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Metallothioneins and trace elements dyshomeostasis induced by exposure to gasoline vapor in mice

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Summary. To investigate the effects of air pollution related with the gasoline/petrochemical industry the expression of metallothionein I (MT-I) mRNA and tissue metals were analyzed in organs of mice, exposed to gasoline (G) vapor in laboratory conditions. Control groups consisted of intact mice and of those exposed in the metabolic chamber to fresh air. The data obtained by RT-PCR and inductively coupled plasma spectrometry have shown that exposure to G vapor leads to upregulation of MT-I mRNA in organs that receive a strong respiratory and olfactory input or participate in gasoline degradation and elimination (lungs, brain, kidney and liver). Besides, in the brain and in the lungs, kidney and liver a decreased tissue content of Zn^{2+} or Cu^{2+} and Mg^{2+} was found ($p<0.001$). Some of these changes were obtained also in mice closed in the metabolic chamber, pointing to the involvement of stress-induced mechanisms in the transcriptional regulation of MTs.

Key words: Gasoline inhalation, Stress, Metallothioneins I, Zinc, Copper, Magnesium, Brain, Lungs, Kidney, Liver

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Introduction

Environmental airborne pollution with gasoline vapors, auto exhaust, chemical production and industrial sources has been repeatedly shown to affect multiple aspects of brain and cardiopulmonary function, leading to cognitive and behavioral changes and to the pronounced inflammatory response in the respiratory airways. The magnitude of exposure might be even greater in individuals involved in chemical manufacturing processes in the oil refining and petrochemical industry, or occupationally exposed to gasoline in filling stations, bulk gasoline terminals and plants, delivery tank trucks etc (Ritchie et al., 2001; Martinez and Ballesteros, 2005; McDonald et al., 2007, 2008; Kobayashi et al., 2008). The damage may be induced by toxic polar fuel components, such as benzene, toluene, ethylbenzene, aniline and phenol, as well as by several heavy metals, such as Pb, Cu, Zn, Hg and Mn, or by oxygenate additives present in the gasoline (Kinawy, 2009).

The major cause of mortality and morbidity is associated with pulmonary aspiration of gasoline vapor, but multisystem organ injuries might be induced by general toxicity and inflammation, leading to neurological and cognitive impairment (Cairney et al., 2004), as well as to reproductive/developmental toxicity and genotoxicity (Reed et al., 2005). The final effect depends on the balance between the damaging and repair processes able to correct the disturbance of homeostasis.

A typical cytoprotective effect consists also in the

enhanced synthesis of metallothioneins (MTs), a family of low-molecular weight cysteine-rich proteins, which have a high affinity for both essential and nonessential metals. *In vivo*, the metal-binding involves mainly Zn(II), Cu(I), Cd(II), and Hg(II), while *in vitro*, additional and diverse metals such as Ag(I), Au(I), Bi(III), Co(II), Fe(II), Pb(II), Pt(II), and Tc (IV) may be bound to apothionein (the metal-free form), regulating the metal ion homeostasis. However, as covered by numerous excellent reviews (Coyle et al., 2002; Mocchegiani et al., 2006a; Penkowa, 2006; Nielsen et al., 2007; Thirumoorthy et al., 2007) these multipurpose proteins might also participate in mitochondrial respiration, thermogenesis, body energy metabolism, angiogenesis, as well as in cell survival, differentiation and apoptosis, having important functions in protein-protein and protein-nucleotide interactions and in cytoprotection against various types of injuries. Besides, owing to their important neurophysiological and neuromodulatory functions, MTs might be involved in brain-endocrine-immune response (Giacconi et al., 2003), in a range of acute and chronic neurological disorders (Hidalgo et al., 2006; Penkowa, 2006), as well as in protection against oxidative damage, drugs and radiotherapy resistance, autoimmunity and in several aspects of the carcinogenic process (Cherian et al., 2003; Theocharis et al., 2003; Pedersen et al., 2009).

