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Rapid Increases in the Steady-State Concentration of Reactive Oxygen Species in the Lungs and Heart after Particulate Air Pollution Inhalation

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***In vitro* studies suggest that reactive oxygen species contribute to the cardiopulmonary toxicity of particulate air pollution. To evaluate the ability of particulate air pollution to promote oxidative stress and tissue damage *in vivo*, we studied a rat model of short-term exposure to concentrated ambient particles (CAPs). We exposed adult Sprague-Dawley rats to either CAPs aerosols (group 1; average CAPs mass concentration, 300 ± 60 μg/m^3) or filtered air (sham controls) for periods of 1–5 hr. Rats breathing CAPs aerosols for 5 hr showed significant oxidative stress, determined as *in situ* chemiluminescence in the lung [group 1, 41 ± 4; sham, 24 ± 1 counts per second (cps)/cm^2] and heart (group 1, 45 ± 4; sham, 24 ± 2 cps/cm^2) but not liver (group 1, 10 ± 3; sham, 13 ± 3 cps/cm^2). Increases in oxidant levels were also triggered by highly toxic residual oil fly ash particles (lung chemiluminescence, 90 ± 10 cps/cm^2; heart chemiluminescence, 50 ± 3 cps/cm^2) but not by particle-free air or by inert carbon black aerosols (control particles). Increases in chemiluminescence showed strong associations with the CAPs content of iron, manganese, copper, and zinc in the lung and with Fe, aluminum, silicon, and titanium in the heart. The oxidant stress imposed by 5-hr exposure to CAPs was associated with slight but significant increases in the lung and heart water content (~5% in both tissues, $p < 0.05$) and with increased serum levels of lactate dehydrogenase (~80%), indicating mild damage to both tissues. Strikingly, CAPs inhalation also led to tissue-specific increases in the activities of the antioxidant enzymes superoxide dismutase and catalase, suggesting that episodes of increased particulate air pollution not only have potential for oxidant injurious effects but may also trigger adaptive responses. *Key words*: CAPs, concentrated ambient particles, oxidative stress, particulate air pollution, reactive oxygen species. *Environ Health Perspect* 110:749–755 (2002). [Online 12 June 2002]**
http://ehpnet1.niehs.nih.gov/docs/2002/110p749-755gurgueira/abstract.html

Ambient air particles are chemically complex and include minerals, organics, and biologic air pollutants. Epidemiologic studies have shown that increased levels of ambient airborne particulate matter (PM) are associated with increased cardiopulmonary morbidity and mortality (reviewed by Kaiser 2000). Particulate air pollution shares some physicochemical properties with mineral dusts known to act through oxidant mechanisms, such as silica and asbestos (reviewed by Churg et al. 1997; Mossman 2000). Known source constituent particles such as oil fly ash, coal fly ash, and diesel exhaust, extensively used as surrogates of PM, are also effective pro-oxidants *in vitro* as well as *in vivo* (Baeza-Squiban et al. 1999; Kadiiska et al. 1997; Nel et al. 2001; Stringer and Kobzik 1998), suggesting that PM toxicity may be due to increased generation of reactive oxygen species (ROSs) in target cells.

In the last few years, the study of the intrinsic toxicity of "real-world" ambient air particles has notably expanded due to the development of the technology to collect, sort, and concentrate PM from urban air samples without altering their physicochemical properties (Sioutas et al. 1995). Some of the epidemiologic findings on the health effects of PM, including inflammation and toxicity, have been successfully reproduced in the laboratory in humans (Ghio et al. 2001), dogs (Clarke et al. 2000b), and rats (Clarke et al. 2000a).

In vitro studies have also showed a variety of biologic responses to concentrated ambient particles (CAPs), including redox regulation and proliferation (Jimenez et al. 2000; Timblin et al. 1998), increased production of proinflammatory cytokines (Imrich et al. 1999; Monn and Becker 1999), increased oxidation of redox-sensitive fluorescent dyes (Baeza-Squiban et al. 1999; Goldsmith et al. 1998; Prahalad et al. 1999; Shukla et al. 2000), and transcriptional activation of redox-sensitive genes (Shukla et al. 2000). Although the *in vitro* findings support the hypothesis that ROSs are mediators of PM biologic effects, we have no direct evidence to date of a particle-driven increased production of oxidants *in vivo*.

In this study, we used inhalation exposure of rats to CAPs aerosols, combined with measurements of *in situ* chemiluminescence (CL), to evaluate the ability of CAPs to increase ROS concentrations in intact animals in real time and in a noninvasive manner. CL is a low-intensity emission in the visible range mainly due to the decay of excited states of molecular oxygen (singlet oxygen and excited carbonyls; Boveris et al. 1980; Cadenas and Sies 1984), which are formed during the termination steps of the chain reaction of lipid peroxidation (Halliwell and Gutteridge 1990). The spontaneous CL of the organs *in situ* correlates with the square of the intracellular concentration of H_2O_2 and with the development of oxidative damage (Boveris and Cadenas 1997; González-Flecha et al. 1993). Measurements of low-level CL have been used to assess the concentration of oxidants in several models of toxicity to the lung, heart, and liver. Acute administration of paraquat, in doses known to cause extensive lung damage (30 and 60 mg/kg body weight), produced > 100% increases in lung CL (Turrens et al. 1988). Perfusion of isolated rat lungs with *tert*-butyl hydroperoxide or activated polymorphonuclear leukocytes resulted in 200–400% increases in CL and were associated with significant edema and accumulation of thiobarbituric acid–reactive substances (TBARSs) (Barnard et al. 1993). In the mouse heart, measurements of CL have been used to study the differential toxicity of the antitumor drugs adriamycin and mitoxantrone. Acute administration of adriamycin to mice resulted in 10-fold increases in spontaneous heart CL and 80% increases in TBARS accumulation. In contrast, administration of mitoxantrone, a functional analog with lower toxicity, did not affect the production of free radicals or the accumulation of oxidized lipids in the heart (Lores Arnaiz and Llesuy 1993).

