Biochemical Pharmacology 95 (2015) 126–132

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Inhibition of cytochrome P450 2B4 by environmentally persistent free radical-containing particulate matter

James R. Reed [a,*], Albert Leo N. dela Cruz [b], Slawo M. Lomnicki [b], Wayne L. Backes [a]

[a] Department of Pharmacology and Experimental Therapeutics and The Stanley S. Scott Cancer Center Louisiana State University Health Sciences Center 533 Bolivar St. New Orleans, LA 70112, USA
[b] Department of Environmental Sciences and LSU Superfund Research Center Louisiana State University A&M College Baton Rouge, LA 70803, USA

ARTICLE INFO

Article history:
Received 20 January 2015
Accepted 17 March 2015
Available online 24 March 2015

Keywords:
Cytochrome P450 2B4
Inhibition
Particulate matter, Environmentally persistent free radicals

ABSTRACT

Combustion processes generate particulate matter (PM) that can affect human health. The presence of redox-active metals and aromatic hydrocarbons in the post-combustion regions results in the formation of air-stable, environmentally persistent free radicals (EPFRs) on entrained particles. Exposure to EPFRs has been shown to negatively influence pulmonary and cardiovascular functions. Cytochromes P450 (P450/CYP) are endoplasmic reticulum resident proteins that are responsible for the metabolism of foreign compounds. Previously, it was shown that model EPFRs, generated by exposure of silica containing 5% copper oxide (CuO–Si) to either dicholorobenzene (DCB230) or 2-monochlorophenol (MCP230) at $\geq$ 230 °C, inhibited six forms of P450 in rat liver microsomes (*Toxicol. Appl. Pharmacol.* (2014) 277:200–209). In this study, the inhibition of P450 by MCP230 was examined in more detail by measuring its effect on the rate of metabolism of 7-ethoxy-4-trifluoromethylcoumarin (7EFC) and 7-benzyloxyresorufin (7BRF) by the purified, reconstituted CYP2B4 system. MCP230 inhibited the CYP2B4-mediated metabolism of 7EFC at least 10-fold more potently than non-EPFR controls (CuO-Si, silica, and silica generated from heating silica and MCP at 50 °C, so that EPFRs were not formed (MCP50)). The inhibition by EPFRs was specific for the P450 and did not affect the ability of the redox partner, P450 reductase (CPR) from reducing cytochrome c. All of the PM inhibited CYP2B4-mediated metabolism noncompetitively with respect to substrate. When CYP2B4-mediated metabolism of 7EFC was measured as a function of the CPR concentration, the mechanism of inhibition was competitive. EPFRs likely inhibit CYP2B4-mediated substrate metabolism by physically disrupting the CPR·P450 complex.

1. Introduction

Particulate matter (PM) represents a ubiquitous form of environmental pollution that is produced largely by combustion processes [1–3]. PM comprising the fine (<2.5 μm) and ultra-fine (<0.1 μM) size range has been associated with a variety of harmful health effects because of their ability to deeply penetrate the lower airways and alveoli of lungs which in turn, facilitates entry into the circulation and distribution to distal tissues [4–6]. Epidemiologic research shows exposure to fine and ultra-fine particles is associated with cardiac morbidities and mortality [7,8] and can lead to impairment of lung development and function in children [9]. Furthermore, inhalation of these nanoparticles exacerbates pulmonary infirmities such as chronic obstructive pulmonary disease [10], asthma [11], and lower tract respiratory infections [12]. In many instances, the adverse effects of exposure to fine and ultra-fine PM can be attributed to oxidative stress and subsequent inflammation [13].

Combustion processes result in the formation of incomplete combustion by-products, including particulate matter, metals, and aromatic hydrocarbons comprised of oxy-aromatic and halogenated-aromatic derivatives. During combustion these organic compounds can undergo chemical reactions with vaporized fuel metal and later condense in the form of chemisorbed oxides in the particulate matter. Such interactions initiate redox processes between the metal center and adsorbed organic molecule resulting in formation of resonance-stabilized semiquinone and phenoxyl type radicals. Due to the association with the metal center and particle, these types of radicals have been shown to have very long lifetimes (>1 week in some cases) in the ambient environment and

* Corresponding author. Tel.: +1 504 568 3351; fax: +1 504 568 2361.
E-mail addresses: rreed@lsuhsc.edu (J.R. Reed), adelac2@tigers.lsu.edu (A.L.N.d. Cruz), slomni1@lsu.edu (S.M. Lomnicki), wbacke@lsuhsc.edu (W.L. Backes).

http://dx.doi.org/10.1016/j.bcp.2015.03.012
0006-2952/

are capable of contributing to oxidative stress in living organisms [14–18] and thus are called Environmentally Persistent Free Radicals (EPFRs). Because of their prolonged ability to initiate oxidation/reduction activities, EPFRs may play an important role in potentiating the harmful effects of PM exposure.

To study exclusive effects of EPFRs, our collaborators have generated model EPFRs by exposure of a particle matrix composed of 5% copper oxide (w/w) and silica (<0.2 μm in diameter) to the aromatic hydrocarbons (2-monochlorophenol (MCP230) and 1,2-dichlorobenzene (DCB230)), at ≥230 °C. This method of EPFR generation provides a simple, well-defined system to study the chemistry and health effects of these pollutants and avoids the complexity and variability inherent in real-world samples. Studies with EPFRs have confirmed the ability of MCP230 to contribute to oxidative stress [17–19], and exposure of animals to this PM by inhalation has led to the development of many of the cardiac [20,21] and pulmonary [22–25] morbidities implicated by epidemiological studies of PM.