Extensive data also point to the possibility that MTs have a special function in sensory organs (Aschner et al., 1997; Shimada et al., 2005), such as in the olfactory mucosa and bulb, since there they might be induced by intranasal instillation of cadmium (Tallkvist et al., 2002), zinc (Persson et al., 2003), mercury vapor (Shimada et al., 2005), as well as by various toxic agents, related to external pollution, occupational exposure or volatile substance abuse. It was, therefore, proposed that MTs might have a protective role particularly in places that provide a direct route of entry into the CNS, where they might also participate in direct olfactory transport of some inhaled chemicals along the cell processes of olfactory neurons to rhinencephalic cortex (Hastings and Evans, 1991; Tallkvist et al., 2002).

Contributing to this field we have previously reported that vapor containing gasoline induces a high upregulation of MT I/II immunoreactivity, particularly in the brain areas that receive a strong olfactory input (dentate gyrus of the hippocampus), as well as in the lungs and in the kidney, suggesting that the expression of MT-proteins was connected with repair mechanisms on sites of injuries induced by toxic components in the gasoline vapor (Grebic et al., 2007). Enlarging these data, in this study we analyzed the MT-I gene expression and mineral tissue content in organs of mice that were exposed to gasoline vapor or to fresh air in a metabolic chamber. The data have shown that both exposure to gasoline and the stress of closure might contribute to the activation of the MT-I gene, as well as to the induction of a marked imbalance of Zn^{2+}, Cu^{2+} and Mg^{2+} homeostasis in the brain, lungs, liver and kidney.

Material and methods

Animals

Mice of strain C57/BL6 aged 2-3 months were selected for the experiment. They were housed in groups of 6-8 animals, kept under standard conditions and exposed to a natural day-night cycle. The mice were bred and maintained according to the guide for Institutional Animal Care, according to the current legal terms (NN 19/99) and used with approval of the Ethical committee of the University of Rijeka.

Exposure to gasoline vapor

The experimental protocol used in this study was previously described (Grebic et al., 2007). Briefly, as shown in Fig. 1, a group of 6 mice was placed in a 1L metabolic cage for small animals (SRI 5056, Ugo Basile, Milano, Italy), in which the temperature and humidity was maintained constant by cold water circulating between the two external layers of the metabolic chamber. In the experimental group, the chamber was ventilated with air containing the gasoline vapor and in control group by fresh air. The ventilation of the chamber was performed automatically by a ventilator, which ensured air flow of 0.4 L/min, using a constant volume, pressure (20 cm H_2O and respiration rate (60/min). The quantity of gasoline (Euro super 95, INA, Rijeka) was determined by Floutec vaporizer (concentration 0.5-5%). The soda lime was used for the absorption of CO_2 from exhaled air. Treatment lasted 1 h/day and the protocol was repeated for 10 subsequent days. One day after the last treatment, the slightly anesthetized mice were sacrified by cervical dislocation. In the freshly dissected organs (lungs, brain, kidneys and liver) the effects of gasoline and stress were evaluated on the MT-I mRNA and metal tissue content. The data were also compared with the findings in a group of untreated mice (N=6).

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from the brain, lungs, kidney and liver tissue homogenates using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions, as we previously described (Jakovac et al., 2006). The RNA yield and purity was assessed spectrophotometrically and its integrity was confirmed by 2% agarose gel electrophoresis. One microgram of total RNA from each sample was reverse transcribed by oligo-p (dT) primers into cDNA using the First the Strand cDNA Synthesis Kit AMV (Roche, Germany) as specified by the manufacturer. cDNA was amplified by polymerase chain reaction (PCR) using *Taq* polymerase and PCR buffer containing 6 mM $MgCl_2$. Amplification of housekeeping gene ß-actin mRNA was always involved as a positive control. The primer sequences

were as follows: MT-I sense: 5'-ATGGACCCC AACTGCTCCTGCTCCACC-3' MT-I antisense: 5'-GGGTGGAACTGTATAGGAAGACGCTGG-3' (cDNA length, 259 bp) ß-actin sense: 5'-GGACTCCTAT GTGGGTGACGAGG-3' ß-actin antisense: 5'-GGGAG AGCATAGCCCTCGTAGAT-3' (cDNA length, 366 bp).

