exacerbation of atherosclerosis in a number of epidemiological and experimental studies [2–4]. The fact that combustion-derived particles (CDP) from various sources, including diesel exhaust (DEP), wood smoke, and cigarette smoke, have partly similar particle characteristics and induce some of the same physiological responses linked to CVD suggests common mechanism(s) of toxicity [5].

There are numerous inter- and intracellular pathways linking CDP to CVD, depending on the vascular endpoint studied. Even though several particle properties are likely to contribute [6, 7], recent studies suggest that organic chemicals (OC) may play an important role in many of the toxic effects of CDPs. Several in vitro studies show that a number of biological effects from CDPs can be reproduced with CDP-organic extracts rather than denuded (washed) CDPs. This includes effects on inflammatory responses [8–11], believed to be central to development of CVDs. In support of this, concentrated ambient particles (CAP) accelerated atherosclerosis development in $ApoE^{-/-}$ mice and reduced cardiac function, while mice exposed to CAP denuded of organic chemicals were not different from controls [12].

CDP, organic extracts from CDP, and specific chemicals found in these extracts, including several polycyclic aromatic hydrocarbons (PAHs), have been shown to increase intracellular concentrations of calcium ($[Ca^{2+}]_i$) in various experimental cell systems [5, 13–15]. Presently, we review the evidence suggesting that CDP and attached PAHs may contribute to the development of CVD by affecting $[Ca^{2+}]_i$. We highlight in vitro research suggesting that extractable organic material of CDP (CDP-EOM) and PAHs could be the main drivers of CDP-induced $[Ca^{2+}]_i$, and evidence linking these responses to cellular processes central in development and exacerbation of CVD.

Biological Mechanisms

Atherosclerosis is initiated by endothelial dysfunction, a pro-inflammatory and pro-constrictive alteration of the endothelium. Various risk factors may cause chemokine-mediated recruitment of immune cells and increased expression of adhesion molecules leading to extravasation of monocytes. Furthermore, macrophages located in the vascular wall develop into foam cells by taking up modified lipoproteins, and defective cholesterol efflux accelerates this process. Accumulating sub-endothelial foam cells gives rise to the early histological hallmark of atherosclerosis, namely fatty streaks. The atheroma formed next may develop into a more fibrous plaque. Several compounds, among others matrix metalloproteinases (MMPs), carry potential to destabilize plaques, ultimately causing rupture and subsequent thrombosis. This leads to ischemia and hence clinical manifestations, such as myocardial and cerebral infarction, as well as peripheral vascular disease [16].

Atherosclerosis is an inflammatory disorder of the arterial wall involving oxidative stress, increased endothelial permeability, leukocyte adhesion, and a number of other inflammatory reactions [17]. Furthermore, inflammation is considered a central link between PM exposure and CVD [18, 19]. Inflammatory reactions may be directly caused by PM-induced chemokine/cytokine release as well as indirectly through cytotoxicity [20, 21]. Oxidative stress seems central to both processes [18, 20, 22]. Reactive oxygen species (ROS) can be generated directly from particles and particle components or more indirectly through various metabolic and inflammatory processes [23–25]. CDP may also affect platelets and coagulation processes, increasing risk of vascular clotting [26–28].

Three major lines of evidence linking air pollution with increased cardiovascular morbidity and mortality have been proposed including effects via i) pulmonary nerve reflexes, ii) direct cardiovascular effects of CDP or CDP constituents that reach the systemic circulation, and iii) atherothrombotic effects related to pulmonary oxidative stress and inflammation [29–31].

The respiratory system is innervated with multiple sensory nerve types such as stretch receptors that may respond to various environmental irritants [32]. Combustion particles such as DEP seem to affect the autonomic nervous system (ANS) by interacting with nerves and/or receptors [33, 34]. More specifically, in hypertensive rats exposed to DEP, increased risk of cardiac arrhythmias was mediated in part by transient receptor potential (TRP) channel, TRPA1 expressed on airway fibers originating in the larynx [35]. Studies link air pollution to heart rate variability (HRV), an indicator of the balance between sympathetic and parasympathetic branches of the ANS. It is further suggested that an ANS imbalance also will affect other functions of the vasculature, blood, and heart and thus aggravate CVD development [31, 32].

