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

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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 dichlorobenzene (DCB230) or 2-monochlorophenol (MCP230) at 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.

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Keywords

cytochrome P450 2B4; inhibition; particulate matter; environmentally persistent free radicals

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 ($< 2.5 \mu\text{m}$) and ultra-fine ($< 0.1 \mu\text{m}$) 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 exacerbate 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 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 ($< 0.2 \mu\text{m}$ 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 b₅, 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 0.1M solution for 24 hours at room temperature. The impregnated silica was then dried at 120°C for 12 hours and subsequently heated for 5 hours 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 hr (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 x 30 sec cycles with 30 sec intervals between each sonication. The suspension was then added to assay mixtures at the desired concentrations (indicated in Results). 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 P450 concentration greater than 5 μ M with an amount of DLPC that was equal to 160 times the amount of P450. Before adding to the enzymes, the DLPC was bath-sonicated to clarity in an 8 mM solution containing 0.05 M HEPES (pH 7.5), 100 mM NaCl, 20% glycerol, and 0.1 mM EDTA. The enzymes and lipid were incubated for 2 hours at room temperature before adding other reaction components and performing the assays. Unless otherwise indicated, the assays were performed at 0.1 μ M CYP2B4, 0.2 μ M CPR, and 16 μ M DLPC.

The reconstituted systems used to measure the rate of *O*-dealkylation of 7BRF by CYP2B4 were prepared as described above except that 0.05 μ M CPR was used instead of 0.1 μ M CPR. This substrate participates in a redox cycle with CPR that interferes with its metabolism by CYP2B enzymes when the protein is in large excess to P450 [45].

The assays were conducted at 37°C in a buffer containing 0.05 M HEPES (pH 7.5) and 15 mM MgCl₂. PM was added at the desired concentrations from a suspension in a solution of 0.9 % NaCl and 0.02% TWEEN 80 as described above. The 0.1 ml (final volume) reactions were initiated with 0.01 ml of 4 mM NADPH and the rates of product formation were determined by measuring the rate of change of fluorescence in real time (Ex: 410 nm, Em: 510 nm) and comparing to a 7-hydroxy-4-trifluoromethylcoumarin standard curve. For the measurement of 7BRF metabolism, the reaction was followed by fluorescence in real time (Ex: 535 nm; Em: 585 nm) and product was quantified by reference to a resorufin standard curve. The effects of the PM were determined by comparison to assays without PM that contained equal concentrations of the saline/TWEEN 80 solution used to suspend the particles.

The rates of cytochrome c reduction by CPR were determined after reconstituting CPR in DLPC at a ratio of 320:1 as described above. The CPR was added at a concentration of 0.005 μ M to the 0.1 ml assay mixtures containing 0.05 M potassium phosphate (pH 7.7) and 1 mg/ml cytochrome c. Reactions at 37°C were initiated with 0.01 ml of 4 mM NADPH and the rate of cytochrome c reduction was determined by monitoring absorbance at 548 nm and using the difference extinction coefficient for reduced vs. oxidized cytochrome c (0.021 μ M⁻¹ • cm⁻¹) [46;47]. The optical references for these incubations contained all components with the exception of CPR.

3. Results

3.1. Inhibition of CYP2B4 activity by different types of PM

The relative abilities of four different types of PM to inhibit the CYP2B4-mediated metabolism of 7EFC were determined by measuring the rates of metabolism over a range of concentrations for each type of particles (Figure 1). In the liver endoplasmic reticulum, P450 is present in excess of the CPR [48]. Thus, in order to assess the relative abilities of the PM to affect P450-mediated metabolism under conditions that are more similar to those observed in vivo, a subsaturating 1:2 CPR:CYP2B4 ratio was used.