The amplified products of the RT-PCR reactions were size-fractionated by 2% agarose gel electrophoresis and visualized under ultraviolet light after staining with ethidium bromide. All experiments included PCR performed with all additives but without cDNA (no template control; NTC) and "no reverse transcription" (NoRT) control to exclude unspecific amplifications of genomic DNA and DNA contamination. The MT-I/ß-actin ratios were calculated after the densitometric analysis of ethidium bromide visualized amplification product bands by a calibrated imaging densitometer (Image Station 446CF Kodak; program Kodak ID3.6). Analyses were done with tissue samples, prepared as a pool from 3 mice.

Tissue sample preparations for liberalization and inductively coupled plasma (ICP) spectrometry

The brain, lungs, kidneys and liver were carefully removed using plastic instrumentation, as previously described (Jakovac et al., 2006). Briefly, 20-50 mg of the tissue was dried at 105°C for 5 hours. After that 3 ml of conc. HNO_3 ("Kemika" d. d., Zagreb, Croatia) and 0.5 ml of 30 % H_2O_2 ("Kemika" d. d., Zagreb, Croatia) were added. The microwave digestion occurred in MLS - 1200 Mega, Microwave Digestion System with MDR Technology, under the following conditions: 5 min at 300 W, 30 seconds at "zero" W, 5 min at 600 W and 1 min of ventilation. After cooling for 15 min and filling with distilled/de-ionised water the sample was prepared for inductively coupled plasma spectrometry (ICP). Liquid samples were introduced into the apparatus by pneumatic nebulisation, and the measurements of zinc, copper and magnesium were performed using a PHILIPS PU 7000-ICP Spectrometer, by the method ASTM D 1976 (power 1kW, coolant 12 l/min, nebulizer 38 psi), at a fixed wavelength of 213.856 nm for zinc, 324.754 nm for copper and 279.078 nm for magnesium.

Statistical analysis

The data were analyzed using Sigma Plot Scientific Graphing System, Version 8.0. Statistical significance was calculated by two-tailed Student's t-test for unpaired samples and by analysis of variances (ANOVA). $p<0.05$ was considered significant.

Results

Enlarging our previously published data (Grebic et al., 2007), we show herein that the repeated exposure of mice to gasoline vapor (1h/day for 10 subsequent days) induces an upregulation of MT-I mRNA expression and a high imbalance in the tissue content of Zn^{2+}, Cu^{2+}, and Mg^{2+} in lungs, brain, kidney and liver. The data were highly significant in comparison with the findings in intact animals, but some of these changes were also found in mice maintained in air-ventilated chamber ("cage control" group), pointing to the high contribution of stress-induced mechanisms in the experimental system used.

Thus, in the lungs we found that both gasoline and stress of closure induced a slight increase of MT-I mRNA (Fig. 2A), as well as a significant hypo-magnesemia in comparison with the intact control (Fig. 2B; $p<0.001$).

Furthermore, the content of MT-I mRNA also increased in the brain (Fig. 3A), where gasoline exposure reduced the concentration of Zn^{2+} (Fig. 3B; $p<0.001$ and $p<0.05$ in comparison to intact and stress-related control, respectively). In addition, in the brains of mice exposed to gasoline vapor and stress reduced levels of Cu^{2+} and Mg^{2+} were found ($p<0.001$).

Upregulation of MT-I gene (Fig 4A) in the kidneys, in comparison with intact control was less expressed, but in gasoline and stress-exposed groups of mice the reduction of Cu^{2+} and Mg^{2+} was observed again. (Fig. 4B; $p<0.001$ versus intact control).