Combustion particles and their constituents may also affect vasculature, blood, and the heart more directly [29, 36, 37]. Inhaled gold nanoparticles were found to accumulate at

sites of vascular inflammation in mice and humans [38]. If CDPs translocate into the circulation in a similar manner, they may deliver their OC-load directly to vascular endothelial cells. However, only a relatively small amount of inhaled nanoparticles (< 50 nm), possibly less than 0.3%, appear to translocate across the epithelial barrier [39]. By contrast, organic chemicals such as PAHs have been shown to rapidly detach from particles, and enter and diffuse throughout epithelial cell membranes in vitro [40]. Exposing dogs to B[*a*]P-coated soot particles, Gerde and co-workers showed that 30% of available B[*a*]P adhered to deposited particles entered the bloodstream within minutes after inhalation, mostly in an un-metabolized state [41, 42]. This suggests that translocation of free B[*a*]P released from the particle surface may exceed the amount of B[*a*]P potentially carried across the alveolar barrier bound to particles by as much as two orders of magnitude. Interestingly, B[*a*]P-DNA adducts have been found in the endothelium of human atherosclerotic lesions [43]. Furthermore, increased expression of aryl hydrocarbon receptor (AhR)-regulated genes has been found in heart. This has been suggested to be due to PAH exposure and to mediate cardiotoxicity [44]. Recently, exposure-relevant concentrations of DEP applied on the epithelial side of an alveolar 3D tri-culture rapidly induced pro-inflammatory and AhR-regulated genes in basolateral endothelial cells [45]. DEP with low levels of organic chemicals gave less effects in this 3D tri-culture model [46], further suggesting that the effects were most likely due to soluble lipophilic constituents rather than particle translocation. In a co-culture model, wood smoke particles and DEP increased the adhesion of monocytes to endothelial cells [47], illustrating how DEP may enhance migration of inflammatory cells from the bloodstream [17]. Other in vitro studies suggest that DEP or chemicals adhered to DEP may impair endothelial function [48] or increase formation of lipid laden foam cells from macrophages [25]. These studies further support in vivo findings linking PM exposure with progression of atherosclerosis [30, 49, 50].

Lastly, a number of studies suggest that CDP trigger oxidative stress in pulmonary macrophages and epithelial cells; and that effects on CVD may result from pro-inflammatory mediators such as cytokines, oxidized lipids/proteins, and/or micro-vesicles from "stressed" or damaged cells that leak into the circulation. This could next cause systemic effects by harming endothelial cells and activating immune cells, a process called "systemic spill over" [4, 51]. Systemic oxidative stress, measured as elevations in biomarkers of protein, lipid, or DNA oxidation, has been demonstrated in young adults after exposure to PM from traffic sources [52, 53]. It should, however, be noted that a review of animal studies on CDP-induced atherosclerosis and vasomotor dysfunction found that this was often triggered in the absence of apparent effects on pulmonary and systemic inflammation [54].

Regulation of $[Ca^{2+}]_i$

Calcium (Ca^{2+}) is a key signaling molecule that regulates cellular homeostasis [55]. In the lungs, Ca^{2+} is involved in regulation of several important functions including cell proliferation, mucus secretion, surfactant secretion, and ciliary beat frequency [56–58]. Furthermore, by interacting with proteins such as calmodulin, enzymes, and various protein kinases, Ca^{2+} regulates a number of processes linked to CVD development, including nerve cell signaling, inflammatory reactions, and vascular tone [59, 60]. Vascular tone is not only regulated by $[Ca^{2+}]_i$ in smooth muscle cells but also by $[Ca^{2+}]_i$ in endothelial cells that communicate with the muscle cells via myoendothelial micro-domains. The later may involve stimulation of Ca^{2+}-sensitive K^+ channels, myoendothelial gap junctions (MEGJ), as well as calmodulin activating endothelial nitric oxide synthase (eNOS) [61–65]. In light of these observations, it is also interesting to note that $[Ca^{2+}]_i$ regulate endothelial permeability, a central step in the pathogenesis of atherosclerosis [66, 67]. Furthermore, Ca^{2+} modify expression of a number of genes regulating pro-inflammatory transcription factors including cyclic AMP response element protein (CREB), nuclear factor-κB (NF-kB), and calcineurin/nuclear factor of activated T-cells (NFAT) [68].

