Anal Bioanal Chem (2012) 403:431–441
DOI 10.1007/s00216-012-5823-z

ORIGINAL PAPER

Glutathione peroxidase inhibitory assay for electrophilic pollutants in diesel exhaust and tobacco smoke

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Received: 19 October 2011 / Revised: 30 January 2012 / Accepted: 1 February 2012 / Published online: 21 February 2012

Abstract We developed a rapid kinetic bioassay demonstrating the inhibition of glutathione peroxidase 1 (GPx-1) by organic electrophilic pollutants, such as acrolein, crotonaldehyde, and *p*-benzoquinone, that are frequently found as components of tobacco smoke, diesel exhaust, and other combustion sources. In a complementary approach, we applied a high-resolution proton-transfer reaction time-of-flight mass spectrometer to monitor in real-time the generation of electrophilic volatile carbonyls in cigarette smoke. The new bioassay uses the important antioxidant selenoenzyme GPx-1, immobilized to 96-well microtiter plates, as a probe. The selenocysteine bearing subunits of the enzyme's catalytic site are viewed as cysteine analogues and are vulnerable to electrophilic attack by compounds with conjugated carbonyl systems. The immobilization of GPx-1 to microtiter plate wells enabled facile removal of excess reactive inhibitory compounds after incubation with electrophilic chemicals or aqueous extracts of air samples derived from different sources. The inhibitory response of cigarette smoke and diesel exhaust particle extracts were compared with chemical standards of a group of electrophilic carbonyls and the arylating *p*-benzoquinone. GPx-1 activity was directly inactivated by millimolar concentrations of highly reactive electrophilic chemicals (including acrolein, glyoxal, methylglyoxal, and *p*-benzoquinone) and extracts of diesel and cigarette smoke. We conclude that the potential of air pollutant components to generate oxidative stress may be, in part, a result of electrophile-derived covalent modifications of enzymes involved in the cytosolic antioxidant defense.

Keywords Air pollution · Electrophiles · Antioxidant enzymes · Oxidative stress

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Introduction

Oxidative stress in biological systems is induced by generation of reactive oxygen or nitrogen species (RONS) that exceed antioxidant defenses (enzymes and other molecules that neutralize RONS). Exposure to reactive chemicals may trigger a cascade of biological oxidative stress responses, including nuclear factor kappa-light-chain enhancer of activated B cells activation and cytokine or chemokine-mediated signaling [1]. They may also irreversibly alter biomolecules leading to tissue injury and inflammation. The resulting oxidative damage of cellular components has been linked to aging [2], chronic disorders including asthma [3], cardiovascular disease [4, 5], and hypertension [6].

Toxicological evidence is mounting that air pollution induces inflammatory and oxidative stress responses mediated by pro-oxidant and electrophilic chemicals in pollutant mixtures from fossil fuel combustion [1, 7–10]. More specifically, conjugated carbonyl compounds, such as α,β-unsaturated carbonyls, 1,2-dicarbonyls, and quinones are of toxicological importance because they can promote oxidative stress and eventually lead to cellular damage by covalently reacting with nucleophilic functions in proteins [11–14]. It is important to note that reactive organic electrophilic compounds do not require metabolic activation but can react readily with proteins or form mutagenic DNA adducts by binding covalently to

nucleic acids (in contrast to carcinogenic PAHs, *N*-nitrosamines, and dioxins) [15, 16].

Unsaturated carbonyls may be present in both the gas and particle phase. Short-chain aldehydes generally have high volatility, for example, the electrophilic α,β-unsaturated aldehyde acrolein (Fig. 4) is frequently detected in large concentrations in the volatile fraction of diesel exhaust and cigarette combustion products [14, 17, 18]. However, larger α,β-unsaturated aldehydes have been observed in particulate matter [19]. Furthermore, molecular compounds with aldehyde functionality can be reversibly adsorbed by inorganic acidic particles [20] and by organic particles [21] in the air, possibly followed by their release in the lung after exposure.

The adverse health effects of diesel exhaust and cigarette smoke emissions may be in part attributed to toxic electrophilic carbonyls. Electrophilic compounds are attracted to electrons and can inactivate the nucleophilic active sites of thiolate or selenocysteine enzymes such as GPx-1 through covalent bonding. We recently showed that diesel particles as well as ultrafine particles (less than 100 nm in diameter) collected at a Los Angeles area location led to the irreversible inactivation of the thiol (cysteine) enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The inactivation was linked to electrophile concentrations in the particle samples [22]. Furthermore, we previously reported that traffic-related air pollution was associated with decreased GPx-1 activity in the blood of elderly subjects followed in a longitudinal study [23, 24].

There is also compelling experimental evidence that cigarette smoke (CS) induces inflammatory and oxidative stress responses by impairing cellular antioxidative defense mechanisms [11, 14, 25, 26]. For instance, exposure of A549 cells to CS from different brands of cigarettes resulted in the depletion of intracellular antioxidant-reduced glutathione (GSH) [27]. A five-fold increase of urinary (3-hydroxypropyl)mercapturic acid levels (3-HPMA) in smokers relative to non-smokers and 78% decrease in the median level of 3-HPMA after smoking cessation demonstrated that smoking is the predominant source of acrolein in humans [28]. Thus, the formation of these conjugates may be an important detoxification mechanism, but also they may potentially disrupt the cellular redox balance by an acrolein-mediated loss of GSH [29]. Most importantly, CS components such as acrolein may exhibit direct inhibitory effects by attacking antioxidant enzymes via Michael addition in the airway epithelium or by being translocated from the lung into the circulation to inactivate enzymes systemically [14, 30, 31]. Acrolein has also been reported to form two major DNA adduct isomers α-OH-Acr-dG and γ-OH-Acr-dG [15]. Both Acr-dG adducts are mutagenic and are found in human lung tissues from current and ex-smokers [16]. A correlation between AdG levels and smoking status could not be established. However, the results of the cited study were based on only 14 subjects and require further investigation [16].