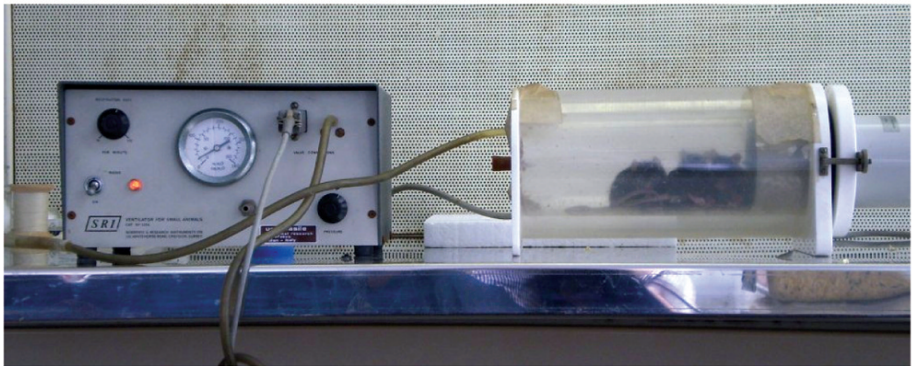
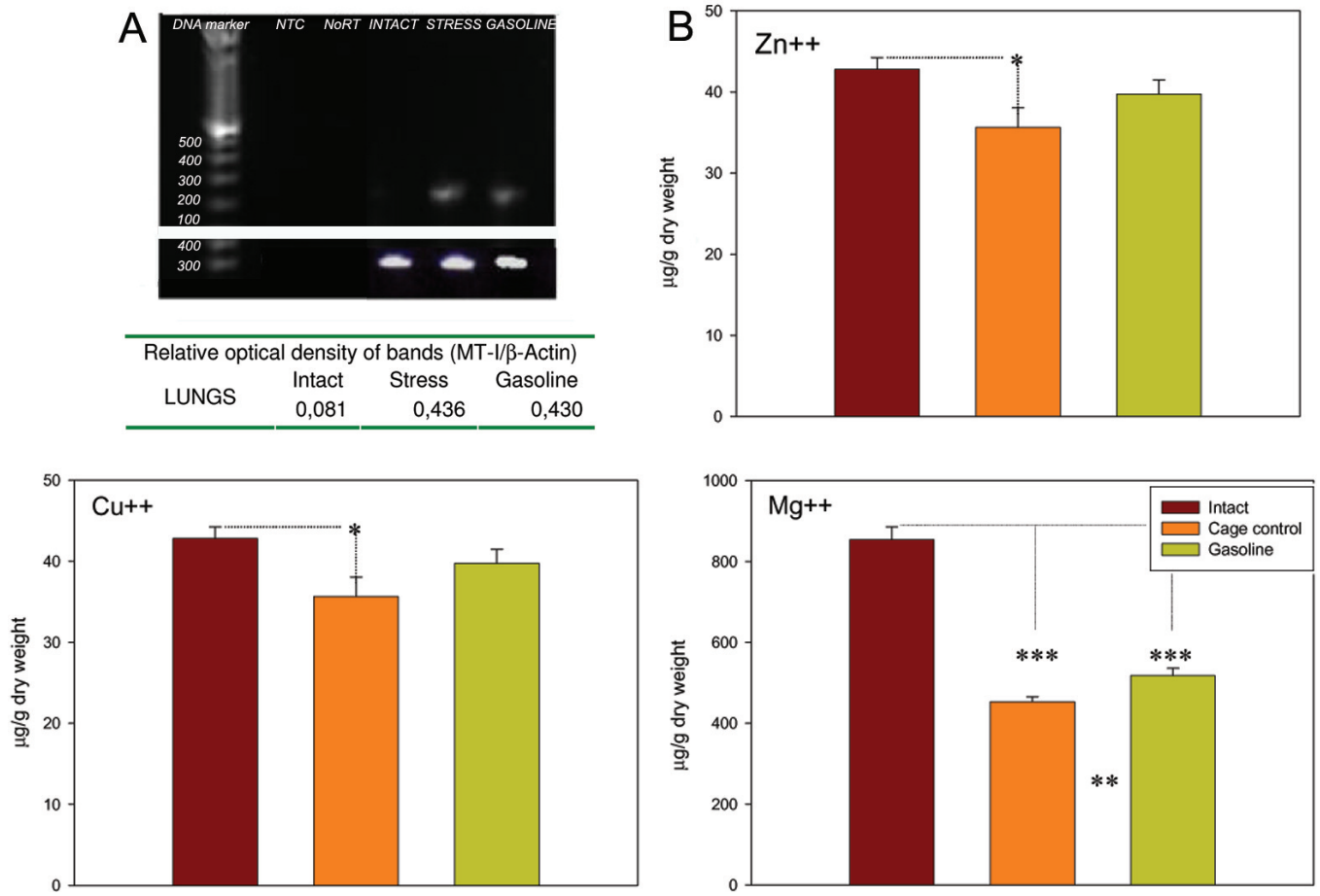


Fig. 1. Metabolic cage for small animals (SRI 5056, Ugo Basile, Milano, Italy) was used to expose a group of mice (N=6) to air containing gasoline vapor. Mice in the control group (N=6) were under the same conditions exposed to fresh air. Treatment lasted 1 h/day and the protocol was repeated for 10 subsequent days. Mice were sacrified one day after the last treatment. As an additional control, the intact mice were used (N=6).