Recently, increased Ca^{2+} flux was found to be a novel regulator of oxidized LDL-induced macrophage foam cell formation [69]. An increase in $[Ca^{2+}]_i$ triggers Ca^{2+}-dependent signaling processes leading to potentiation of agonist-induced platelet aggregation [70]. Generation of extracellular vesicles (notably micro-vesicles) from monocytes and endothelial cells may also partly be mediated by Ca^{2+} mobilization [71]. These micro-vesicles are involved in coagulation and inflammatory reactions. A well-functional cardiovascular system thus depends on well-regulated $[Ca^{2+}]_i$.

TRP channels constitute a family of six transmembrane channels regulating influx of Ca^{2+} and other cations. Some TRP channels may be triggered via receptors in the plasma membrane, a process called receptor-operated calcium entry (ROCE) [72], while others such as vanilloid receptor TRPV1 may be triggered directly by ligands [73]. Increased influx of $[Ca^{2+}]_i$ from the extracellular space may also secondarily result from a depletion of internal Ca^{2+} stores such as the endoplasmic reticulum. The latter is referred to as store-operated calcium entry (SOCE) [74]. Stromal interaction molecule 1 (STIM 1) senses lowering of Ca^{2+}-levels in endoplasmic reticulum and activates a secondary Ca^{2+} entry, i.e., SOCE, through ion channels in the plasma membrane, primarily the ORAI channels [75], but TRPC may also be secondary activated [76].

Besides reacting directly with TRP channels thereby leading to ROCE, membrane spanning G protein-coupled receptors (GPCRs) or receptor tyrosine kinases (RTKs) may

also trigger SOCE [77, 78]. GPCRs are involved in CVD development. More specifically, beta 1- and beta 2-adrenoceptors (β1- and β2ARs) are expressed in the lung, heart, vasculature, and peripheral tissues regulating cardiopulmonary function and immune responses [79, 80]. Other GPCRs such as protease-activated receptor 2 (PAR-2) have also been implicated in Ca^{2+}-signaling in primary human bronchial cells, and are also known to be expressed in the vascular endothelium, where they regulate tone, permeability, and coagulation as well as inflammation [81, 82]. Furthermore, PAR activation may promote conversion of endothelial cells into a pro-inflammatory phenotype, and in conditions associated with endothelial dysfunction PARs mediate contraction, thus potentially contributing to atherosclerosis and hypertension [82].

Aryl Hydrocarbon Receptor

The aryl hydrocarbon receptor (AhR), a basic helix-loop-helix PAS transcription factor, plays a central role in regulating toxicity of environmental chemicals including PAHs [83, 84]. 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 the so-called xenobiotic response elements (XREs) in the promotor region of target genes including the cytochrome P450 enzymes CYP1A1 and CYP1B1. AhR can also mediate signals via non-classical pathways independent of ARNT activation, including crosstalk with NF-κB family of transcription factors as well as other transcription factors and/or signaling molecules [85–88]. An activation of AhR non-genomic signals may imply an increase of focal adhesion and Src kinases, and in the present context most importantly increased $[Ca^{2+}]_i$ [89, 90]. Several studies indicate that Ca^{2+} signaling is central to AhR-induced gene expression, including CYP enzymes and pro-inflammatory cytokines [89, 91, 92]. AhR-mediated changes in $[Ca^{2+}]_i$ affect phospholipase A2 (PLA2), Src as well as protein kinase C (PKC), extracellular signal-regulated kinases (ERK), finally inducing inflammation-associated genes via activator protein 1 (AP-1) [89]. Thus, increase in $[Ca^{2+}]_i$ via AhR non-genomic signaling may have implications for cardiovascular function (Fig. 1).

CDP-/CDP-EOM-/PAH and Calcium

There are a number of studies suggesting possible triggering mechanisms involved in CDP and PAH-induced Ca^{2+} influx, focusing on Ca^{2+} channels like TRP channels, and receptors including GPCRs (βARs and PAR-2) and AhR. However, before discussing these studies in more detail, there are also some unspecific effects that may result in an increased $[Ca^{2+}]_i$ that should be considered.

The plasma membrane is formed of various liquid-ordered phase micro-domains defined by their lipid composition, which is required for proper functioning of various membrane-bound proteins [93–95]. The activity of various ion channels, transporters, and pumps as well as receptors are affected by their presence inside or outside such ordered domains [94, 96–99]. Caveolae and other membrane microdomains appear to be central to regulation of calcium signaling, and have been suggested to provide a platform for pro-inflammatory signals in the vascular endothelium [99, 100].