The antioxidant selenoenzyme GPx-1 catalyzes the reduction of hydrogen peroxide or organic hydroperoxides to water or to the corresponding alcohols (R-OH), thus shielding cells from oxidative stress (Fig. 1). It may be noted, that the family of human glutathione peroxidases (GPx) comprise different selenoproteins including GPx-1. Whereas cellular GPx-1 consists of the classic cytosolic-mitochondrial cGPx, the lung contains intracellular and extracellular Se-dependent GPx-2 [32]. GPx-2 was initially identified as gastrointestinal GPx with the structure and substrate specificity similar to that of GPx-1 [33]. Because of its low pK_a value the selenol group at the enzyme's active location easily ionizes at physiologic pH to the reactive selenolate, which is highly susceptible to electrophilic attack [33]. Therefore, part of the ability of air pollutants to generate oxidative stress may be by electrophile-derived covalent modifications of seleno enzymes involved in the cytosolic defense against reactive oxidative and nitrogen species. This will add to the endogenous burden of oxidative stress because electrophiles can also be produced endogenously in cells (e.g., acrolein is a byproduct of lipid peroxidation) [29, 34, 35], possibly resulting in a vicious cycle of electrophilic stress-induced protein damage [36].

In this work, we demonstrate a novel rapid kinetic bioassay for screening organic electrophilic compounds (such as acrolein, crotonaldehyde, and *p*-benzoquinone (BQ); Fig. 4) in traffic-related air pollutants and tobacco smoke using the selenoenzyme GPx-1 as a probe. One particular benefit of the

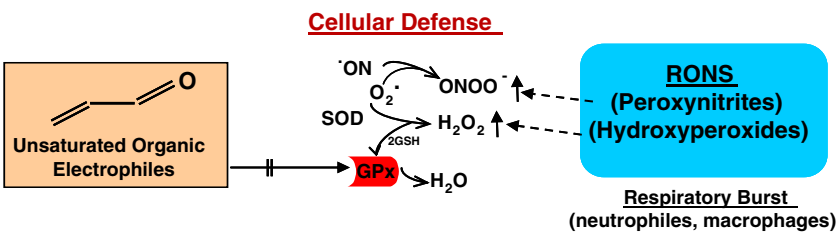

Fig. 1 GSH peroxidase (*GPx*) and Cu/Zn-SOD are linked together in the cytosolic defense against RONS. Cu/Zn-SOD catalyzes the dismutation of superoxide to oxygen and hydrogen peroxide (H_2O_2). H_2O_2 and other hydroperoxides are subsequently reduced by the selenoenzyme GPx. GPx and Cu/Zn-SOD team up with a complex cellular antioxidant system that includes catalase, glutathione transferase, and reduced GSH (not shown). Environmental exposure to reactive electrophiles may add to the endogenous burden of oxidative stress by direct inactivation of GPx

described assay is that it may be used as a high-throughput method for screening many environmental air samples simultaneously and in replicate.

Experimental

Materials

Bovine erythrocyte GPx-1, acetaldehyde, formaldehyde, acrolein, crotonaldehyde, glyoxal, methylglyoxal (MG), *N*-ethylmaleimide (NEM), BQ, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), cupric sulfate, and potassium cyanide were purchased from Sigma-Aldrich Co. (St. Louis, MO). 3-morpholin-osydnonimine *N*-ethylcarbamide, hydrochloride (SIN-1) was obtained from EMD Biosciences, Inc. (San Diego, CA). GPx cumene hydroperoxide and GPx co-substrate mixture with lyophilized powder of NADPH, reduced GSH, and glutathione reductase (GR) were purchased from Cayman Chemical (Ann Arbor, MI). Chemicals were of the highest grade available and used without further purification. Nunc MaxiSorp 96-well plates were obtained from Fisher Scientific (Pittsburgh, PA). Diesel exhaust particles (DEPs) were obtained from US EPA's National Risk Research Laboratory (RTP, NC) and carbon black Vulcan® XC72 particles from Cabot Corp. (Billerica, MA).

GPx activity

High protein binding, polystyrene 96-well microtiter plates (NUNC MaxiSorp) were coated overnight at 4 °C with bovine erythrocyte GPx-1 (0.5-μg protein/well in 50-mM phosphate-buffered saline (PBS), pH=7.4 containing 5 mM EDTA). DTT (5 mM) was added to the coating buffer in order to convert all of the immobilized GPx into its active form. After coating, plates were washed with PBS three times to remove excess enzyme. After washing, the immobilized enzyme was incubated with assay buffer (control), standards or environmental samples (in DTT-free PBS buffer containing 5 mM EDTA; pH=7.4) on a horizontal shaker for 1 h at room temperature, after which the excess of inhibitory compounds was washed away. A mixture of 180-μl GPx co-substrate solution (GSH + NADPH + GR) was added. The reaction was then initiated by adding cumene hydroperoxide (20 μl/well). GPx-1 activity was then determined in duplicate or triplicate by monitoring the linear decrease of absorbance at 340 nm (reflective of the oxidation of NADPH to $NADP^+$) for 6 min at 25 °C (Fig. 2). No cumene hydroperoxide was added to the blank wells. For development purposes, the activity rates (ΔA_{340}/min) of GPx-1 free in solution (rather than immobilized) was tested by applying a commercial test kit (Cayman Chemical, Ann Arbor, MI) and then comparing to the new assay format (Fig. 2).