Cytochromes P450 (P450 or CYP) represent a ubiquitous superfamily of enzymes that are widely expressed in various tissues of plants and animals [26]. These enzymes use molecular oxygen and electrons provided by a separate redox partner, either cytochrome P450 reductase (CPR) or cytochrome b_5, to catalyze the mixed-function oxidation of lipophilic substrates [27,28]. Although some P450s have evolved to participate in endogenous reactions such as steroid biosynthesis [29], most of the P450s, and particularly the ones from families 1, 2, and 3, are responsible for the metabolism of lipophilic xenobiotics [30]. Oxidation by the P450s makes lipophilic substrates more water soluble and thus, the enzymes play a critical role in eliminating exogenous compounds from the body. However, P450s also can bioactivate some compounds forming reaction products that bind to protein and DNA and as a result, can lead to toxicity and/or carcinogenesis [31].

Given the importance of P450 enzymes in xenobiotic metabolism and elimination, we previously assessed the ability of different types of PM to influence the activities of these enzymes [32]. Using rat liver microsomes from animals treated with various chemicals to induce different forms of P450 and substrates that were specifically metabolized by individual forms of P450, we determined that MCP230 and DCB230 potently inhibited six different forms of P450 relative to other types of PM such as pure silica, copper oxide-silica (CuO-Si), and silica that was exposed to either 2-monochlorophenol or 1,2-dichlorobenzene at lower temperatures so that free radicals are not formed (MCP50 or DCB50). The inhibition of P450-mediated metabolism by EPFRs was a specific effect on P450s as the EPFRs did not inhibit the ability of CPR to reduce cytochrome c or the activity of heme oxygenase-1 in rat liver microsomes. When the activity of a CYP2D2 was measured in rat liver microsomes as a function of the concentration of a selective probe substrate for this enzyme, 3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin [33,34], the inhibition by both types of EPFRs was shown to be noncompetitive.

In the present study, the purified, reconstituted system for rabbit CYP2B4 was used to study the inhibition by different types of PM including the EPFR, MCP230. CYP2B4 was the first mammalian P450 enzyme [35] to be purified and is probably the most well studied P450. Virtually every novel biophysical and biochemical technique that has been applied to the study of P450s has involved CYP2B4, many of these occurring before the human forms of P450 were readily available [36–39]. Furthermore, the enzyme is still used as a prototype in many recent P450 studies [40–42]. Thus, this enzyme provides a useful starting point from which to study the effects of EPFRs on P450-mediated metabolism. By using the purified, reconstituted P450 system, components and conditions were modulated to specifically determine the mechanism by which PM inhibit CYP2B4. Our results indicate that the EPFR and each of the control particles inhibited CYP2B4 noncompetitively. Furthermore, the mechanism of inhibition is consistent with the MCP230 physically interfering with the requisite complex between CPR and P450.

2. Materials and methods

2.1. Chemicals

The reagents used were of the highest commercial quality available. TWEEN 80, HEPES sodium salt, EDTA, potassium phosphate, NADPH, sodium chloride, glycerol, resorufin, and dilauroylphosphatidylcholine (DLPC) were purchased from Sigma (St. Louis, MO). 7-hydroxy-4-trifluoromethylcoumarin was purchased from Molecular Probes (Eugene, OR). 7-ethoxy-4-trifluoromethylcoumarin (7EFC) and 7-benzyloxyresorufin (7BRF) were purchased from Anaspec (Fremont, CA).

2.2. Particle preparation

Fumed Silica (CAB-O-SIL EH-5) was purchased from Cabot Corporation (Billerica, MA) and was used to make silica impregnated with 5% copper oxide (w/w) (CuO–Si); MCP230; and silica with physisorbed MCP (MCP50) as described previously [16]. Briefly, silica was first impregnated with copper nitrate hemipentahydrate by incubation in a 0.1 M solution for 24 h at room temperature. The impregnated silica was then dried at 120 °C for 12 h and subsequently heated for 5 h in air at 450 °C to complete the calcination process. The prepared particles were placed in vacuum ($<10^{-2}$ torr) and heated to 230 °C before being dosed with vapors of the organic constituents at 10 torr in a custom-made vacuum exposure chamber for 5 min. The samples were cooled to room temperature and evacuated for 1 h (10^{-2} torr). The radical contents of the EPFRs were analyzed by electron paramagnetic resonance (EPR) spectroscopy as described previously [17], and the samples were then weighed in 15 mg portions and sealed in ampoules under vacuum. Only EPFRs that contained greater than 1×10^{17} spins/g were used in the experiments described herein and were used within one week after synthesis. The PM suspensions were previously characterized by flow cytometry and transmission electron microscopy and were shown to consist of disaggregated particles approximately 200 nm in diameter [14].

Each type of PM was added to the assay mixtures from a suspension in 0.9% NaCl containing 0.02% TWEEN 80 (v/v). The pH of the saline/TWEEN 80 solution was adjusted to pH 7.0 with 1% sodium bicarbonate before adding to the PM. The nanoparticle suspensions were prepared at a concentration of 2 mg/ml by vortexing vigorously for one minute followed by probe sonication (15 watts) on ice for four × 30 s cycles with 30 s intervals between each sonication. The suspension was then added to assay mixtures at the desired concentrations (indicated in Section 3). The PM suspensions were vortexed intermittently to keep the particles from settling before they were added to the reaction mixtures.

2.3. Enzymes

CYP2B4 was expressed in *E. coli* and purified as described previously [43]. Rabbit CPR was expressed in *E. coli* and purified as described previously [44].

2.4. Enzymatic assays

The *O*-dealkylation of 7EFC mediated by CYP2B4 was measured after reconstituting purified CPR and CYP2B4 with dilauroylphosphatidylcholine (DLPC). The proteins were pre-incubated at a