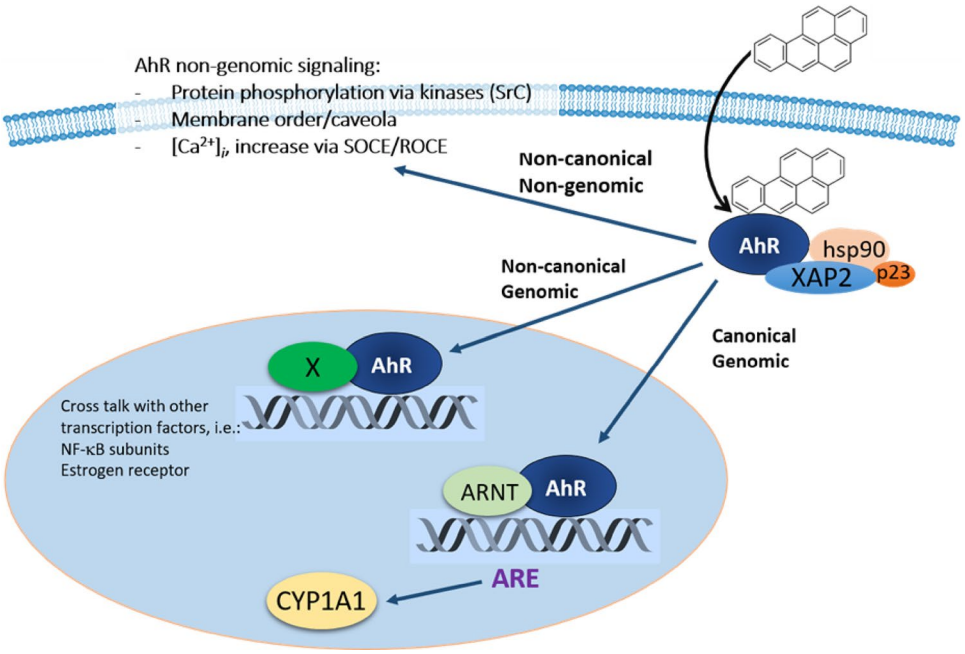

Fig. 1 *AhR signaling pathways*. AhR ligands such as PAHs may enter the cells, and bind and activate the AhR. In the canonical pathway, AhR sheds its chaperones (hsp90, XAP2, and p23), translocates to the nucleus, and binds AhR nuclear translocator (ARNT). AhR–ARNT then activates transcription of classical AhR-regulated genes such as CYP1A1 and CYP1B1. AhR also activates non-canonical pathways, affecting genes via other transcription factors, such as NF-κB and estrogen receptor. Finally, AhR activation may lead to non-genomic signaling, affecting protein phosphorylation, membrane microstructure, and calcium

Indeed, cardiovascular dysfunction has been linked to direct effects on membrane fluidity, enzyme activities, and cation transporters in endothelial cells, vascular smooth muscle cells, and cardiomyocytes. Furthermore, alterations in membrane composition have been shown to affect myocardial contractility, excitability, and conduction properties [101]. Thus, it is interesting that several environmental pollutants including PAHs such as B[*a*]P and phenanthrene have been found to affect membrane microstructure, thereby modulating cellular signaling pathways [102, 103].

As many of the environmental compounds affecting the plasma membrane are very lipophilic, accumulating chemicals in the membrane as such could at least theoretically alter membrane microstructure. On the other hand, we recently found that pyrene [104] and lipophilic organic extracts from DEP [13] induced reductions in the global plasma membrane order in human endothelial cells. These effects were dependent on AhR non-genomic signaling. Most interestingly, they were accompanied by increased $[Ca^{2+}]_i$.

Several studies have found that CDP and/or chemicals adhered, increase $[Ca^{2+}]_i$ by interacting with central calcium channels and/or receptors, and suggested that this may have important implication for CVD [5, 105–107]. Organic chemicals from DEP seem to activate TRPA1, specifically by modification of an interaction in the electrophile/oxidant sensing site of TRPA1 [108]. Furthermore, SOCE activation [109] and increased production of inositol 1,4,5-trisphosphate (IP3) [110] have been suggested to occur upon exposure of cells to oxidative stress. Thus, besides an activation of TRP channels, increased $[Ca^{2+}]_i$ could potentially also result from unspecific damage of Ca^{2+} pumps responsible for transporting Ca^{2+} into internal stores or out of cells. There are in fact studies showing that exposure to ROS or reactive electrophilic metabolites can inhibit these pumps by oxidation/binding to sulfhydryl groups needed for their activity [111].