In a previous study we used this technique to quantify the increases in the steady-state concentrations of oxidants associated with the development of oxygen tolerance in a model of adaptation to mild hyperoxia (Evelson and González-Flecha 2000). We show here that inhalation of

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Data presented in this article were presented in part at the 2001 FASEB meeting held 31 March–4 April 2001 in Orlando, Florida.

We thank E. Diaz for help in setting up and carrying out the ROFA exposures, R. Streans for carrying out the X-ray fluorescence analysis of carbon black particles, and L. Kobzik, J. Godleski, and P. Koutrakis for many insightful discussions.

S.A.G. is supported in part by a fellowship from the FAPESP (Brazil). This work was supported by grants from the National Institutes of Health (P01 ES08129-03 and P50 ES00002-39) and by the Francis Families Foundation.

Received 12 December 2001; accepted 17 January 2002.

ambient air particles, but not control inert particles, rapidly increases the steady-state concentrations of oxidants in the lung and heart but not in the liver. The oxidative stress imposed by CAPs is associated with the metal content in particles in a tissue-specific manner and leads to mild increases in lung and heart edema as well as in serum levels of lactate dehydrogenase (LDH). Animals breathing CAPs for 5 hr also show an increase in the activity of several antioxidant enzymes in both heart and lung.

Materials and Methods

CAPs. We used the Harvard Ambient Particle Concentrator (HAPC) to concentrate ambient air particles for subsequent aerosol exposure of animals (Sioutas et al. 1995). The principle of virtual impaction was used to concentrate ambient particles in the size range of 0.1–2.5 μm (fine particles; concentration factor, 26 ± 4; Sioutas et al. 1995). CAPs remained in suspension without physical or chemical alteration for inhalation exposures or for collection onto filters for mass and composition analysis. During the operation of the HAPC, we continuously monitored mass concentrations (gravimetrically determined) and the size of the particles (using a microorifice impactor) (Godleski et al. 2000). We determined trace metal concentrations using X-ray fluorescence (Chester LabNet, Tigrad, OR, USA).

Exposure to CAPs. We used pathogen-free male Sprague-Dawley rats (Taconic Farms, Germantown, NY, USA) weighing 250–300 g. Animals were fed a conventional laboratory diet and water *ad libitum.* We exposed rats to CAPs aerosols (CAPs group) or filtered air (control group) in the chamber of the HAPC (Clarke et al. 2000a). The animals were awake and unrestricted during the exposures, and we exposed and tested the CAPs and control groups simultaneously. We carried out each exposure with groups of six animals: three were exposed to CAPs aerosols and three to filtered air (sham controls). At 1, 3, and 5 hr, we removed two animals (one exposed to CAPs and one sham control) from the chamber to be assessed for oxidative stress, tissue damage, and antioxidant enzymes as described below. We conducted each experiment with groups of two animals. The temperature in the room and chamber was 25°C.

Exposure to carbon black and ROFA. We carried out exposures to carbon black and residual oil fly ash (ROFA) in a 40 × 25 × 60 cm chamber. We used a Wright dust feeder (model MK-II; L. Adams Ltd., London, UK) to generate carbon black and ROFA aerosols. Carbon black (catalog no. C198) was purchased from Fisher Scientific (Pittsburgh, PA, USA). We analyzed the elemental composition of carbon black by X rays using a LEO 1450 VP scanning electron microscope with an Oxford Si detector (Leo Microscopy, Inc., Thornwood, NY, USA). Carbon black particles consisted of 85.9 ± 0.2% carbon, 13.0 ± 0.2% oxygen, and 1.17± 0.02% sulfur. We detected no transition metals. We obtained fly ash from a Boston, Massachusetts, area oil-fired power plant (Killingsworth et al. 1997). The metal content of ROFA was as reported by Killingsworth et al. (1997). We packed ROFA or carbon black particles into the dust feeder and flushed it with a stream of air at 14 L/min (6 pounds per square inch gauge). We passed the air stream containing the aerosols through a size-selective impactor (to eliminate particles > 2.5 μm) and then fed it into the exposure chamber isokinetically. We determined particle concentration from the mass change on the filter and the total volume of air sampled. We also monitored the particle concentration during exposures using a real-time aerosol monitor (model RAS-1; MIE Inc., Bedford, MA, USA).

Exposure to filtered air. For the experiments in which rats were exposed for 3 days to either room air or filtered air, long-term continuous exposures to filtered air took place in a 40 × 40 × 60 cm chamber. We used a Millipore 0.2 μm filter to retain PM, and filtered air was humidified with sterile water before delivery to the chamber at a flow rate of 12 L/min. The temperature in the room and chamber was maintained at 25°C.

