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Short Communication

Homocysteine and cysteine concentrations are modified by recent exposure to environmental air pollution in São Paulo, Brazil

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ABSTRACT

Millions of people worldwide are affected by anthropogenic air pollution derived from the combustion of fossil fuels. In this work, we tested the effects of fetal, lactation and post-weaning ambient air pollution exposure on total homocysteine (tHcy) concentrations and on a downstream pathway element, the plasma cysteine (Cys) concentration. Two similar exposure chambers (polluted and filtered chamber) were located near an area with heavy traffic in São Paulo, Brazil, and male Swiss mice were housed there from the pre-natal period until 3 months of age. Groups during fetal, lactation and adult periods of exposure were apportioned, and tHcy and Cys plasma concentrations were assessed when the animals were 3 months old. In our study, both the tHcy and Cys concentrations were decreased in groups that spent their final stage of life in polluted chambers, suggesting recent alterations in tHcy and Cys concentrations due to air pollution exposure. The possible relationship of these data with cardiovascular dysfunction is still a matter of controversy in animals; nevertheless, epigenetic mechanisms emerge as a possible issue to consider in the investigation of the link between air pollution and Hcy measurement.

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1. Introduction

Many lines of independent research have demonstrated that ambient air pollution is associated with adverse effects on cardiovascular function (Katsouyanni et al., 2001; O'Neill et al., 2005; Schwartz et al., 2005). In addition, air pollutants, especially particulate matter (PM), have also been correlated with heritable mutation and changes in methylation that could have repercussions for chromatin structure, gene expression and genome stability (Somers et al., 2002; Yauk et al., 2008). Large-scale epidemiological studies have been carried out on levels of homocysteine (Hcy), a thiol containing amino acid derived from the metabolism of methionine, demonstrating that a mild increase in total Hcy (tHcy) in the general population is associated with increased risk of several age-related diseases, including coronary artery disease, stroke, osteoporosis, and dementia (Perry et al., 1995; van Meurs et al., 2004). Hcy metabolism is also linked with methylation and folate, important metabolic pathways required for optimal health (Williams and Schalinske, 2007). The

short-term effects of particles have been the main focus of study, as well as the relationship between Hcy and air pollution. This issue has been studied primarily in humans and in acute conditions (Baccarelli et al., 2007; Park et al., 2008), resulting in a shortage of literature using experimental models. In the present study, we investigate the effects of air pollution on tHcy concentrations in the short and long-term in a mouse model of air pollution exposure and verify the relationship between air pollution and Hcy concentrations previously found in clinical studies.

2. Materials and methods

2.1. Ethical considerations

The institutional review board (Comissão de Ética em Pesquisa da Universidade Federal de São Paulo) approved the present study, ensuring that animal care and evaluation were performed according to humane standards.

2.2. Exposure chambers and measurement of pollutants

The experiments were performed 20 m from a street with heavy traffic in São Paulo. The animals were housed concurrently in two open-top chambers; one of the two chambers received ambient air, called the polluted chamber, whereas the

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other (control), placed in the same location, received filtered air and was designated the filtered chamber. In the filtered chamber, three stages of filters (Purafil, São Paulo, Brazil) were arranged serially. The first eliminates large particles (plain and bag filters), and the second and third stages (model JFL-90 and a High Efficiency Particulate Air (HEPA), respectively) trap fine particles, from Purafil™ (Sao Paulo, SP, Brazil). Inside the chambers, animals were kept under the same conditions of temperature and humidity. The chambers were used in other published reproductive (Mohallem et al., 2005; Veras et al., 2009) and respiratory studies (Mauad et al., 2008). Ambient levels of nitrogen dioxide (NO₂), PM₁₀ (PM < or = 10 μm in aerodynamic diameter), carbon monoxide (CO) and sulfur dioxide (SO₂) were continuously measured by The Environmental Agency of the City of São Paulo (CETESB) in the vicinity of the inhalation chambers. Gaseous pollutants were not retained by the filtering system, and thus the concentrations were assumed to be similar in both chambers. There are no industries or biomass-burning sources in this area (CETESB, 2006), and the main source of pollution is predominantly vehicular at this site (Castanho and Artaxo, 2001).