Recent studies indicate that B[*e*]P, an analog of B[*a*]P that does not bind covalently to DNA or other macromolecules, may increase Ca^{2+} influx [15]. Pyrene as well as organic DEP extracts [13, 104] triggered an increased $[Ca^{2+}]_i$ that declined towards resting state within one hour. Furthermore, this increased Ca^{2+} flux was reduced by specific pharmacological inhibitors and siRNA-mediated knockdown targeting the AhR. Overall, these data suggest that the effects might be due to AhR binding. These and other examples illustrating the effect of combustion-derived PM, extracts from PM as well as PAHs on selective receptors/channels linked to calcium influx will be discussed in more detail below.

TRP Channels

The link between CDP and activation of TRP channels with implication for lung injury (TRPA1, V1, V4, and M8) have recently been elegantly reviewed by Farris and co-workers [5]. This will be briefly summarized below.

Various combustion particles have been found to activate different TRP channels: DEP activate TRPA1, V1, V4, and M8, while cigarette and wood smoke particles activate TRPA1 [108, 112]. In the airways, substance P-positive C-fibers and epithelial cells of conducting bronchi and alveoli express various TRP channels [112]. Provided that PM physically interact with TRP channels on nerve termini, an activation would be predicted to elicit neuronal responses as well as inflammation. Conversely, particles that may activate TRP also have ability to initiate epithelial cell-mediated immune responses, often described in PM toxicology studies. More specifically, TRPV1, V4, and M8 mediate Ca^{2+} influx caused by various CDP including DEP and wood smoke particles. This has been shown by use of selective inhibitors or knockdown of specific TRP channels, and in some cases, by site-directed mutagenesis against specific binding sites within the TRP structure.

Notably, TRPV channels may regulate $[Ca^{2+}]_i$ in response to a variety of exogenous stimuli and is involved in both SOCE and ROCE. Thus, it should be considered that these Ca^{2+} channels may also be activated through more indirect mechanisms triggered by CDP. In fact, this seems to be the case for TRPV4. Exposure of human bronchial epithelial cells to an organic extract of DEP resulted in TRPV4-mediated Ca^{2+} influx, increased RAS expression, mitogen-activated protein kinase (MAPK) signaling, and activation of matrix metalloproteinase-1 (MMP-1). Furthermore, the authors hypothesized that secretion of MMP-1, or other MMPs, could then activate PAR-2 [5, 113].

In humans, exposure to DEP from urban driving cycles reduced vaso-dilatory effects of the calcium blocker verapamil [114]. This was considered related to vaso-constrictive effects of increased $[Ca^{2+}]_i$ in vascular smooth muscle cells. Interestingly, verapamil has been found to have direct effects on endothelial cells [115]. Thus, the inhibitory effect of DEP towards verapamil may also relate to endothelial effects of DEP exposure. We have recently found that rather low concentrations of organic extracts from DEP and various PAHs increase $[Ca^{2+}]_i$ in endothelial cells, thus disturbing calcium regulation [13, 15, 104]. Several TRP channels are important to endothelial homeostasis, and seem to play a role in development of CVD, especially by affecting endothelial function [116–121]. Furthermore, findings of increased Ca^{2+}-influx through TRPC channels in monocytes from patients and rats with hypertension, indicate a role of dysregulated $[Ca^{2+}]_i$ in CVD [122, 123].

GPCRs

βARs regulate cardiopulmonary function and immune responses, and are among the main drug targets in CVD

treatment [124–126]. In some recent studies, B[*a*]P caused a transient increase $[Ca^{2+}]_i$ in human microvascular endothelial cells (HMEC-1) independently of AhR [15]. The increased $[Ca^{2+}]_i$ was prevented by β2AR antagonists, blocking antibodies, or siRNA. Furthermore, increased $[Ca^{2+}]_i$ was strongly potentiated by β2AR overexpression in human kidney HEK293 cells [106]. B[*a*]P was shown to directly bind β2AR, as assessed by in vitro binding assays and molecular modeling; and various signals acting downstream of β2AR, such as G protein, adenylyl cyclase, Epac-1 protein, and IP3/IP3 receptor were demonstrated to be involved in the Ca^{2+} influx. Further studies showed that exposure to B[*a*]P decreased β2ADR responsiveness. Analysis of β2ADR demonstrated that B[*a*]P rapidly decreased expression and level of β2ADR in the plasma membrane [127].