The additional data also showed that gasoline vapor and stress of closure had a high impact on the liver, since in this organ we found a marked upregulation of MT-I mRNA and reduced concentrations of Cu^{2+} and Mg^{2+} (Fig. 5A,B; $p<0.001$).

Discussion

In an immunohistochemical study we previously demonstrated that short term exposure of mice to gasoline vapor was followed by a high upregulation of MT-I/II proteins in the brain, lungs and kidney (Grebic et al., 2007). The changes were significantly greater than those induced by stress provoked by the closure of mice into the metabolic chamber, but were also regionally distributed. Thus, in the brain an increased number of MT-I/II positive glial, ependymal and macrophages-like cells was found, particularly in subventricular zones and perivascular spaces, as well as in the hippocampus (in subgranular layer of the dentate gyrus and the CA3-CA1 fields of the Ammon's horn), which receives a strong olfactory input from the olfactory bulb and the entorhinal cortex. Besides, a high gasoline-induced enhancement of MT-I/II proteins was found on pneumocytes type I and type II, and in the kidneys on the parietal wall of Bowman capsule and on proximal and distal tubules, implying that MT-I/II proteins were upregulated particularly at the sites that were directly irritated by gasoline vapor or by its metabolites (Grebic et al., 2007). The hypothesis is supported by reports describing the function of MTs in sensory organs (Aschner et al., 1997) and by evidence showing that agents that are normally excluded by the blood-brain barrier (BBB), such as cadmium (Tallkvist et al., 2002),



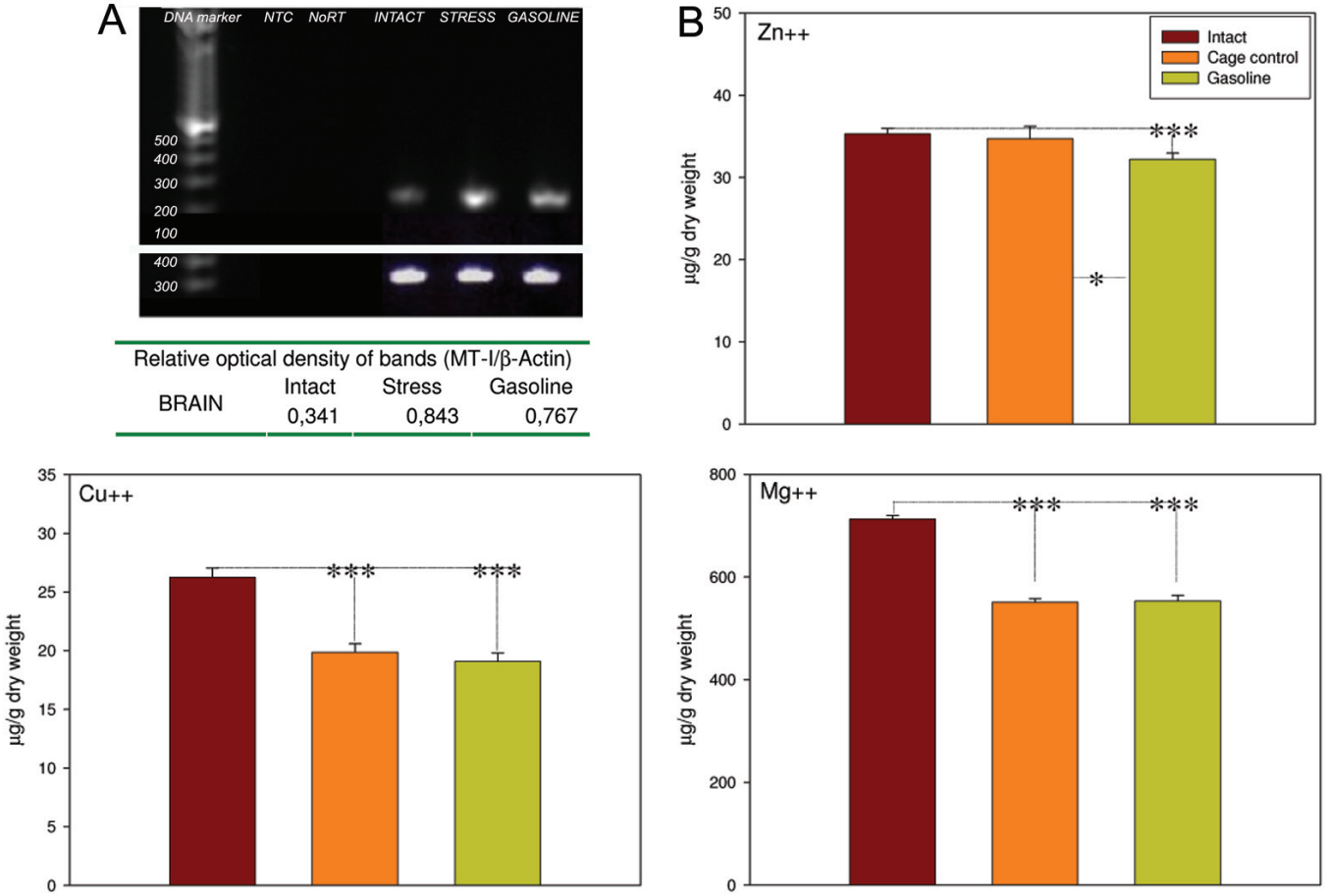
Relative optical density of bands (MT-I/β-Actin)			
	Intact	Stress	Gasoline
LUNGS	0,081	0,436	0,430