Organ CL. We measured CL of the lung, heart, and liver *in situ* as previously described (Evelson and González-Flecha 2000) using a Thorn EMI CT1 single-photon counting apparatus with an EMI 9816B photomultiplier (Electron Tubes, Inc., Rockaway, NJ, USA) cooled at –20°C. Rats were anesthetized (sodium pentobarbital, 50 mg/kg body weight) and connected to an animal ventilator (5 mL/breath, 60 breaths/min; Harvard Apparatus, Cambridge, MA, USA). Once we intubated and ventilated the animal, we opened the chest and placed the animal in the measurement compartment. We carried out the surgical procedure and measurements in < 10 min, allowing analysis to begin within 15 min of CAPs exposure. We kept rats at ~37°C using isothermal pads (Braintree Scientific, Braintree, MA, USA). The emission data is expressed as counts per second per unit of tissue surface (cps/cm^2). We placed a high-pass cutoff filter (Wratten no. 25; Eastman Kodak, Rochester, NY, USA), which allows wavelengths > 600 nm, in the optical path to avoid hemoglobin interference. Photon counting decreased only by 15–20%, thus indicating that 80–85% of the emitted light could be regarded as singlet oxygen emission (singlet oxygen dimol emission, 634 and 703 nm; Cadenas and Sies 1984).

Tissue preparation. After measuring CL, we removed the animals from the ventilator; we then rapidly removed the lungs, liver, and heart, and froze them in a dry ice bath. We took separate samples from each tissue

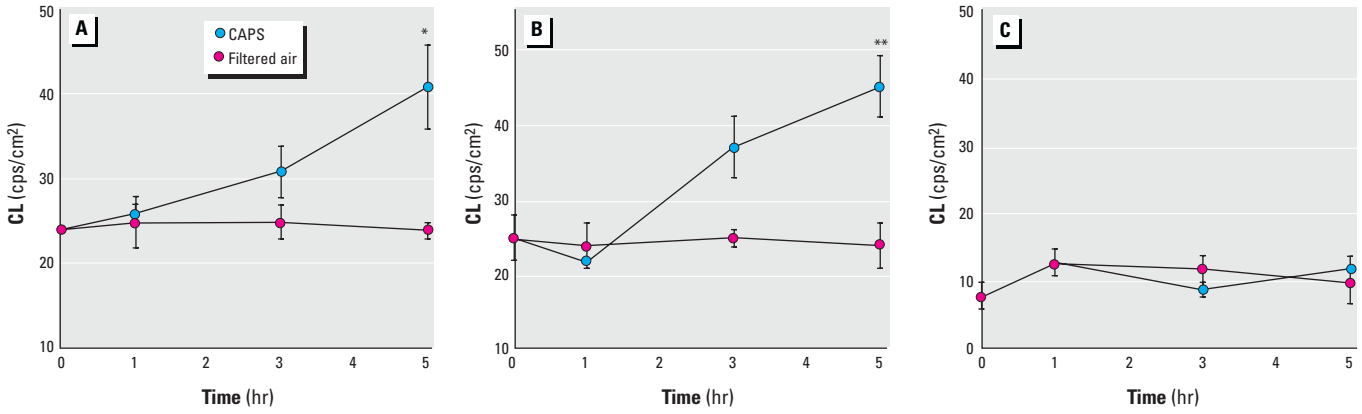

Figure 1. Time course of increase of *in situ* CL from the lung (*A*), heart (*B*), and liver (*C*) of rats exposed to CAPs (average mass concentration, 300 ± 60 μg/m^3) or filtered air for 1, 3, and 5 hr. See "Materials and Methods" for details. Each point represents the mean ± SEM (n = 10 determinations).
Compared with their sham controls or with time 0, *p < 0.001 and **p < 0.005.

and time point to determine water content or enzymatic activities. We also withdrew blood samples from the inferior vena cava to determine serum markers of tissue damage [LDH and creatine phosphokinase (CPK) activities]. We homogenized samples for the determination of enzymatic activities in 5 volumes of 120 mM KCl, 30 mM phosphate buffer (pH 7.2) with added protein inhibitors (1 µg/mL leupeptin, 1 µg/mL aprotinin, 10 µg/mL soybean trypsin inhibitor, 1 µg/mL pepstatin, and 0.5 mM phenylmethyl sulfonyl fluoride) at 0–4°C. We centrifuged the suspensions at 600 × *g* for 10 min at 0–4°C to remove nuclei and cell debris. We discarded the pellets and used the supernatants as homogenates.

Enzymatic measurements. We measured fumarase activity by following the increase in absorbance at 240 nm at 25°C in a reaction mixture containing 30 mM phosphate (pH 7.4), 0.1 mM EDTA, and 5 mM L-malate (Racker 1950; Sigma Chemical Co., St. Louis, MO, USA). We determined total superoxide dismutase (SOD) activity from the rate of inhibition of the oxidation of 20 µM ferrocytochrome *c* at 550 nm in a reaction mixture consisting of 50 mM phosphate buffer (pH 7.8), 50 µM xanthine, and 5 mU xanthine oxidase (McCord and Fridovich 1969). We measured MnSOD activity after inhibition of the Cu/Zn isoenzyme by addition of 1 mM KCN (Beauchamp and Fridovich 1973). We determined catalase activity by measuring the decrease in absorption of H_2O_2 at 240 nm in a reaction medium containing 2 mM H_2O_2 (Nelson and Kiesow 1972). We determined hemoglobin on rat lung homogenates using a standard kit (Sigma Chemical Co., St. Louis, MO, USA) and quantified it by comparison with standard hemoglobin solutions. SOD activity attributable to hemoglobin represented 1–5% of the total SOD activity in both lung and heart. We measured protein concentration in homogenates by the method of Lowry et al. (1951) using bovine serum albumin as standard. We carried out measurements in a Perkin Elmer Lambda 40 spectrophotometer (Perkin-Elmer, Norwalk, CT, USA).