All of the assays were analyzed using a temperature controlled, monochromatic plate reader (VERSAmax™; Molecular Devices LLC, Sunnyvale, CA) for high sample throughput. Data acquisition of the time-dependent inactivation of GPx by standards and environmental samples was provided by SoftMax Pro Software (Molecular Devices, LLC, Sunnyvale, CA). GPx activity of 0.5 μg protein/well was calculated using the following formula and an extinction coefficient for NADPH 340 nm of 0.00373 μM^{-1} at 0.6 cm pathlength (pathlength adjusted for the 200-μl reaction mixture solution in the well):

$$(\Delta A_{340}/\min)/(0.00373\mu M^{-1}) = 1\ nmol\ NADPH/\min/ml = 1\ mU/mL$$

Cigarette smoke sample preparations and measurements

To demonstrate the abundance of toxic carbonyl compounds in cigarette smoke emissions, single puffs of smoke from one cigarette (Marlboro Seventy-Twos Red™) were collected with a 25-mL pipette pump and directed into a clean inflatable Teflon bag by manually mixing CS with about 200 L of dry zero air. The volatile organic compounds (VOC) of the cigarette smoke in the Teflon bag were sampled through a heated (80 °C) PEEK inlet and detected in real time using a high-resolution proton transfer reaction time-of-flight mass spectrometer (PTR-ToF-MS) from Ionicon Analytik GmbH (Innsbruck, Austria; instrument described by Jordan et al. [37]. Zero air from the bag was sampled for 15 min as a background and diluted CS was sampled for 10 min. PTR instruments rely on soft-ionization of VOC in the sampled air by proton transfer from H_3O^+ reagent ions, $VOC+H_3O^+ \rightarrow VOC\text{-}H^+ + H_2O$. All VOC that have gas-phase proton affinity exceeding that of water can be ionized by PTR, e.g., all oxygenated and unsaturated volatile organics. The resulting protonated VOC ions are then detected by a ToF (5,000 mΔ/m at *m/z* 70) mass spectrometer with 7-s time resolution.

To test the assay, aqueous cigarette smoke extracts (CSE) from the same brand of cigarettes used for real-time PTR-ToF-MS (Marlboro Seventy-Twos Red™) were prepared by applying a modification of the method previously described [11]: Mainstream whole smoke was bubbled from a total of five cigarettes into a glass impinger with 10 mL of 2 mM Dulbeccos's phosphate-buffered saline for 5 min (one cigarette/min). Aliquots of the extracts were centrifuged at 18,000 g for 15 min at 4 °C, adjusted to pH 7.4 by diluting the concentrated supernatant solutions with 50 mM PBS (1:5) and then tested for their inhibitory effects on the newly developed GPx-1 assay as described before. A blank sample was generated following the same procedure using ambient air instead of cigarette smoke.

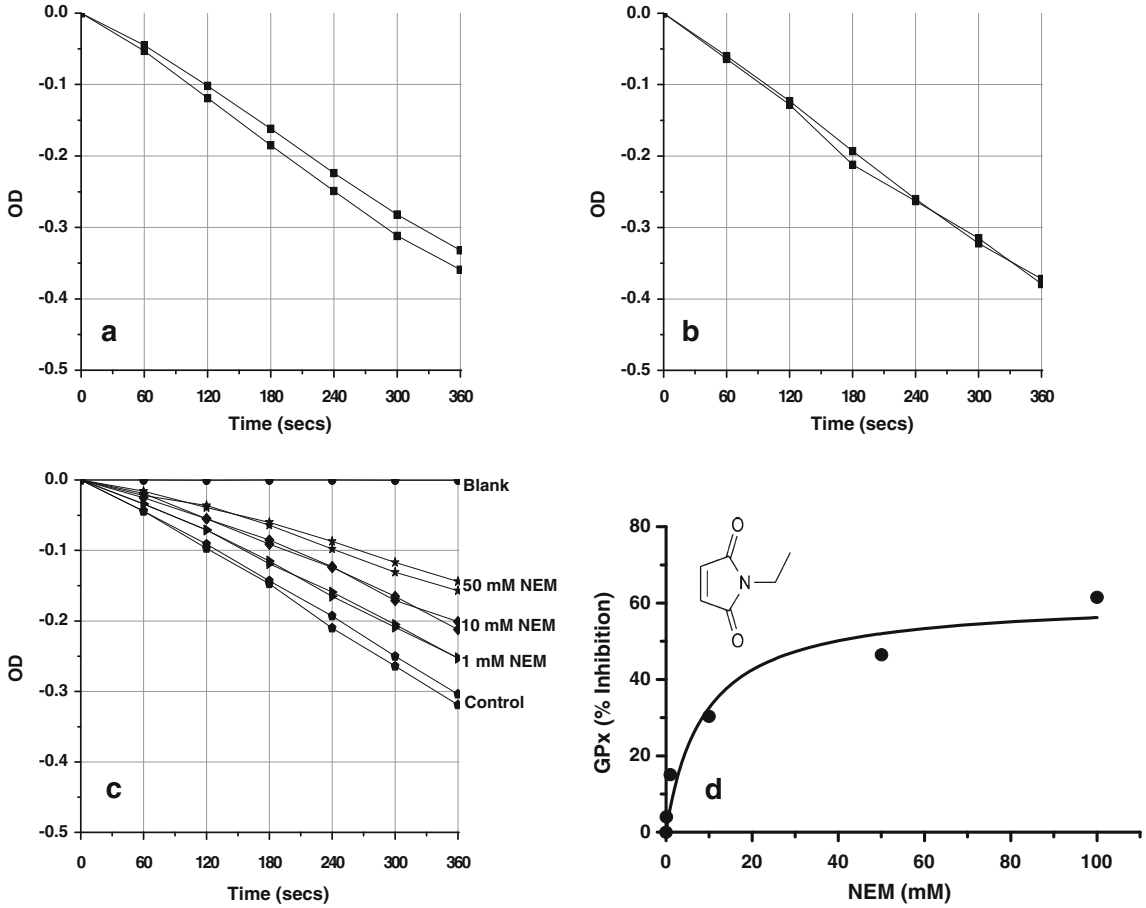

Fig. 2 Well Graph Raw Data; decrease of absorbance (normalized optical density, OD at 340 nm reflective of the oxidation of NADPH to $NADP^+$, blank corrected) was recorded every 60 s for 6 min in duplicate wells. A_{340}/min activity rates of erythrocyte bovine glutathione peroxidase (GPx-1; 0.5 μg protein per duplicate well): **a** free GPx-1; **b** immobilized GPx-1; **c** immobilized GPx-1 plus *N*-ethylmaleimide (*NEM*); **d** inactivation of GPx-1 by direct reaction with the indicated concentrations of NEM. Data are the average of duplicate samples (variation between duplicates, <10%) and were fit to a one-site binding hyperbola (for more details, see "Experimental")