2.3. Experimental groups

Male and female Swiss mice were apportioned into filtered and polluted chambers and were mated. After the mating period, females were housed in separate cages until the offspring's birth, and pups were weaned at post-natal day (PND) 21. After this period, male pups were housed inside chambers until 3 months of age. We had six groups depending on the type of chamber in the three different periods: mice that spent their entire life inside a single chamber (filtered or polluted), those that spent only the fetal period inside the filtered or polluted chamber and were then moved to the other, and those that spent both the fetal and lactation periods in a single chamber and were moved to the other chamber after that. All animals had reached adulthood (3 months) when the biochemical analyses were performed, and they received the same balanced diet (Nuvital-Nutrients Ltda, Colombo, Brazil) and water *ad libitum* throughout the entire protocol. All animals spent one or more of the three periods of their lives inside one of the chambers (filtered-F or polluted - P; Table 1) and did not come back into the laboratory, except for the day of euthanasia.

To confirm the data, a group of animals that spent their adult period in the polluted chamber was moved to a filtered chamber, and another group that lived inside the filtered chamber during the adult period was moved to a polluted chamber, where they were housed for six more months before blood collection for measurement of tHcy and Cys.

2.4. Homocysteine and cysteine measures

The animals were euthanized at 3 and 9 months by decapitation between 08:00 and 11:00 h, and their blood was collected in heparin tubes. The total plasma Hcy and cysteine (Cys) concentration was measured according to the method described by Pfeiffer and co-authors (Pfeiffer et al., 1999), which utilizes High Performance Liquid Chromatography with fluorimetric detection and isocratic elution (de Oliveira et al., 2002).

2.5. Statistical methods

The data were analyzed with the statistical software *Statistica* (SAS-Statistical Analysis System for Windows, version 6.12, SAS Institute Inc., Cary, NC), and a general linear model was applied to the results when the animals reached 3 months of age. The study design incorporated three different periods of exposure to air pollution or filtered chambers: fetal, lactation exposure (PND 1-21) and adult exposure (PND 21-90) and consisted of six groups for the analysis by the generalized linear model. The group that lived their entire life in the filtered chamber is represented in all sections of the results by columns denoting the filtered chamber results. All groups that spent the fetal period in the filtered chamber are represented in the first column of the results (Table 1). The group that

spent only the adult period in the filtered chamber and the other periods in the polluted chamber is represented in the polluted columns in the fetal and lactation period results and by filtered columns in the adult period (second, fourth and fifth columns, respectively).

For the validation experiment (after 6-months), a student's *t*-test was applied, and the level of significance for both tests was $P < 0.05$.

3. Results

3.1. Measurements of pollutants

The ambient levels of air pollutants measured by CETESB (CETESB, 2006) throughout the protocol were as follows (mean \pm SEM): PM₁₀, $31.2 \pm 1.2 \mu\text{g}/\text{m}^3$; NO₂, $72.1 \pm 2.5 \mu\text{g}/\text{m}^3$, SO₂, $4.8 \pm 0.2 \mu\text{g}/\text{m}^3$ and CO 1.33 ± 0.07 ppm. The PM_{2.5} was significantly lower in the filtered chamber ($P < 0.00001$), with a mean reduction of 70% in PM_{2.5}.

3.2. Homocysteine and cysteine measures

The Hcy and Cys concentrations are presented in Table 1. We were able to measure the Hcy and Cys concentration in adult mice after a specific exposure protocol, including fetal, lactation and adult periods of life with (P) or without (F) exposure to air pollution. Mice exposed to air pollution during adulthood had significantly diminished tHcy and Cys concentrations, suggesting a recent effect on these biochemical parameters owing to their exposure.

To confirm the effect of air pollution on tHcy and Cys concentrations, the groups that spent the final phase of development in the pollution chamber were moved to a filtered chamber. The same occurred with the controls, which were moved to a polluted chamber, and they all remained there for 6 months. Following this treatment, we still observed a persistent decrease in tHcy and Cys concentrations in the polluted exposure group when compared to the controls, even though this group had spent a significant amount of time in the filtered chamber earlier in their life (Fig. 1).