Serum markers of tissue damage. We measured LDH and CPK activity and hemoglobin content in serum samples spectrophotometrically using standard kits (Sigma Chemical Co.).

Water content. We weighed lung and heart samples (~100 mg) and then dried them in a conventional oven (~80°C). We reweighed tissues after 24 hr to obtain the wet/dry ratios.

Statistics. Values in tables and figures are mean ± SEM. We analyzed data statistically by factorial analysis of variance followed by Fisher's test for comparison of means. For elemental composition correlation analyses, we fitted separate linear regression models using actual elemental concentration univariately as predictors. We performed all statistical analyses using Statview software (Abacus Concepts, Inc., Berkeley, CA, USA) for Macintosh. We carried out graphical diagnostics of model adequacy and outlier detection using the S-Plus statistical package (Mathsoft, Inc., Seattle, WA, USA) (Venables and Ripley 1994).

Animal care. Animals were handled humanely in the performance of this project to minimize the use of animals and to prevent animal distress. All protocols of exposure and other procedures used in this study were approved by the Harvard Animal Use Committee. The Harvard School of Public Health is accredited by the American Association for the Accreditation of Laboratory Animal Care, meets National Institutes of Health standards as set forth in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources 1996), and accepts as mandatory the National Institutes of Health's *Principles for the Use of Animals* (NIH 2000).

Results

CAPs increases the steady-state concentration of oxidants in the lung and heart. Inhalation exposure to CAPs increases the steady-state concentration of oxidants in the rat lung and heart. *In vitro* studies suggest that biologic effects of particulate air pollution are initiated by an increased generation of ROS in cells exposed to particles. To determine whether PM affects ROS production in intact animals, we exposed adult Sprague-Dawley rats to aerosols of CAPs and monitored the steady-state concentration of oxidants in the lung, heart, and liver by measuring their spontaneous *in situ* CL. We chose to study the lung and heart because they are the major targets of particulate air pollution (Kaiser 2000). The central role of the liver in the detoxification of a wide range of xenobiotics suggests that this organ may also be a target for the soluble fractions of inhaled particles.

Figure 1 shows the mean values of *in situ* CL in the lung, heart, and liver of rats exposed to CAPs or filtered air for 1–5 hr. Our data show a significant increase in lung and heart CL at 5 hr of exposure. The time courses of increase in CL in the lung and heart follow slightly different patterns. Lung CL increased linearly with the time of exposure (Figure 1A). On days of high pollution (CAPs concentration > 500 µg/m^3), differences between the CAPs and filtered air groups were apparent after only 1 hr of exposure (data included in Figure 1A). In contrast, heart CL showed a lag phase of about 1 hr before any increase could be detected (Figure 1B). The increases in lung and heart CL were specifically due to inhaled CAPs because they were absent in the control animals breathing filtered air under the same experimental conditions (Figure 1). Liver CL was unchanged throughout the 5 hr of exposure to CAPs (Figure 1C).

To determine if the oxidant effect of CAPs in lung and heart tissue depended on particle composition, we tested model environmental particles of different composition for their pro-oxidant effects in lung and heart (Table 1). ROFA is composed of fugitive oil combustion particles, which contribute to PM in urban air and have been shown to cause pulmonary injury and inflammation (Kodavanti et al. 2001; Madden et al. 1999; Nadadur et al. 2000). The large batch of ROFA particles available allows a constant composition of particles during experimental exposures. ROFA is rich in transition metals, specifically vanadium, Fe, and nickel (Killingsworth et al. 1997). Carbon black fine particles resemble the carbonaceous core of PM, but because of their synthetic origin, they do not carry significant levels of adsorbed metals or organic compounds. Consistent with this lack of active components, fine carbon black particles are mostly inert in *in vivo* systems; therefore, we used them as negative control particles (Killingsworth et al. 1997; Murphy et al. 1998).

Table 1 shows that exposure to inert carbon black particles does not exert oxidant effects on the heart or lung. In contrast, ROFA aerosols produced a strong increase in lung and heart CL after exposures as short as 30 min (Table 1). These results strongly suggest that the oxidant effect of environmental particles (CAPs and ROFA) is due to specific components not present in the chemically inert carbon black particles.

To further test this thesis, we took advantage of the day-to-day variations in CAPs composition, which provided a set of samples

Table 1. Lung and heart CL in rats exposed to particles of different composition.