Each type of nanoparticle had differing abilities to inhibit the reaction. The data show that MCP230 was clearly the most potent inhibitor of the reaction as the rate was 50% decreased at a concentration of only 0.018 mg/ml. The EPFR completely inhibited the reaction at a concentration of 0.05 mg/ml. The next most potent particle was CuO-Si, which caused 50% inhibition of the P450 activity at a concentration of 0.13 mg/ml and completely inhibited at a concentration of 0.25 mg/ml CuO-Si. Silica and MCP50 were very similar in potency as the CYP2B4-mediated reaction was approximately 50% inhibited by each particle at a concentration of 0.4 mg/ml. These data show that MCP230 is more than 7-fold more potent than CuO-Si as an inhibitor of CYP2B4. The EPFR, MCP230, is 22-fold more potent than silica and MCP50.

3.2. Effect of PM on CPR activity

To assess the possibility that the PM inhibited the P450-mediated metabolism indirectly through effects on CPR, the rates of reduction of cytochrome c catalyzed by CPR were measured with and without the various types of PM at concentrations that resulted in greater than 50% inhibition of the metabolism of 7EFC by CYP2B4 (Figure 2). The data show that none of the PM significantly altered the ability of CPR to reduce cytochrome c. Thus, the inhibitory effects of the PM on CYP2B4 are caused by a direct interaction with the P450.

3.3. PM inhibit CYP2B4 noncompetitively

In order to determine the manner by which the different types of PM inhibited CYP2B4-mediated metabolism, the rates of 7EFC *O*-dealkylation were measured as a function of 7EFC concentration in the presence of different concentrations of MCP230 (Figure 3), CuO-Si (Figure 4), and MCP50 (Figure 5). In order to accurately characterize the effects of the PM on P450-mediated metabolism, the CPR was added in excess of P450 for these experiments (see Materials and Methods). The B panels of each figure show the double reciprocal plots derived from the corresponding data in panel A. The lines drawn through the double reciprocal data for each type of particle intersect at a common point on the x-axis indicating that the type of inhibition by each particle is noncompetitive. The only difference in the mechanisms of inhibition by the particles pertains to the effects of silica and MCP50 as these particles seemed to only be partial noncompetitive inhibitors of the CYP2B4-mediated metabolism. When the CPR was present in excess to P450, higher particle concentrations were required to obtain the same degree of inhibition when compared to the subsaturating CPR used in Fig 1. However, the relative differences in particle potencies were similar under the two conditions. Despite the similarity in the type of inhibition, it is important to keep in mind that the concentrations of EPFR-containing MCP230 was much lower than the other control particles due to its more potent inhibition (Fig. 1). From the data shown in Figures 3-5, the relative K_i constants for MCP230, CuO-Si, and MCP50 were calculated to be 3 $\mu\text{g/ml}$, 160 $\mu\text{g/ml}$, and 243 $\mu\text{g/ml}$, respectively, showing that the EPFRs were more than 50 times more potent than the non-EPFR particles. In order to determine that the effects of EPFRs on CYP2B4-mediated metabolism were consistent, we also examined the mechanism of inhibition for metabolism of another CYP2B4 substrate 7-benzyloxyresorufin. Inhibition of this reaction was also characterized as noncompetitive (Figure 6). Thus, the EPFR and other particles inhibit CYP2B4-mediated metabolism by a consistent mechanism.

3.4. MCP230 competes with CPR for binding to CYP2B4

The data discussed above showed that PM inhibited CYP2B4 noncompetitively with respect to the substrate, 7EFC. It was suspected that the EPFRs might be inhibiting this reaction by disruption of the complex between CPR and CYP2B4, therefore depriving CYP2B4 of the electrons necessary for substrate oxygenation. In order to test this possibility, we measured the rate of 7EFC metabolism by CYP2B4 as a function of the CPR concentration at several MCP230 concentrations (Figure 7). The lines generated from the double reciprocal plots of the data for different MCP230 concentrations (panel 6B) intersect at the same point on the Y-axis, which is consistent with a competitive interaction between CPR and CYP2B4.