In human bronchial epithelial BEAS-2B cells, the calcium chelator BAPTA-AM attenuated CXCL8 responses by a panel of environmental pollutants, including 1-nitropyrene (1-NP) [128]. Further studies showed that exposure to 1-NP increased $[Ca^{2+}]_i$ and expression and release of CXCL8 (IL-8) [107]. The early but not the later phase of 1-NP-induced $[Ca^{2+}]_i$ was hampered by inhibitors of β2AR. Moreover, inhibition of β2AR by antagonists, blocking antibody, or siRNA transfection attenuated CXCL8 responses induced by 1-NP. These results suggest that β2AR may induce Ca^{2+} signaling at least partly also in BEAS-2B cells, and that both β2AR and Ca^{2+} signaling may be involved in 1-NP-induced CXCL8 responses. It should, however, be noted that these effects of 1-NP were first observed at high concentrations.

Other GPCRs have also been implicated in DEP-induced Ca^{2+} signaling and inflammation. Li and colleagues [105, 129] described a role of PAR-2 in DEP-induced responses in BEAS-2B and primary bronchial epithelial cells. Upon exposure to DEP or DEP–OE, PAR-2 activated TRPV4 channels leading to up-regulation of MMP-1. In another study on BEAS-2B, DEP increased expression of IL-6 and CXCL8 [130]. SiRNA towards PAR-2 attenuated IL-6 responses without affecting CXCL8, suggesting that these two pro-inflammatory responses may be independently regulated. Furthermore, a heptane extract of DEP containing the most lipophilic chemicals induced both IL-6 and CXCL8 release.

The above studies may seem to indicate a central role for GPCRs in mediating health effects caused by CDP. However, care should be taken when considering relevance, as in real life we are exposed to varying combinations of CDP, that all contain complex mixtures of various PAHs as well as other compounds. Recently, we exposed HMEC-1 to DEP-extractable organic material (EOM) fractionated by sequential extraction with solvents of increasing polarity: *n*-hexane (*n*-Hex-EOM), dichloromethane (DCM-EOM), methanol (Methanol-EOM), and water (Water-EOM) [131]. The two fractions containing the more lipophilic chemicals including various PAHs, i.e., *n*-Hex-EOM and DCM-EOM, enhanced $[Ca^{2+}]_i$ and expression of inflammation-associated genes (IL-1α, IL-1β, COX-2, and CXCL8). The βAR inhibitor carazolol suppressed the increase in $[Ca^{2+}]_i$ induced by both *n*-Hex-EOM and DCM-EOM, and reduced DCM-EOM-induced COX-2 expression [131]. Furthermore, PAR-2 seemed involved in the up-regulation of some genes induced by both lipophilic fractions [45]. However, the PAR-2 inhibitor ENMD-1068 only attenuated $[Ca^{2+}]_i$ responses to DCM-EOM. As the overall picture obtained was complex, it was concluded that βAR and PAR-2 only partly modulate effects of DEP-EOM on $[Ca^{2+}]_i$ and inflammation-associated genes in HMEC-1.

AhR

The classical AhR ligand, dioxin (TCDD), increases $[Ca^{2+}]_i$ in vitro [132–134]. Furthermore, AhR is involved in the development of the vasculature and AhR activation is linked to CVD, although any specific role of non-genomic AhR-mediated calcium responses is uncertain [84, 135–138]. Exposure of U937 cells to native urbane dust, DEP, their corresponding organic extracts, and stripped particles showed that native PM, their extracts, and TCCD led to a greater increase of IL-8, TNF-α, and COX-2 mRNA expression than stripped particles [25]. Effects of particles and organic extracts on COX-2 expression were significantly suppressed by AhR antagonist, indicating that these effects are mainly due to organic components activating AhR. Further studies from the same group showed that TCDD-induced expression of pro-inflammatory genes (cPLA2, COX-2 and IL-8), but not CYP1A1 expression, in U937 macrophages was abrogated by pretreatment with the L-type Ca^{2+}-channel blocker nifedipine and by 2-APB which blocks Ca^{2+} release from endoplasmic reticulum [89, 139]. Thus, Ca^{2+} signaling may represent an initial triggering mechanism regulating TCDD-induced inflammatory responses in macrophages. Notably, the ability of AhR ligands to induce non-genomic $[Ca^{2+}]_i$ appears to vary considerably between experimental models, both with respect to time course and magnitude of response, and AhR-independent $[Ca^{2+}]_i$ increases have also been reported [132, 133, 140–142]. Another characteristic of AhR non-genomic pathway has been the lack of involvement of ARNT. This has been confirmed at several stages of the action of TCDD including the initial stage of signaling as well as later stages, using siRNAs against ARNT [89].