Treatment (concentration/time)	CL (cps/cm^2)	
	Lung	Heart
Filtered air (0 µg/m^3, 5 hr)	24 ± 1	24 ± 3
ROFA (1.7 mg/m^3, 30 min)	90 ± 10*	50 ± 3*
Carbon black (170 µg/m^3, 5 hr)	22 ± 3	25 ± 5

Values indicate mean ± SEM (n = 4–6).
*p < 0.001, compared with filtered air control.

with a range of metal concentrations sufficient for statistical analyses (Table 2). Using univariate regression analyses, we identified several components with significant associations to increased CL in the lung or heart (Table 3). Because of the strong effect of CAPs inhalation on both lung and heart CL, many elements show positive correlations with the CL levels (Table 3). However, we found the stronger and more significant associations for Mn, Fe, Cu, and Zn in the lung and for total mass, Al, Si, Ti, and Fe in the heart (Table 3).

The effect of CAPs on the steady-state levels of oxidants could be due to reversible or irreversible interactions of the particles with cellular components. To determine what type of interaction was responsible for the observed increases in CL in this model, we used two complementary approaches. In one approach we simulated transient increases in particulate air pollution by exposing rats to CAPs for 5 hr and then allowing them to recover in ambient air (room air) for 24 hr. In the other approach, we exposed rats to particle-free (filtered) air for 3 days, which is known to decrease the concentrations of oxidants in the lung and heart (Evelson and González-Flecha 2000), and then allowed them to recover in room air for up to 8 hr. Table 4 summarizes the effects of both treatments on the steady-state concentrations of oxidants in the lung. Rats breathing CAPs for 5 hr showed a significant increase in their *in situ* lung CL. However, CAPs-initiated oxidative stress is not detectable in rats allowed to recover in room air after the simulated "peak" in particulate air pollution (Table 4). Similarly, the second approach showed that decreases in ambient particle levels also affect ROS production in the lung, and this effect is also reversible. Rats breathing particle-free filtered air for 3 days had significantly lower levels of oxidants in their lungs (Table 4). As in the model of acute exposure to increased pollution, the effect of chronic "depletion" in PM is reversed shortly after reexposure to room air.

CAPs-induced oxidative stress is associated with mild damage to the lung and heart. To assay for lung and heart damage in this model, we measured water content (edema) and serum levels of LDH and CPK in rats exposed to CAPs for 5 hr. We measured these markers in blood and tissue samples collected from the same rats assayed for CL (Figure 1). The water content of both lung and heart increased significantly upon exposure to CAPs but not to filtered air (Table 5). The wet/dry ratio in both tissues showed significant increases as a function of the length of exposure and with respect to the control (filtered air) animals for exposure times longer than 3 hr (Table 5). In agreement with the lack of oxidative unbalances in the liver, the wet/dry ratio in this tissue remained unchanged throughout the exposure (filtered air sham, 3.5 ± 0.1; CAPs 3 hr, 3.5 ± 0.1; CAPs 5 hr, 3.3 ± 0.1). Because we collected these samples immediately after the end of the exposure, the observed increase in water content indicates an almost immediate interaction (and toxicity) of environmental particles with lung and heart cells. To evaluate longer-term responses, we also studied rats exposed to CAPs or filtered air for 5 hr and tested for lung and heart edema and serum markers of tissue damage 24 hr after the end of the exposure (Tables 5 and 6). Interestingly, the wet/dry ratio in rats breathing room air for 24 hr after 5 hr of exposure to CAPs returned to control values in the lung but not in the heart (Table 5). These results indicate that the lung can readily compensate for transient increases in the levels of ambient air particles. In contrast, the effects of CAPs on the heart tissue are more pronounced and longer lasting.

Rats breathing CAPs also showed increases in the serum levels of LDH and CPK, as a function of the length of exposure and compared with filtered air controls (Table 6). LDH activity increased approximately 2-fold in rats exposed to CAPs for 5 hr and returned to control values 24 hr after the end of the exposure. CPK activity also

Table 3. Statistical parameters for the regression of lung and heart CL and the mass CAPs elemental components.

Element	Lung CL		Heart CL	
	r^2	*p*-Value	r^2	*p*-Value
Al	0.14	0.140	0.67*	0.001
Si	0.31	0.020	0.61*	0.002
S	0.14	0.140	0.08	0.360
Cl	0.14	0.070	0.08	0.360
K	0.39	0.008	0.30	0.500
Ca	0.41*	0.007	0.36	0.030
Ti	0.38	0.008	0.59*	0.002
V	0.02	0.620	0.42	0.020
Cr	0.04	0.440	0.32	0.046
Mn	0.51*	0.001	0.43	0.010
Fe	0.46*	0.003	0.50*	0.007
Ni	0.00	0.840	0.27	0.070
Cu	0.42*	0.005	0.29	0.060
Zn	0.48*	0.002	0.38	0.020
As	0.31	0.020	0.08	0.340
Se	0.05	0.410	0.06	0.430
Br	0.24	0.040	0.13	0.230
Cd	0.01	0.680	0.38	0.030
Ba	0.36	0.011	0.22	0.110
Pb	0.31	0.020	0.38	0.020
Total mass	0.03	0.450	0.51	0.003

The most significant associations are indicated by asterisks (*).

Table 2. Elemental composition of CAPs (μg/m^3).