Fig. 2. Expression of MT-I gene **(A)** and metal tissue content **(B)** in the LUNGS of three groups of mice: 1) untreated mice ("intact"), 2) mice closed in the metabolic chamber, ventilated with fresh air ("cage control") and 3) mice closed in the metabolic chamber, ventilated with gasoline vapor ("gasoline"). In RT-PCR **(A)** the data obtained with all additives but without cDNA (NTC and those obtained without the reverse transcription (NoRT) are also shown. The ß-actin mRNA was used as a loading control. The Table shows the ratio of MT-I/ß-actin mRNA, calculated from the optical density of bands on the Northern blot membranes. Analyses were done on tissue samples, prepared as a pool from 3 mice. In **B**) the metal concentrations are expressed as micrograms per gram on dry organ weight. The values represent the mean ± SE of four to six mice. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

zinc (Persson et al., 2003) or mercury (Shimada et al., 2005) might be transported directly into the CNS by olfactory epithelium after intranasal instillation.

In the present study, using the same experimental model, we intended to enlarge our previous findings on analyses of MT-I mRNA and tissue metals content. The data revealed that that exposure of mice to gasoline vapor in laboratory conditions is also followed by the upregulation of MT-I mRNA, as well as by a marked systemic deprivation of copper and magnesium, but these changes were less specific than those found in the expression of MT-I/II proteins (Grebic et al., 2007), since similar findings were seen also in mice closed into the metabolic cage. The discrepancy might be related with our inability to detect the regionally expressed changes by the use of homogenates of the whole organs, but the similarity of changes found in both groups of mice closed in a metabolic chamber, point also to the possibility that animals were not well adapted to the experimental system. Trying to prevent the latter, we extended the experiments to 11 days, but since the animals were not conditioned to the metabolic cage prior to the experiment, the participation of stress-induced events in mice exposed to gasoline should be taken into account. Furthermore, the discrimination between the toxic and stress-induced events in our model was additionally complicated by the fact that MTs belong to phylogenetically ancient stress-proteins, which might be transcriptionally activated by a number of similar mediators and stimuli.