Diesel exhaust sample preparations and measurements

The five DEP samples tested in this study were generated and collected at the US EPA's National Risk Research Laboratory and analyzed at the University of California Los Angeles for their chemical, physical, and toxicological properties (details described by Shinyashiki et al. 2009 [38]). The DEP samples were stored at minus 80 °C in glass sample jars prior to sample extraction and analysis. DEP samples were weighed into 2 mL microcentrifuge tubes and a 50 mM PBS assay buffer solution (containing 5 mM EDTA; pH: 7.4) was added to achieve aqueous particle mass concentrations of 0.5–1.0 mg/mL. The tubes were loaded into a high speed, reciprocating FastPrep™ instrument (MP Biomedicals, Inc., Solon, OH) and processed at 6.5 m/s for 60 s to efficiently suspend DEP samples. The extraction tubes were then sonicated for 15 min in a water bath and centrifuged (at 18,000 g for 15 min, 4 °C). GPx-1 inhibition by the obtained aqueous DEP extracts was then directly measured following a 1-h incubation.

Results and discussion

Assay development

GPx catalyzes the reduction of hydroperoxides by reduced GSH to protect cells from oxidative damage. Quantitative measurements of GPx activity is based on a coupled enzymatic reaction with GR: GR reduces GSSG (generated upon reduction of hydroperoxide) by using NADPH as its electron donor, thus replenishing the GSH pool. At this step, NADPH is recycled back to $NADP^+$, resulting in a decrease of absorbance at 340 nm. The absorbance change can be monitored and is indicative of GPx activity [39]. Although the described assay principle is widely used in commercial test kits to measure GPx activity in biological systems, it cannot be applied for testing GPx inhibitors directly. More specifically, the assay component GR itself may be susceptible to the inhibitory effects of compounds tested in this study. For instance, to demonstrate the inactivation of GPx by electrophiles such as NEM, a potent inhibitor of GR, excess NEM must be removed

from each and every sample before measuring GPx enzyme activities (coupled to the oxidation of NADPH by GR). This may be accomplished by dialysis, a time-consuming and potentially error-prone procedure.

We modified a commercially available kinetic GPx-1 assay (Cayman Chemicals) by immobilizing purified bovine erythrocyte GPx-1 (Sigma-Aldrich) onto the wells of a 96-well microtiter plate through passive physical adsorption. The system allows the removal of residual inhibitory reagents from the assay after incubating immobilized GPx-1 with organic electrophilic chemicals or environmental samples. Initially, the inhibitory effect of the commonly used sulfhydryl blocking reagent NEM was evaluated to test the feasibility of the new assay system. The compound's highly electrophilic α,β-unsaturated carbonyl structure covalently alkylates thiols via the Michael addition reaction. Because NEM is not redox-active under physiological conditions [22], inactivation of GPx-1 by NEM can then solely be contributed to Michael adduct formation with the enzyme's seleno function (Fig. 3).

In order to compare free vs. immobilized GPx kinetics, we used the GPx-1 assay kit from Cayman Chemicals to determine the activity of (free) bovine erythrocyte GPx-1 protein (0.5 μg per well), which was approximately 16 mU/mL (one milliunit was defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to $NADP^+$ per minute at 25 °C).

The results from the GPx-1 assay kit indicate that the immobilization of enzyme protein onto the surface of 96-well microtiter plates by passive physical adsorption did not result in a decrease of activity rates (expressed as the change of absorbance at 340 nm per minute (Fig. 2a vs. b). This is important because physical adsorption can denature the enzyme depending on the surface properties of the carrier [40]. The somewhat lower A_{340}/min rates of GPx (control, Fig. 2c) may be due to the added 1 h incubation time in the inhibition experiment before the remaining active enzyme was determined. The increased assay time followed by an additional washing step may subject the selenofunction of GPx to prolonged autooxidation, thus reducing its overall activity. Furthermore, it cannot be ruled out that the applied adsorption method allows leaching of the enzyme when washing away residual inhibitors. On this note, covalently binding the enzyme to the (functionalized) surface of microtiter plates is a common technique and might minimize leaching, but is also known to dramatically decrease enzyme activities [40]. In this study, measurements of the inhibitory effect of different millimolar

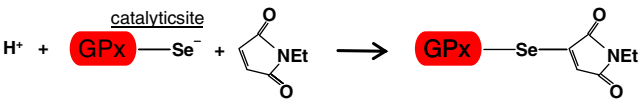

Fig. 3 The nucleophilic selenofunction of GPx-1 is viewed as a cysteine analogue and vulnerable to electrophilic attack by organic unsaturated electrophiles to form a covalent bond, thereby irreversibly inactivating the enzyme

NEM concentrations on non-covalently immobilized GPx have been proven feasible. Specifically, after treatment, the activity rates of the microtiter plate-adsorbed enzyme decreased in a dose–response manner following exposure to the organic electrophile, but remained stable and linear at all activity rate levels throughout a 6 min time course (Fig. 2c). Figure 2d reflects the inactivation of GPx-1 activity by different concentrations of NEM (0.1–100 mM). The line represents the fit of the data by non-linear regression analysis to a one site-binding hyperbola (r^2=0.94; Kd=8.77 mM) using GraphPad Prism 5 (available from www.graphpad.com). The inhibitory effect of NEM on GPx-1activity was observed in the low mmolar range with an estimated detection limit of approximately 0.2 mM.