Date	Total mass	Al	Si	S	Cl	K	Ca	Ti	V	Cr	Mn	Fe	Ni	Cu	Zn	As	Se	Br	Cd	Ba	Pb
7 Jul 2000	130.7	4.986	10.19	3.673	0.209	2.171	4.690	0.392	0.026	0.013	0.130	5.936	0.012	0.079	0.521	0.005	0.000	0.012	0.018	0.376	0.099
9 Aug 2000	957.5	1.513	9.634	110.7	0.000	3.480	2.720	0.441	0.075	0.028	0.136	7.596	0.094	0.156	0.537	0.037	0.111	0.207	0.014	0.709	0.180
10 Aug 2000	334.0	0.534	3.730	29.5	0.051	2.121	3.408	0.163	0.016	0.011	0.096	3.628	0.010	0.112	0.421	0.019	0.020	0.050	0.000	0.469	0.082
11 Aug 2000	352.8	8.938	21.11	17.3	0.005	4.030	8.962	0.803	0.157	0.038	0.320	11.171	0.035	0.232	0.677	0.018	0.000	0.052	0.000	1.222	0.177
17 Aug 2000	99.6	0.901	2.729	4.630	0.230	1.024	2.721	0.140	0.016	0.013	0.059	3.608	0.012	0.084	0.187	0.006	0.002	0.025	0.004	0.466	0.062
23 Aug 2000	328.8	2.208	7.850	32.48	0.148	2.021	2.111	0.371	0.110	0.027	0.158	6.889	0.089	0.150	0.699	0.025	0.022	0.082	0.015	0.545	0.240
8 Sep 2000	229.5	1.437	5.253	20.31	0.156	1.528	1.891	0.258	0.053	0.028	0.106	4.944	0.044	0.145	0.436	0.007	0.082	0.071	0.000	0.468	0.155
15 Sep 2000	219.3	0.000	1.165	27.58	0.434	0.634	1.451	0.087	0.033	0.028	0.050	2.370	0.029	0.056	0.299	0.000	0.022	0.065	0.010	0.369	0.075
29 Nov 2000	226.6	0.449	3.597	29.70	0.390	1.013	2.299	0.251	0.041	0.014	0.101	6.167	0.030	0.188	0.327	0.018	0.013	0.034	0.002	0.504	0.080
11 Dec 2000	465.0	0.417	7.133	76.59	7.015	2.860	8.987	0.488	0.624	0.024	0.453	13.618	0.518	0.346	2.169	0.028	0.062	0.282	0.000	0.992	0.262
18 Dec 2000	121.0	0.159	1.007	5.120	0.124	0.255	0.550	0.023	0.006	0.012	0.017	0.829	0.011	0.021	0.056	0.005	0.000	0.008	0.005	0.293	0.027
8 Feb 2001	255.0	1.319	5.816	14.29	7.210	1.306	3.644	0.228	0.036	0.014	0.073	4.965	0.035	0.113	0.239	0.017	0.002	0.032	0.000	0.416	0.073
22 Feb 2001	219.0	1.678	5.522	10.04	3.549	1.438	4.640	0.210	0.048	0.015	0.085	4.721	0.057	0.093	0.211	0.007	0.001	0.118	0.003	0.436	0.086

showed a significant increase in the serum activity as a function of the time of exposure to CAPs but not to filtered air (Table 6). However, because of a slight increase in the CPK values of the control group, the differences between CAPs and filtered air control groups for the same times of exposure did not reach statistical significance (Table 6). As in the case of LDH release, CPK activity returned to control levels in animals tested 24 hr after the end of a 5-hr exposure to CAPs. For both enzymes, the increase in serum activity is mild and compatible with reversible tissue damage.

Short-term exposure to CAPs up-regulates antioxidant activities. In addition to their effect on the intracellular production of ROS, some xenobiotics can also affect antioxidant defense systems (Lissi et al. 1991). To test whether this was the case for CAPs, we measured the activity of SOD and catalase (the main detoxifying systems for superoxide and hydrogen peroxide, respectively) in tissue samples collected from rats exposed to CAPs or filtered air for 5 hr (Figure 1). Because of the short exposure and the relatively low toxicity of CAPs, we expected to see no change in these antioxidants. However, our data showed an increase in SOD and catalase activities in both lung and heart (Table 7). The pattern of increase in these activities was tissue specific. In the lung, we found the higher level of induction (70%) for MnSOD, the mitochondrial isoform of SOD (Table 7). Cu/ZnSOD, the cytosolic isoform, was unchanged, and catalase was increased by 30%. In the heart, in contrast, Cu/ZnSOD showed the highest level of induction (100%), MnSOD was increased by 40%, and catalase by 20% ($p > 0.05$).

To confirm that the increases in SOD and catalase activities were due to specific regulatory effects and not to a global effect on proteins, we measured the activity of fumarase, an essential tricarboxylic acid cycle enzyme resistant to oxidants (Evelson and González-Flecha 2000). Fumarase activity was unchanged in rats exposed to CAPs compared with their filtered air controls.

Table 4. Effect of changes in the levels of ambient air particles on rat lung CL (cps/cm^2).

Treatment	Lung CL
Exposure to concentrated ambient particles	
Filtered air (5 hr)	24 ± 1
CAPs[a] (5 hr)	41 ± 4**
CAPs[a] (5 hr)/room air (24 hr)	25 ± 4
Filtered air (5 hr)/room air (24 hr)	20 ± 2
Exposure to particle-free ambient air	
Room air (3 days)	27 ± 3
Filtered air (3 days)	16 ± 1**
Filtered air (3 days)/room air	
3 hr	20 ± 7
5 hr	32 ± 8
8 hr	32 ± 2

Values indicate mean ± SEM (n = 4–6).
[a]Average CAPs concentration = 300 ± 60 μg/m^3. **$p < 0.001$ compared with the control values.