The issue is covered by numerous reviews in this field (Moffatt and Denizeau, 1997; Coyle et al., 2002; Cousins et al., 2006; Mocchegiani et al., 2006a; Penkowa, 2006; Nielsen et al., 2007; Thirumoorthy et



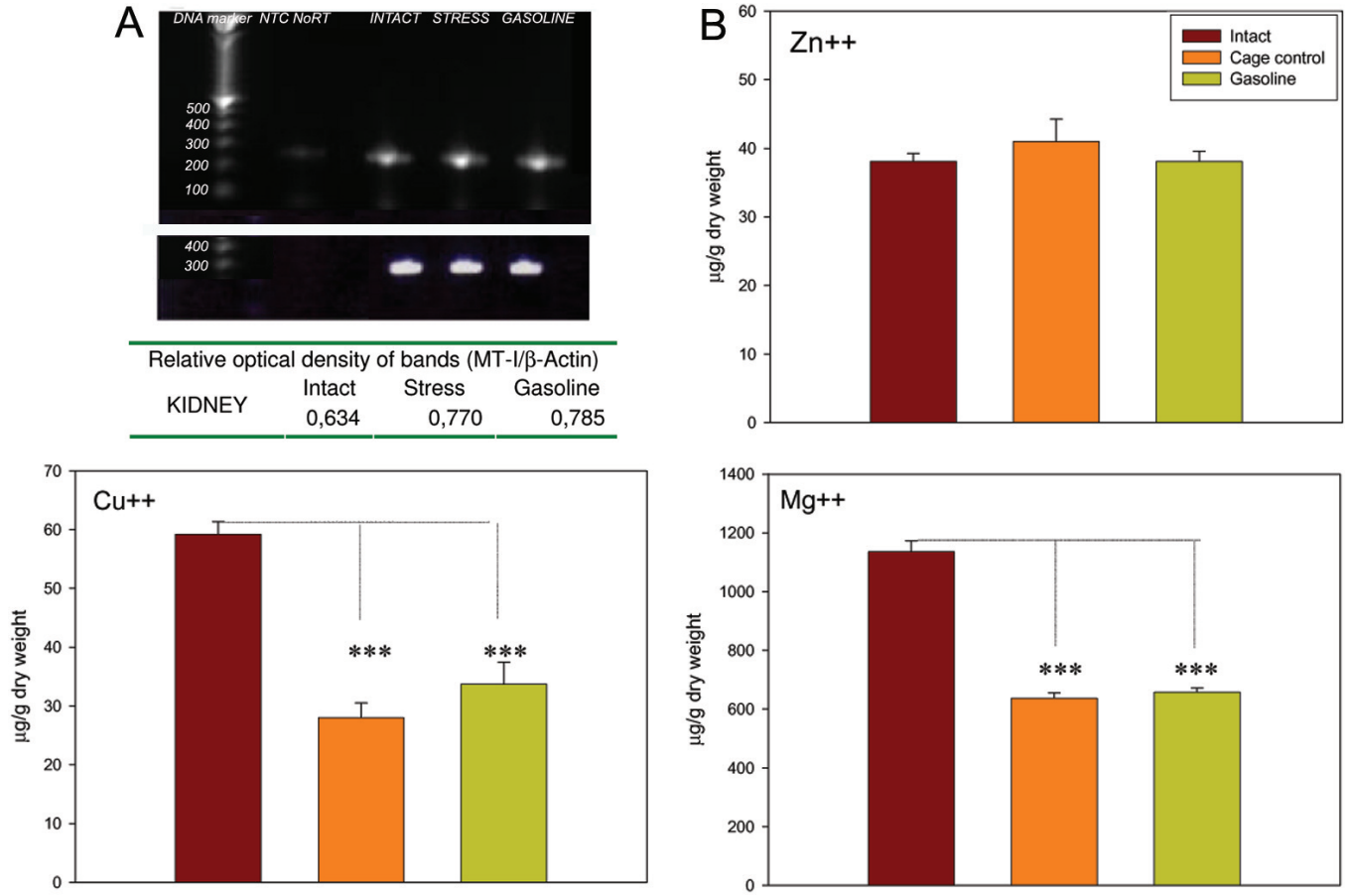
Relative optical density of bands (MT-I/β-Actin)