4. Discussion

We previously showed that EPFRs acted as general inhibitors of P450 [32]. In this study, we examined this inhibition in more detail using the purified, reconstituted CYP2B4 system. In addition, we also compared the inhibition of the CYP2B4-mediated metabolism of 7EFC by the EPFR, MCP230, to the inhibition observed with other non-EPFR PM. Our findings showed MCP230 to be a much more potent inhibitor than CuO-Si which in turn, was significantly more potent than either silica or physisorbed MCP50. We showed that the inhibition by each of the PM was attributable to a specific interaction with the P450 as the rate of cytochrome c reduction by CPR was not affected by the PM at concentrations that dramatically inhibited the P450-dependent reaction. Furthermore, our previous study demonstrated that the EPFRs did not inhibit another CPR-dependent activity, the metabolism of heme by heme oxygenase-1, in rat liver microsomes [32]. Thus, the EPFRs interact with P450 but not heme oxygenase.

The inhibition by each type of PM was noncompetitive with respect to the substrate, 7EFC. Thus, the PM did not inhibit CYP2B4 by binding to the active site of the enzyme. The other region of CYP2B4 molecule that could be involved in the inhibition by the PM is likely to occur would be at the interface of the CPR•CYP2B4 complex. This would prevent the flow of electrons to the P450 that are needed for oxygen activation and substrate oxidation. To test this possibility, the inhibition of CYP2B4-mediated 7EFC *O*-dealkylation by MCP230 was measured as a function of the CPR concentration. Under these conditions, the inhibition was competitive indicating that the PM did indeed interfere with the ability of CYP2B4 to form a complex with CPR. The potency of the MCP230 relative to the other types of PM must reflect a higher affinity for binding to the P450. However, it is not clear why the EPFR would bind to CYP2B4 with higher affinity than CuO-Si, MCP50, or silica. A potential explanation for this greater potency is stabilization of the EPFR•CYP2B4 complex by the radical species.

Other studies also have demonstrated that PM inhibit P450s [49;50], albeit at higher concentrations than those we have observed with EPFRs, and have postulated mechanisms that involved both interfering with the formation of the P450/CPR binding complex [49] and binding of substrate to P450 [50]. Using the purified, reconstituted P450 system, we have for the first time, elucidated a mechanism by which the PM inhibit a P450 enzyme and have compared different types of PM to show that they inhibit by a common mechanism.

The size of CYP2B4 is about $8.75 \times 10^5 \text{ \AA}^3$ [51] with an active site of about $350 \text{ \AA}^3$, depending on the substrate bound [52]. Based on these sizes it would be surprising if a nanoparticle with a volume approximately $2.9 \times 10^9 \text{ \AA}^3$ was able to access the active site of CYP2B4. It is much more likely that the particle associates with the surface of CYP2B4 in the region that binds to CPR.

The harmful health effects of nanoparticle pollution have been attributed to inflammatory and altered immune responses [23-25] and oxidative stress [13;19]. PM containing metal oxides have been shown to directly contribute to oxidative stress [53], and the effects of EPFRs in this regard are even more dramatic [19]. Our studies suggest that PM and EPFRs

in particular, may also be deleterious by inhibiting P450s and in turn, compromising the ability of an organism to metabolize and eliminate xenobiotics. This would make individuals exposed to PM more susceptible to the harmful effects of toxins and carcinogens present in the environment. Furthermore, there may be a greater likelihood of individuals exposed to EPFRs also being exposed to other harmful pollutants at sites that are involved in the combustion of hazardous wastes. More work is needed to better understand how the variables in EPFR composition affect the ability to inhibit P450s.

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Figure 1. Effects of PM on metabolism of 7EFC by CYPB4

Reconstituted systems containing 0.1 μM CYP2B4, 0.05 μM CPR, and 16 μM DLPC were prepared and assayed to measure the rate of 7EFC metabolism as described in Materials and Methods. The reactions were performed at 25 μM 7EFC which is approximately the K_m for metabolism by CYP2B4 (see figure 3). The relative rates of activity as a function of nanoparticle concentration were expressed as the percentages of the rates measured in the absence of particles. These reactions were performed in the presence of 0.23% saline and 0.005% TWEEN 80. The data points represent the average $\pm$ the standard error of three separate determinations.