Discussion

Epidemiologic studies have shown that exposure to airborne PM is associated with increased cardiopulmonary morbidity and mortality. The mechanisms by which particulate air pollution causes cardiopulmonary effects at the cellular level are poorly understood. The ability of PM to increase the intracellular production of ROS, although assumed to be essential for their biologic effects, has never been tested *in vivo*. In the present study, we have taken advantage of two unique tools, the HAPC and the measurements of organ CL, to begin to investigate the postulate that particle toxicity operates through oxidant-dependent mechanisms derived from specific particle components. We found that inhalation of CAPs increases by 2-fold the steady-state concentration of ROS in the rat lung and heart. Organ CL measures the steady-state concentration of singlet oxygen ($^1O_2$), a by-product of lipoperoxidation, in intact organs. In this way, organ CL provides an accurate measure of the redox status of the tissue. The spontaneous CL of the organs *in situ* has been successfully used to assess oxidant stress in several models of toxicity to the lung, heart, and liver. The magnitude of increase in lung and heart CL observed in rats breathing CAPs for 5 hr is similar to that previously reported by us in rats exposed to 85% O_2 for 3–5 days, a treatment associated with mild and transient lung and heart injury (Evelson and González-Flecha 2000), and by Turrens et al. (1988) in rats treated with sublethal doses of paraquat (30 mg/kg, intraperitoneal).

In our model, *in situ* CL of the lung increases shortly after exposure to CAPs (Figure 1) and returns to control values a few hours after removal of the animals from the HAPC (Table 4). The rapid increase in the lung concentrations of ROSs upon exposure to CAPs indicates an almost immediate effect of particles, or particle components, on the intracellular sources of free radicals. Furthermore, the transient nature of these increases points to a reversible interaction of particle components with cellular targets. Both observations would be compatible with Fenton-type reactions catalyzed by transition metals, redox-cycling processes, or biochemical changes triggered by noncovalent binding to membrane receptors. Single-component regression analyses showed a strong association of the oxidant effect of CAPs aerosols generated on different days and their content of transition metals, specifically to the CAPs content of Mn, Zn, Fe, and Cu (Table 3). The notable lack of association with the total mass of particles strongly supports a

Table 5. Wet/dry ratios in the lung and heart of rats exposed to CAPs or filtered air.

Time of exposure	Lung		Heart	
	Filtered air	CAPs	Filtered air	CAPs
1 hr	4.81 ± 0.06	4.77 ± 0.04	4.15 ± 0.02	4.11 ± 0.03
3 hr	4.72 ± 0.03	4.90 ± 0.04 *,#	4.20 ± 0.02	4.33 ± 0.04 *,##
5 hr	4.84 ± 0.04	4.92 ± 0.02#	4.12 ± 0.04	4.29 ± 0.01**,##
24 hr after 5 hr exposure	4.75 ± 0.08	4.8 ± 0.1	4.21 ± 0.02	4.27 ± 0.02##

Values indicate mean ± SE (n = 6–10).
*$p < 0.05$ compared with control values for the same time of exposure. **$p < 0.002$ compared with control values for the same day of exposure. #$p < 0.02$ compared with values after 1 hr exposure. ##$p < 0.0001$ compared with values after 1 hr exposure.

Table 6. Serum markers of tissue damage in rats exposed to CAPs or filtered air.

Time of exposure	Serum markers			
	LDH (IU/mL)		CPK (IU/mL)	
	Filtered air	CAPs	Filtered air	CAPs
1 hr	440 ± 40	570 ± 100	260 ± 40	360 ± 30
3 hr	570 ± 60	700 ± 140	420 ± 50	510 ± 60##
5 hr	530 ± 60	950 ± 180*,#	440 ± 30	550 ± 50##
24 hr after 5 hr exposure.	520 ± 20	580 ± 10	420 ± 10	430 ± 10

Values indicate mean ± SE (n = 10).
*$p < 0.03$ compared to the control values for the same time of exposure. #$p < 0.03$ compared to the values after 1 hr exposure. ##$p < 0.01$ compared to the values after 1 hr exposure.

Table 7. Activity of antioxidant enzymes in the lung and heart of rats exposed to CAPs or filtered air.

Activity	Lung		Heart	
	Filtered air	CAPs	Filtered air	CAPs
Cu/ZnSOD (U/mg protein)	38 ± 7	38 ± 10	170 ± 20	340 ± 60*
MnSOD (U/mg protein)	6 ± 3	10 ± 2*	11 ± 1	15 ± 2 *
Catalase (mU/mg protein)	43 ± 4	55 ± 5*	0.28 ± 0.03	0.34 ± 0.02
Fumarase (U/mg protein)	0.17 ± 0.01	0.19 ± 0.01	1.7 ± 0.1	1.8 ± 0.1

Values indicate mean ± SE (n = 10).
*$p < 0.05$ compared to control values.

cause–effect relationship between the presence of these metals and the oxidant capability of CAPs aerosols, as opposed to a nonspecific effect caused by the physical interaction of foreign particles with lung cells. The association of the CAPs oxidant effect with their content of redox-active metals also supports the idea of an increased occurrence of Fenton-type reactions in the lung of CAPs-exposed animals.