Effect of carbonyls and benzoquinone on GPx activity

The newly developed assay format enabled us to test for the inhibitory properties of highly electrophilic α,β-unsaturated aldehydes and BQ, which are frequently found as components of tobacco smoke, diesel exhaust, and other combustion products. Highly reactive electrophilic carbonyls such as acrolein or crotonaldehyde are particularly abundant in CS (1,000-fold higher amounts than CS polycyclic aromatic hydrocarbons, PAHs) [18, 41, 42]. Furthermore, acrolein can reach 80 μM in the respiratory tract fluid of smokers and is magnitudes more toxic than the two major carbonyl combustion products formaldehyde or acetaldehyde [43]. Quinones can exhibit both redox cycling activities and Michael acceptor properties, i.e. they can form Michael addition products with cellular nucleophiles [14]. Specifically, the arylating BQ is known for its strong electrophilic character and rapid Michaels adduct formation with cellular nucleophiles [44]. BQ is a major oxidative product of aromatic hydrocarbons from air pollution and also found in the tar-phase from CS extracts [45, 46]. Figure 4 shows the direct inhibition of GPx-1 activity by two α,β-unsaturated carbonyls and BQ in the low millimolar range confirming the feasibility of the 96-well plate assay system to screen for the inhibitory capacity of electrophilic air pollutant components on antioxidant GPx-1 enzyme activities.

In addition to toxic α,β-unsaturated aldehydes, harmful low molecular weight carbonyl compounds include acetaldehyde, formaldehyde, and dicarbonyls (such as glyoxal and MG) and are formed in cigarette smoke (CS) or other anthropogenic emission products [18, 47]. Figure 5 reports the effects of different short-chain carbonyls on GPx-1 activity. Compared with control conditions, GPx-1 activity decreased substantially when treated with the electrophilic chemicals acrolein, glyoxal, MG, and BQ. Differences in electrophilic potency of the carbonyls and quinones are reflected by the decrease in GPx-1 activity which may also indicate differential in vitro toxicities of the tested compounds. Specifically, the very strong observed inhibitory effect of the pure arylator BQ can be explained by its four quinone ring positions available for direct interaction with

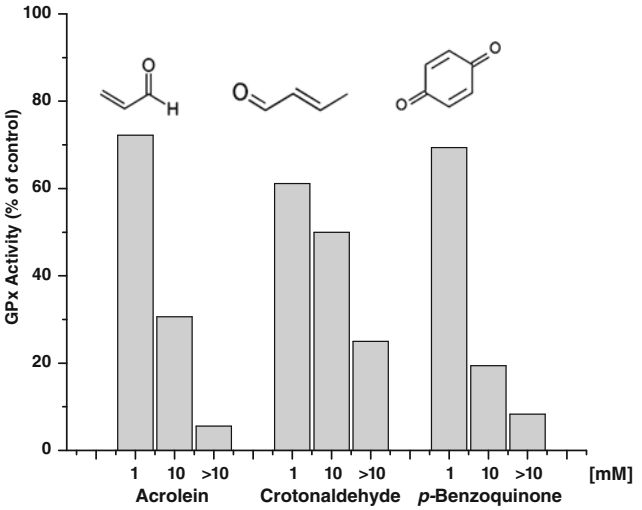

Fig. 4 Inactivation of GPx-1 by three different organic electrophiles with α,β-unsaturated carbonyl structure at 1, 10, and >10 mM concentration levels. GPx-1 results are the average of duplicate samples (variation between duplicates, <25%)

thiols, which makes BQ more reactive than acrolein [48, 49]. Among direct-acting Schiff base formers, electrophilic α,β-dicarbonyls (such as glyoxal) are more reactive than acetaldehyde [49], which is supported by results in Fig. 5.

It needs to be emphasized that the observed direct inhibitory effect of dicarbonlys on GPx-1 activity has been reported before with data indicating that MG does not react with the selenofunction at the active center of GPx-1, but binds instead to its GSH binding sites Arg 184 and 185 [50]. As a result, we propose the mechanism shown in Fig. 6 for the inactivation of GPx-1 by dicarbonyls in accordance with a previous study describing the binding and irreversible modification of proteins by MG under physiological conditions [51].

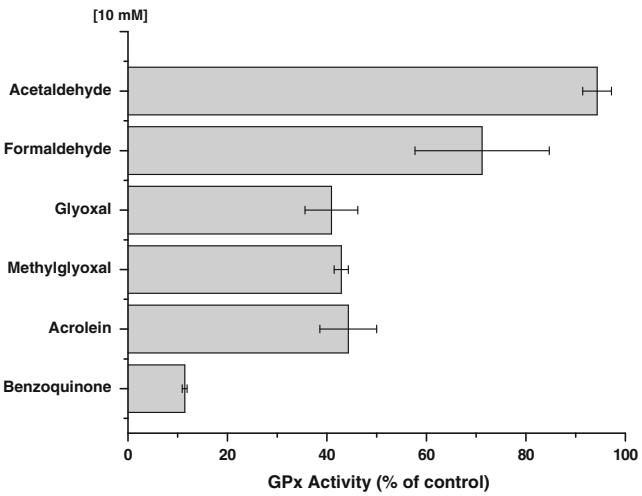

Fig. 5 Inactivation of GPx-1 by low molecular weight carbonyls and the arylating *p*-benzoquinone (*BQ*; positive control). GPx-1 results are the average of triplicate measurements±1 standard deviation

Electrophilic dicarbonyls can also be formed endogenously as a result of glucose autooxidation, DNA oxidation, or lipid peroxidation and then react non-enzymatically with the free amino groups of amino acid residues (e.g. arginine) or with DNA guanyl nucleotides, thus irreversibly altering biomolecules [52]. As a consequence, and similar to the effects of α,β-unsaturated aldehydes, dicarbonyls may inactivate antioxidant enzyme activities under physiological conditions and accelerate oxidative stress-induced impairment of cellular proteins. Glyoxal and MG may also function as tumor promotors by forming DNA adducts [53].