Figure 2. Effects of PM on the rate of cytochrome c reduction by CPR

CPR and DLPC were reconstituted and the rate of cytochrome c reduction was measured as described in Materials and Methods. The PM was present at the concentrations indicated. These concentrations of particles resulted in at least 50% inhibition of CYP2B4-mediated metabolism in the presence of silica and MCP50 and more than 90% inhibition for MCP230 and CuO-Si. These reactions were performed in the presence of 0.61% saline and 0.014% TWEEN 80. The data points represent the average $\pm$ the standard error of three separate determinations.

Figure 3. Rates of CYP2B4-mediated metabolism as a function of 7EFC concentration in the presence of different concentrations of MCP230

The rates of 7EFC metabolism by CYP2B4 were determined as described in Materials and Methods. These reactions were performed in the presence of 0.7% saline and 0.015% TWEEN 80. The data points represent the average $\pm$ the standard error of at least three separate determinations. Panel B shows the double reciprocal plot of the data shown in panel A. Legend: Saline/TWEEN 80 control, circles; 0.005 mg/ml MCP230, open squares; 0.01 mg/ml MCP230, triangles; 0.02 mg/ml MCP230, open diamonds; and 0.03 mg/ml MCP230, stars.

Figure 4. Rates of CYP2B4-mediated metabolism as a function of 7EFC concentration in the presence of different concentrations of CuO-Si

The rates of 7EFC metabolism by CYP2B4 were determined as described in Materials and Methods. These reactions were performed in the presence of 0.7% saline and 0.015% TWEEN 80. The data points represent the average $\pm$ the standard error of at least three separate determinations. Panel B shows the double reciprocal plot of the data shown in panel A. Legend: Saline/TWEEN 80 control, circles; 0.125 mg/ml CuO-Si, open squares; 0.175 mg/ml CuO-Si, triangles; 0.25 mg/ml CuO-Si, open diamonds; and 0.35 mg/ml, stars.

Figure 5. Rates of CYP2B4-mediated metabolism as a function of 7EFC concentration in the presence of different concentrations of physisorbed MCP50

The rates of 7EFC metabolism by CYP2B4 were determined as described in Materials and Methods. These reactions were performed in the presence of 0.7% saline and 0.015% TWEEN 80. The data points represent the average $\pm$ the standard error of at least three separate determinations. Panel B shows the double reciprocal plot of the data shown in panel A. Legend: Saline/TWEEN 80 control, circles; 0.3 mg/ml MCP50, squares; 0.45 mg/ml MCP50, triangles; and 0.45 mg/ml MCP50, diamonds.

Figure 6. Rates of CYP2B4-mediated metabolism as a function of 7BRF concentration in the presence of different concentrations of MCP230

The rates of 7BRF metabolism by CYP2B4 were determined as described in Materials and Methods. These reactions were performed in the presence of 0.11% saline and 0.003% TWEEN 80. The data points represent the average $\pm$ the standard error of at least three separate determinations. Panel B shows the double reciprocal plot of the data shown in panel A. Legend: Saline/TWEEN 80 control, circles; 0.01 mg/ml MCP230, open squares; 0.015 mg/ml MCP230, triangles; 0.025 mg/ml MCP230, open diamonds.

Figure 7. Rates of CYP2B4-mediated metabolism as a function of CPR concentration in the presence of different concentrations of MCP230

The rates of 7EFC metabolism by CYP2B4 were determined as described in Materials and Methods. The reconstituted systems contained 0.025 μM CYP2B4, 4 μM DLPC and CPR (ranging from 0-0.25 μM). The 7EFC concentration for these assays was 500 μM . These reactions were performed in the presence of 0.11% saline and 0.0025% TWEEN 80. The data points represent the average $\pm$ the standard error of at least three separate determinations. Panel B shows the double reciprocal plot of the data shown in panel A. Legend: Saline/TWEEN 80 control, circles; 0.01 mg/ml MCP230, open squares; and 0.02 mg/ml MCP230, triangles.