These results agree with previous reports showing associations of different biologic readouts with the levels of transition metals in CAPs. Intratracheal instillation of ROFA particles with high Mn content induced bronchoalveolar lavage (BAL) eosinophilia *in vivo* (Jiang et al. 2001). Inhalation of soluble Fe, V, and Ni sulfates showed substantial cardiopulmonary toxicity in rats with acute or subacute ozone-induced pulmonary inflammation (Watkinson et al. 2001). Addition of surface iron converts nonreactive titanium dioxide particles into fibrinogenic particles (Dai et al. 2001). Finally, in a model similar to ours but with exposure time of 6 hr/day on 3 consecutive days, CAPs inhalation in rats elicited acute lung inflammation and the intensity of the inflammation was associated with the levels of metals (Saldiva et al. 2001).

The effect of CAPs on the ROS concentrations in the heart seems to operate through a different series of mechanisms. In contrast to lung CL, heart CL showed a 1-hr lag phase (Figure 1B) that may reflect the time required for the lung cells to signal the heart of the presence of an oxidant insult. The oxidant effect of CAPs on the heart is associated with CAPs components different from those associated with the development of oxidative stress in the lung. Heart CL strongly correlated with the CAPs content of Al, Si, Ti, and Fe, as well as with the total mass. The common association of lung and heart CL with Fe content suggests that at least some of the effects may be due to direct mechanisms, probably Fenton-type reactions. However, indirect mechanisms are also suggested by the associations of heart CL with nonredox active components such as Si and Ti and with the total CAPs mass. Reports by Clarke et al. (2000a, 2000b) also show association of increased pulmonary neutrophil percentages with the content of Al and Si in CAPs.

We also found that 5-hr inhalation exposure to CAPs causes significant tissue edema (Table 5) and increased release of LDH (Table 6). These results agree with the increased levels of BAL neutrophils and circulating lymphocytes reported in rats exposed to CAPs for 3 days (Clarke et al. 2000a). The slight increases in water content (≤ 5% for both tissues; Table 5) and release of LDH (≤ 80%; Table 6) and CPK (≤ 50%; Table 6) reported here for the short-term exposures tested are compatible with mild, reversible damage. As for the increases in lung and heart CL, the magnitude of increase in the lung and heart water content was similar to that reported in rats breathing 85% O_2 for 3 days (Evelson and González-Flecha 2000), a treatment associated with significant, although not lethal, injury to the lung and heart. In contrast, the increases in LDH and CPK values observed in animals breathing CAPs, although indicative of an increased permeability in cellular membranes, are below the levels associated to massive morphologic changes (González-Flecha et al. 1993).

One of the most striking findings of this study is that particulate air pollution has the ability to up-regulate antioxidant enzymes. We found that 5-hr exposures to 100–500 $\mu g/m^3$ CAPs increased SOD and catalase activities in a tissue-specific manner (Table 7). MnSOD induction was more marked in the lung than in the heart (70% vs. 40%), whereas increases in Cu/ZnSOD are observable only in the heart (100%). On the other hand, catalase was increased by 30% in the lung and showed a trend of increase that did not reach statistical significance in the heart. These patterns of antioxidant enzyme induction agree with results from gene array studies of the responses to inhaled CAPs in the rat lung (Godleski JJ. Personal communication). In these experiments, rats exposed to CAPs aerosols for 6 hr/day on 3 consecutive days showed increased lung mRNA levels of MnSOD and catalase. In contrast, expression of Cu/ZnSOD was slightly decreased after exposure. Taken together, these results suggest transcriptional regulation of antioxidant enzymes by short-term exposures to CAPs. Up-regulation of antioxidant enzymes has been described in other models of oxidant inhalation. Rats breathing 85% oxygen for > 5 days develop resistance to 100% oxygen, and this increased tolerance is associated with higher levels of MnSOD and Cu/ZnSOD in the lung (Clerch and Massaro 1993; Crapo and McCord 1976). Long-term exposure to ozone has also been reported to cause site-specific increases in the activities of antioxidant enzymes (Plopper et al. 1994).

There is abundant data that MnSOD can be induced by ROS (reviewed by Crawdford 1999) and proinflammatory cytokines *in vitro* (reviewed by Valentine and Nick 1999). Cu/ZnSOD, although found unresponsive to ROS levels and many cytokines, can be up-regulated by interleukin-1 (Tannahill et al. 1997), during cell differentiation (Valentine and Nick 1999), and in response to oxygen in very premature newborn baboons (Morton et al. 1999). Finally, catalase regulation by hydrogen peroxide has been documented in several systems (Csonka et al. 2000; Rohrdanz et al. 2001). CAPs exposure increases ROS concentrations (Figure 1, Table 4) as well as the levels of proinflammatory cytokines (Calderon-Garciduenas et al. 2001; Shukla et al. 2000). Therefore, the observed increases in activity of SODs and catalase could be due to transcriptional activation mediated by these factors.

In summary, our data show for the first time that short-term inhalation exposure to increased concentrations of particulate air pollution promotes oxidative stress and mild damage to the lungs and heart *in vivo*. The observed up-regulation of antioxidant defenses and the reversibility of the CAPs-mediated oxidative stress and toxicity strongly suggest that the lung and heart can readily adapt to rapid increases in the intracellular levels of oxidants. Further experimentation is warranted to establish the causal role of oxidants in CAPs toxicity as well as to confirm transcriptional regulation of antioxidant enzymes and establish the mechanism operating this regulation.

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