Determination of volatile cigarette smoke components

We measured VOC in CS in real-time with a PTR-ToF-MS instrument (Ionicon Analytik GmbH) and observed toxic low molecular weight carbonyl compounds such as acetaldehyde, formaldehyde, and the highly reactive electrophiles acrolein and crotonaldehyde. Figure 7a shows the PTR mass spectrum of CS after subtraction of spectrum corresponding to background dry zero air. Greater than 50 compounds were detected with PTR-ToF-MS (representative compounds are labeled in Fig. 7a). The advantage of the direct trace analysis is that it reduces the uncertainties associated with the measurements of reactive aldehydes which are subject to decomposition when sampled indirectly. Most importantly, and in contrast to low resolution versions of PTR instruments with mass resolving power of $m/\Delta m \sim 200$ (ratio of the peak position to its width), the instrument used in this study is equipped with a ToF mass analyzer capable of mass resolving power exceeding 5,000. This major improvement made it possible to unambiguously resolve peaks of isobaric compounds, i.e. compounds having the same nominal molecular weights such as acrolein (C_3H_4O, 56.0262 Da) and *n*- and *i*-butenes (C_4H_8, 56.06260 Da) as shown in Fig. 7b. In previous PTR-MS studies of cigarette smoke [54], these two compounds could not be separated, and the protonated ion detected at *m/z* 57 corresponded to a convoluted sum of acrolein, 1-butene, 2-butene, and *i*-butene. With the higher mass resolving power, protonated acrolein ($C_3H_5O^+$, *m/z* 57.0340) could be cleanly separated from protonated butenes ($C_4H_9^+$, *m/z* 57.0704). The unambiguous detection of acrolein in our work is important, because the presence of C_4H_8 isomers was previously considered a major obstacle for the analysis of acrolein by PTR-ToF-MS in a complex matrix like cigarette smoke [54]. In addition to the high resolving power, the PTR-ToF-MS instrument is characterized by high sensitivity (<10 ppt), large mass range, and fast time response (1 s). It should be noted, however, that the identification capability of the PTR-TOF is not based on its mass accuracy and mass resolution alone [55]. The tentative assignments to volatile organic compounds (VOC) in Fig. 7 were based on previous identification of these compounds with separation or spectroscopy techniques [18]. These VOC

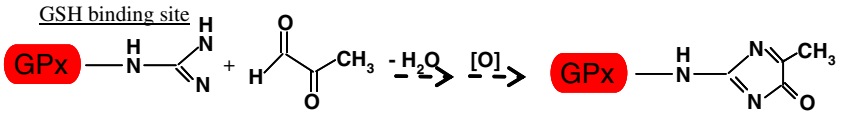

Fig. 6 Proposed reaction mechanism of methylglyoxal (*MG*) with arginine guanidinium moieties: MG irreversibly modifies Arg 184 and Arg 185 located at the GSH binding sites of GPx-1, thereby inactivating the enzyme under physiological conditions [50, 51]

may represent the dominant species present at certain accurate masses, although structural isomers cannot be fully excluded. Assigned candidates are VOC known to undergo non-dissociative proton transfer under analytical conditions employed.

Effect of CSE on GPx-1 activity

The PTR-ToF-MS method confirmed the presence of highly reactive chemicals in CS and is in good agreement with previous findings [14, 18]. As indicated above, organic electrophilic compounds present in CS may inactivate the nucleophilic active sites of thiolate or selenocysteine enzymes through covalent bonding. Thus, we tested whether aqueous

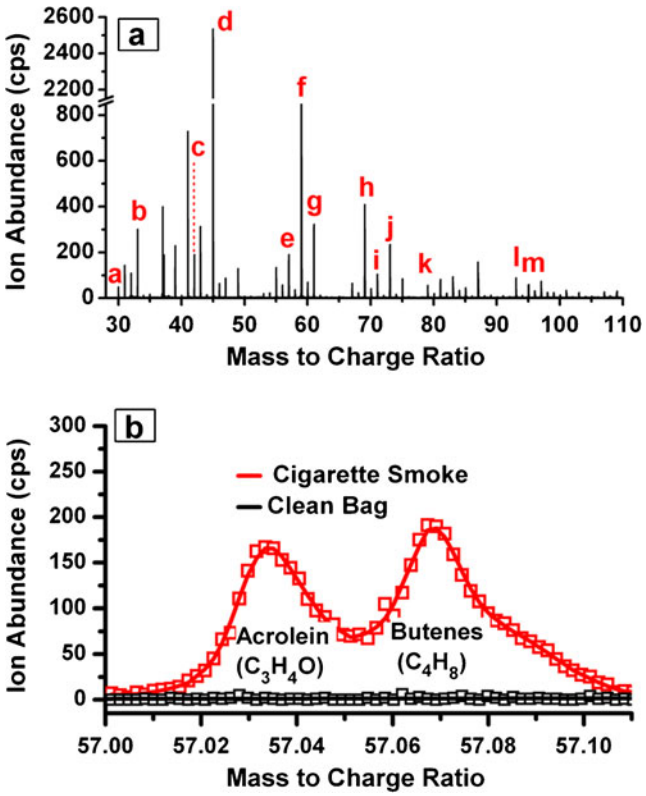

Fig. 7 PTR-ToF-MS detection of volatile organic compounds (*VOC*) in cigarette smoke. **a** The full background-subtracted mass spectrum with peak assignments: *a* formaldehyde, *b* methanol, *c* acetonitrile, *d* acetaldehyde, *e* acrolein/butenes, *f* acetone, *g* acetic acid, *h* isoprene, *i* crotonaldehyde, *j* MG/tetrahydrofuran, *k* benzene, *l* toluene, and *m* phenol. **b** A magnified version of the mass spectrum to demonstrate clear spectral resolution of isobaric acrolein and butenes