4. Discussion

We have demonstrated that a recent period of exposure of inbred mice to ambient air pollution caused a decrease in both tHcy and Cys concentrations, which was confirmed after moving adult groups to the opposite chamber (Fig. 1). In the presence of Vitamin B6, Hcy is converted to cystathionine and then to Cys (Fowler, 2005); hence Cys concentrations were used to confirm downstream alterations in the Hcy pathway. In the literature, there is no consensus on the relationship between air pollution and Hcy concentrations in humans. A recent study involving older men (mean age, 73.6 ± 6.9 years) showed statistically significant

Table 1
Homocysteine and cysteine concentrations in plasma of adult mice after air pollution exposure.

	Fetal period		Lactation period		Adult period	
	F	P	F	P	F	P
Homocysteine	4.10 ± 0.14	4.15 ± 0.16	4.09 ± 0.15	4.12 ± 0.15	4.43 ± 0.17	3.80 ± 0.11^a
P-value		0.404		0.186		0.013
Cysteine	189.33 ± 3.10	196.68 ± 3.89	191.89 ± 3.93	186.35 ± 4.93	199.46 ± 4.01	179.21 ± 4.20^a
P-value		0.142		0.664		0.032

The data are represented as the mean (μM) \pm SEM. The general linear model was used for this analysis. The measurements were performed on PND 90 for all groups. F-filtered chamber and P-polluted chamber, in the relevant life period (fetal, lactation (PND 1–21) and adult life (PND 21–90)).

^a Significantly different ($P < 0.05$) from the respective control group (F).

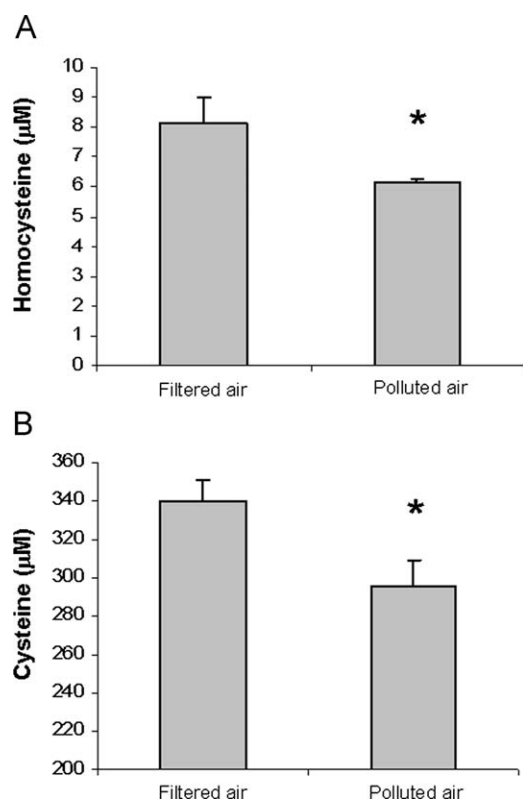


Fig. 1. Validation of the effect of air pollution on tHcy and Cys concentrations. The adult group that presented with a statistical difference was moved to the opposite chamber to confirm the latest exposure effect. **A.** Hcy concentrations after 6 months in the opposite chamber – the group that spent the adult period in the polluted chamber was exchanged with those in the filtered chamber for an additional 6 months. **B.** Cys concentrations acquired from the same groups as **A.** The data are presented as means (μM) \pm SEM. The student's *t*-test was applied and *denotes a significant difference compared to the control group.