In a recent study, we have further explored non-genomic AhR signaling using pyrene. Pyrene is a PAH which binds to AhR, but causes little or no AhR-genomic responses. We found that exposure of HMEC-1 to pyrene induced a relatively rapid transient AhR-dependent increase in $[Ca^{2+}]_i$, consistent with activation of AhR non-genomic signaling. In

silico molecular modeling confirmed that pyrene may dock in the human AhR-PAS-B binding pocket in the antagonist, but not the agonist conformation [104].

The precise link between CDP/CDP-EO/PAHs exposure, non-genomic AhR signaling such as $[Ca^{2+}]_i$, and expression of inflammatory genes including cPLA2 and COX-2 is far from well established. However, in support of a connection, we have found that DEP-EOM containing the most lipophilic compounds increased $[Ca^{2+}]_i$ via AhR non-genomic signaling in HMEC-1 [13], and that the AhR inhibitor CH223191 attenuated up-regulation of inflammation-associated genes induced by the same lipophilic DEP-EOM in HMEC-1 and primary human endothelial cells [45].

Non-genomic pathways mediate important signaling from various nuclear receptors including the estrogen, androgen, and cortisone receptors as well as PPARα [143–145]. Interestingly, estrogen receptor (ER) non-genomic signaling appears to be important to endothelial function [146, 147], and the ER is functionally divided into nuclear and membrane receptors [146, 148]. Accordingly, steroid receptor-related non-genomic signaling has been linked to caveolae [149]. In line with this, caveolae have also been implicated in AhR non-genomic signaling [100]. AhR-mediated gene expression appears to depend on caveolin-1 (Cav-1) [100]. AhR and Cav1 were found to co-localize in caveolae, and distribution of Cav1 to these micro-domains was AhR regulated. Adding exogenous cholesterol induced an "$AhR^{-/-}$-phenotype" illustrating the importance of cholesterol in caveolar structure and function [150]. Furthermore, AhR has been shown to mediate effects non-genomically via both phospholipase A2/Ca^{2+}, and FAK/Src [90, 132, 151], which also have been shown to interact with Cav-1 [97, 100, 152]. Thus, it seems likely that caveolae might play a central role in AhR non-genomic signaling, and that the mechanisms involved may resemble those described for nuclear receptors.

Dichloromethane-extracted organic material (DCM-EOM) from DEP previously washed with n-Hexane, increased Ca^{2+} entry and membrane re-shuffling via AhR non-genomic signaling in HMEC-1 [13]. These effects were inhibited by cholesterol addition and may thus relate to interplay between AhR and caveolae. In support of this, caveolin has been shown to bind cholesterol and regulate endothelial calcium signaling [153, 154]. Furthermore, Ca^{2+} entry via STIM1, Orai1, and TRPs seems to depend on membrane structure/caveolae [75, 99, 154, 155], and actually, impaired calcium influx in endothelial cells from caveolin knockout mice was restored upon reconstitution with endothelial Cav-1 [154]. Finally, polychlorinated biphenyls (PCBs), that have inflammatory effects on the endothelium, seem to accumulate in caveolae [156]. PCB exposure increased AhR binding to caveolin, and Cav silencing attenuated the effects of PCB on CYP1A1, thus indicating a tight interplay between PCBs, caveolae, and AhR [156]. It therefore seems plausible that lipophilic organic chemicals from DEP may interact with caveolae and AhR in a similar manner.

Conclusion and Future Perspectives

Several CDP, CDP-EOM, and PAH have been shown in vitro, to disturb $[Ca^{2+}]_i$ and affect various cellular processes linked to vascular disease. Suggested triggering mechanisms include TRP channels, βAR, and PAR-2 as well as AhR non-genomic pathway. Clarifying any possible role of disturbed $[Ca^{2+}]_i$ and mechanisms involved may aid in understanding the relationship between CDP and vascular disease. Thus, further studies both in vitro and in vivo addressing the possibility that CDP and adhered organic chemicals may increase risk of CVD by disturbing cellular regulation of calcium are warranted.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interests.

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