CSE exhibits direct inhibitory effects by using the antioxidant enzyme GPx-1 as a probe. Interestingly, the inhibitory effects of electrophilic carbonyls on GPx-1 activity (Fig. 5) found in CS (Fig. 7) was mimicked by fresh aqueous CS whole phase extracts obtained from a commercial Marlboro Seventy-Twos Red™ cigarette (Fig. 8). However, to demonstrate the potential interferences of water soluble *inorganic* CS components, we also show the inhibitory effects of Cu^{2+}, CN^{-}, and peroxynitrite on the GPx-1 assay system (Fig. 8). Cigarette smoke contains elevated concentrations of water-soluble transition metal particles including copper (~1,000 ng/m^3) [56] as well as cyanide (CN^{-}) and peroxynitrite. Besides catalyzing the generation of reactive oxygen species via the Fenton reaction [14], copper ions may inhibit the catalytic activity of antioxidant enzymes by directly interacting with sulfhydryl groups. For example, the inactivation of GR activity by copper is seen as the inability of GSSG to react with the distal protein sulfhydryl group complexed with copper [57]. Interestingly, the observed sulfydryl activities of CS [58–61] may not only be attributable to CS particulate phase transition metals, but also in part be due to the rapid oxidation of sulfhydryls by peroxynitrite. Peroxynitrite (the reaction product of superoxide and nitric oxide) has been suggested to be formed as an oxidative stress-inducing component of aqueous CSE [62]. More importantly, recent data have provided evidence that peroxynitrite inactivates GPx-1 by oxidative cross-linking of the selenocysteine and cysteine residue at the catalytic center. [63, 64]. Finally, cyanide present in CS has also been known to affect the activity of heme- and other peroxidase enzymes, including ovine erythrocyte GPx-1 [65, 66]. Different mechanisms for cyanide inactivation of GPx-1 have been discussed and may involve the direct formation of a selenocyanate derivative [66, 67].

The demonstrated inhibition of GPx-1 activity by both highly reactive electrophilic chemical standards (Fig. 5) and CS extract (Fig. 8) suggests that CS may generate oxidative stress by posttranslational modifications of antioxidant enzymes. However, different reactions can drive the inactivation of GPx-1: irreversible inactivation of GPx-1 activity under aerobic conditions may be due to covalent binding of organic electrophiles with the enzyme's selenofunction and arginine residues shown in Figs. 3 and 6, transition metal complex formations, reaction with cyanide ions, or the oxidation of selenocysteine groups at its active center by, for example, peroxynitrite. Although the mechanisms of action are different, each one may contribute to the decreased activity

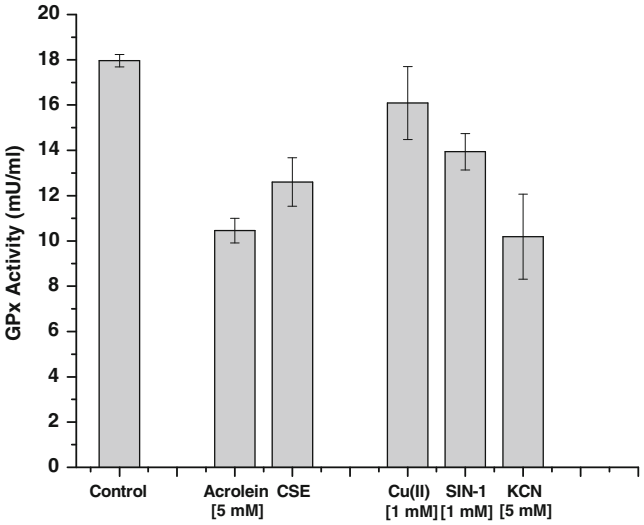

Fig. 8 Partial inactivation of GPx-1 by mainstream whole cigarette smoke extract (*CSE*) in comparison with the inhibitory effects of acrolein (positive control) and water-soluble inorganic components found in CS (data represent means±1 standard deviation of triplicate measurements); *SIN-1* 3-morpholin-osydnonimine *N*-ethylcarbamide, peroxynitrite precursor

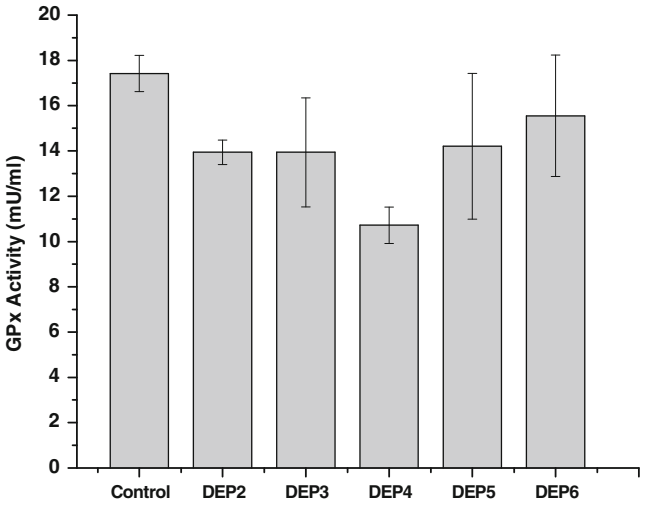

Fig. 9 Effect of five different aqueous DEP extracts on GPx-1 activity as compared with a control sample (GPx-1 treated with PBS assay buffer). Average values along with standard deviations of triplicate measurements are shown (see "Experimental" for procedure details)

of GPx-1 after treatment with CSE (Fig. 8). The extracts in this study were not analyzed for tobacco smoke components, but previous chemical analyses have estimated the content of hydrogen cyanide (HCN), acrolein, acetaldehyde, formaldehyde, glyoxal, MG, and total carbonyl compounds in aqueous CSE. The carbonyl constituents were in the range of several tens to hundreds of micrograms per cigarette (238–468 μg for acrolein and 81±5.5 μg for HCN) corresponding to millimolar concentrations of individual compounds in CSE [18, 68]. It should be noted that the preparation of cigarette smoke in our study did not simulate human smoking behavior. This is important because human smoking behavior might change for different types of cigarettes and may alter the composition and eventually the toxicity of the generated combustion products. The employment of a computer controlled smoking machine in future studies programmed with various puff regimens will help to better reflect smoker practices.

Effects of DEPs on GPx-1 activity

We examined the GPx-1 inhibitory activity of diesel exhaust particles, which were characterized for their electrophilic and redox properties in a previous report [38]. Our test results clearly confirmed the inhibitory potential of aqueous diesel particle extracts on enzymatic systems as reflected by sample DEP2 and DEP4 (Fig. 9). Correspondingly, sample DEP4 with the strongest inhibition on GPx-1 activity had also the highest levels of organic species including PAHs and quinones previously described by Shinyashiki et al. [38]. Most importantly, in the previous study aqueous DEP suspensions exhibited greater inhibitory effects on the electrophile-susceptible enzyme GAPDH compared with dichloromethane extracts, indicating that a significant amount of electrophiles are water soluble [38].