positive associations of tHcy and traffic-related particles (Park et al., 2008). Nevertheless, no association was observed with sulfate or $\text{PM}_{2.5}$ ($\text{PM} < \text{or} = 2.5 \mu\text{M}$ in aerodynamic diameter). Another study that included a large age range (11–84 years) found that older subjects had higher plasma tHcy than younger ones, which is in accordance with our results in mice (table and Fig. 1). In this human subject study however, air pollution levels measured 1 week before the study did not show overall consistent associations with tHcy. The authors found increased tHcy concentrations only when they crossed PM_{10} with cigarette smoking. PM_{10} levels, especially 24 h before the study, were associated with increased tHcy in smokers but not in nonsmokers (Baccarelli et al., 2007). Although hyperhomocysteinemia is commonly associated with cardiovascular risk in humans (Antonades et al., 2008), that association is not clear in animals. Hyperhomocysteinemia is usually induced in mice through both genetic and dietary manipulations because concentrations of Hcy are regulated by both the methionine and folate cycles (Dayal and Lentz, 2008). However, experimental manipulations that induce hyperhomocysteinemia in mice do not reflect real human scenarios and do not inform the question of whether Hcy is an independent risk factor developed in the body or a marker of vascular disease. Exposure to air pollution is known to be associated with an increased risk of cardiovascular dysfunction in humans (Brook et al., 2004), and acceleration of atherosclerosis and vascular inflammation has been shown in mice after 6 months of exposure to concentrated air pollutants (Sun et al., 2005). Nevertheless, our results showed a decrease in tHcy and Cys concentrations when no changes were expected based on

previous studies in humans, suggesting that, at least in a mouse model, Hcy is not a good marker or does not indicate independent risk of cardiovascular disease. The relationship between Hcy concentration and vascular dysfunction remains to be elucidated in mice. A decrease in tHcy concentration could result due to the intense catabolism of this molecule, characterized as hypohomocysteinemia, which is commonly found in the early stages of type 1 diabetes (Williams and Schalinske, 2007).

Another point to consider is that in our mouse model of exposure, air pollutants interact dynamically with Hcy, and modifications do not persist when the animal is removed from the pollution source. These dynamic modifications of molecules suggest the involvement of epigenetic mechanisms such that environmental factors can interact with the organism, inducing nonpermanent modifications in a pathway that is linked with Hcy and Cys concentrations in a chronic exposure scenario (Williams and Schalinske, 2007). In an animal model of air pollution exposure, Yauk and co-authors demonstrated that there was no significant change in methylation in germ-line cells at 3 weeks of exposure; however, after 10 weeks, hypermethylation arose in the mice exposed to particulate air pollution near two integrated steel mills. In addition, the methylation persisted until 6 weeks even without further exposure (Yauk et al., 2008). This exposure occurred before fecundation; the alterations observed in germ-line cells indicate that pollution exposure is able to modify epigenetic patterns in mouse models and that some modifications in DNA methyl-transferases (DNMTs) and global DNA methylation may also occur after zygote formation. Moreover, it has long been known that air pollution causes DNA damage and mutations (Somers et al., 2002) and that DNMTs are upregulated by toxicants and bind with high affinity to many DNA lesions (James et al., 2003). In theory, elevated DNA damage due to exposure to air pollutants can result in upregulation of DNMT, leading to persistent but transient hypermethylation (Yauk et al., 2008).

An Italian study in an adult population with a common polymorphism in 5,10-methylenetetrahydrofolate reductase showed that these individuals had elevated plasma Hcy concentrations, which correlated inversely with global DNA methylation (Friso et al., 2002). In our study, we found decreased concentrations of tHcy, suggesting a possible correlation with increased global DNA methylation in our model. Conversely, Tarantini et al., (2009) recently found that global DNA methylation estimated in Alu and LINE-1 repeated elements were negatively associated with individual PM_{10} exposure. Similarly, the same group found that LINE-1 methylation in human blood decreased after recent exposure to higher black carbon (Baccarelli et al., 2009).

The link between phases of development, Hcy, methylation and physiologic effects, is still unknown, and data in humans and animals are controversial. More information and studies are needed to increase knowledge of these issues. In conclusion, this is the first time that tHcy concentrations were measured after various phases of development in a mouse model of ambient air pollution exposure; however, correlation of these findings with previous human data and cardiovascular dysfunction remains unclear. New insight involving Hcy concentrations, epigenetics and air pollution may be forthcoming, as epigenetic mechanisms have an important role in the developmental origins of health and disease (Waterland and Michels, 2007) and offer new avenues in the exploration of the link between air pollutants and disease.

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Competing interests

The authors declare they have no competing interests.

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