Figure 10 confirms and further evaluates the observed strong inhibitory impairment of GPx-1 by DEP4. As before (Fig. 9), the treatment of GPx-1 with aqueous extract of DEP4 (1 mg/mL) resulted in an approximately 40% loss of enzyme activity. No significant effect of 0.5 mg/ml of DEP4 on GPx-1 activity was observed, reflecting a possible dose–response

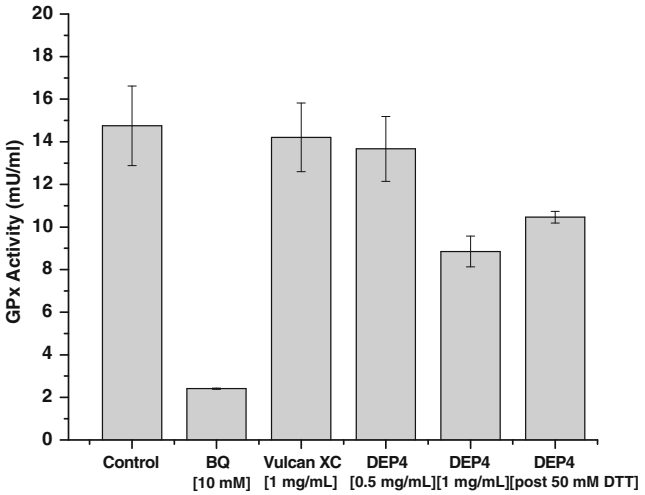

Fig. 10 Effect of DEP4: GPx-1 activity was measured following a 1-h incubation with two different DEP4 concentrations. Reversibility of inhibition by DEP4 (1 mg/ml) was tested after incubation of GPx-1 with excess DTT (50 mM) following DEP4 treatment. Inhibitory effects were compared with treatments with PBS assay buffer (control), aqueous extracts of Vulcan XC-72® (negative control particles), and *p*-BQ as control electrophilic inhibitor. The average ± standard deviations of triplicate measurements are reported

relationship between different concentrations of particle extracts. In order to investigate interferences on the GPx-1 assay solely based on the amount of particles used for extraction, inert black carbon (Vulcan-XC 72®) was employed as a negative particle control. No effect of black carbon particle extracts (1 mg/ml) on the GPx-1 assay could be observed. In an attempt to measure the reversibility of inhibition by DEP4, DTT was added after inactivation. Recovery was minimal but detectable, indicating several key mechanisms were at work: inactivation by irreversible covalent binding of electrophiles to the catalytic and/or GSH binding sites of GPx-1 or the oxidation of the enzyme's active locations. Since the GPx-1 assay is performed under aerobic conditions, it is not possible to strictly distinguish between the redox and electrophilic activities of the tested particles. However, the observed recovery in enzyme activity may be due to DTT-based reduction of selenenic acid (GPx-SeOH) back to the selenol (GPx-SeH) with the remainder of lost activity caused by electrophilic addition and/or the formation of a sulfur-seleno bridge which might be resistant to reducing agents [69].

Conclusions

The results of this study provide evidence that the GPx-1 bioassay is a rapid high throughput assay to screen for the potential inhibitory effects of electrophilic and/or redox-active conjugated carbonyls on antioxidant enzymes. Toxic unsaturated α,β-aldehydes, dicarbonyls, or alkylating quinones tested in this study directly modify cellular macromolecules and inactivate antioxidative enzymes such as GPx-1. These highly reactive chemicals are detected in large concentrations in cigarette and diesel exhaust combustion products as shown here (Fig. 7) and elsewhere [38]. Pulmonary oxidative stress may be induced by the resultant dysfunction of antioxidative enzymes as exemplified by our results in addition to the release of reactive oxygen species either through endogenous cellular processes (respiratory burst) or by inhalation of environmental pollutants (cigarette smoke contains more than 10^{14} free radicals per puff) [70]. The repeated impairment of peroxide scavenging enzymatic mechanisms may intensify cigarette smoke-induced systemic vascular oxidative stress and inflammation, thus promoting accelerated aging and a variety of chronic diseases including atherosclerosis [1, 71]. The direct inhibitory response of the GPx-based bioassay to both highly reactive organic electrophiles and aqueous extracts prepared from tobacco smoke and diesel exhaust emissions demonstrates the feasibility of the newly developed method. Irreversible inhibition of GPx-1 activity under aerobic conditions may be due to different modes of action including the covalent binding of organic electrophiles with the enzyme's selenofunction (Michael adduct formation) or oxidation of the seleno group by RONS. Future optimization of the test system is planned and will be accomplished by conducting the GPx-1 assay in the presence of different thiol-containing or thiol-free antioxidants to better differentiate between the GPx-1 inhibitory activities of electrophilic and redox active compounds [44]. Similarly, the effect of cyanide in CS extracts on GPx-1 activity may be adjusted by adding cyanide scavengers such as cobalamin or cobinamide [72]. Furthermore, the potential interference of metal ions with GPx-1 activity may be determined in the presence and absence of the metal chelator diethylenetriamine pentaacetic acid. Moreover, a standard addition approach will be employed in future research to reveal the magnitude of any matrix interferences. Future research will also be aimed at comparative chemical analyses by real-time PTR-Tof-MS and off-line liquid or gas chromatography of sample extracts for electrophlic carbonyls present in air pollutants. Because high-performance liquid chromatography (HPLC) methods can concentrate sample extracts as well as remove materials interfering with the GPx assay, a hyphenated HPLC-GPx technique is planned in future research to improve the specificity and sensitivity of the overall method.

Acknowledgments This work was supported by the US Environmental Protection Agency (USEPA) STAR Grant No. RD83241301, National Institute of Environmental Health Sciences (grant no. R01 ES12243), and UCI Multi-Investigator Faculty Research Grant MI 7 2008–2009. We are grateful to Professor Arthur Cho, University of California, Los Angeles, for useful comments after reading this manuscript, helpful discussions, and for the kind gift of DEP samples.

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