	Intact	Stress	Gasoline
BRAIN	0,341	0,843	0,767

Fig. 3. Expression of MT-I gene **(A)** and metal tissue content **(B)** in the BRAIN of three groups of mice: 1) untreated mice ("intact"), 2) mice closed in the metabolic chamber, ventilated with fresh air ("cage control") and 3) mice closed in the metabolic chamber, ventilated with gasoline vapor ("gasoline"). In RT-PCR **(A)** the data obtained with all additives but without cDNA (NTC) and those without the reverse transcription (NoR) are also shown. The ß-actin mRNA was used as a loading control. The Table shows the ratio of MT-I/ß-actin mRNA, calculated from the optical density of bands on the Northern blot membranes. Analyses were done on tissue samples, prepared as a pool from 3 mice. In **B**) the metal concentrations are expressed as micrograms per gram on dry organ weight. The values represent the mean ± SE of four to six mice. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

al., 2007) showing that the expression of MT-I/II may be driven through several response elements in the MT gene promoter. Among them are metal response elements (MRE), glucocorticoid-responsive elements (GRE), elements activated by signal transducers and activators of transcription (STAT) proteins, and the antioxidant (or reducing) response element (ARE) activated in response to redox status. Owing to this, MTs have been considered as stress - or acute phase response proteins, whose induction dramatically increases after tissue injury, infection, inflammation, and carcinogenesis, as well as in physiological processes that regulate cell proliferation and apoptosis. The effects, however, depend on the cell specific expression of different MTs forms, showing that they belong to specific cellular defense mechanisms (Miles et al. 2000).

Since a range of different stimuli induce MTs, the molecular mechanisms of MT regulation and their effects on the essential metals after inhalation of gasoline vapor and exposure to stress remain to be elucidated. We can, however, speculate that toxic pollutants from gasoline vapor (such as hydrocarbon compounds, including benzene, n-hexane, toluene, xylenes, naphthalene) or heavy metals (such as Pb, Cu, Zn, Mn) induced the expression of the MT-I gene and the synthesis of MT-I/II proteins after activation of metal-responsive transcription factor-1 (MTF-1), since it was shown that in mice it plays a central role in transcriptional activation of the MT-I gene in response to zinc and cadmium, as well as in response to agents which cause oxidative stress and/or inflammation (Andrews, 2000). Moreover, since the DNA-binding activity is reversibly activated by zinc interactions with the zinc-finger domain, it has been postulated that MTF-1 might also regulate numerous cellular proteins, enzymes, DNA and RNA polymerases and transcription



Relative optical density of bands (MT-I/β-Actin)

	Intact	Stress	Gasoline
KIDNEY	0,634	0,770	0,785

Fig. 4. Expression of MT-I gene **(A)** and metal tissue content **(B)** in the KIDNEY of three groups of mice: 1) untreated mice ("intact"), 2) mice closed in the metabolic chamber, ventilated with fresh air ("cage control") and 3) mice closed in the metabolic chamber, ventilated with gasoline vapor ("gasoline"). In RT-PCR **(A)** the data obtained with all additives but without cDNA (NTC) and those without the reverse transcription (NoRT) are also shown. The ß-actin mRNA was used as a loading control. The Table shows the ratio of MT-I/ß-actin mRNA, calculated from the optical density of bands on the Northern blot membranes. Analyses were done with tissue samples, prepared as a pool from 3 mice. In **B**) the metal concentrations are expressed as micrograms per gram on dry organ weight. The values represent the mean ± SE of four to six mice. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

factors that require Zn for their biological activity, acting as a sensor of free zinc pools in the cell (Moffatt and Denizeau, 1997; Lichtlen and Schaffner, 2001; Coyle et al., 2002). Among them are also the genes responsible for cellular response to oxidative stress and acute phase response and those necessary for the synthesis of glutathione, maintenance of embryonic colloid-osmotic pressure and angiogenesis. Furthermore, for herein presented data, it might be relevant that MTF-I is a crucial transcriptional regulator for basal expression of zinc transporter ZnT-1, which affects zinc metabolism and numerous Zn functions in the brain, where zinc has a considerable role in the stress response and acts as a neuromodulator at excitatory synapses (Carrasco et al., 1998; Tapiero and Tew, 2003).

Besides, there is also a high possibility that exposure to gasoline and stress of closure induced the activation of MT-I gene expression by distinct or overlapping signals from STAT, ARE and GRE pathways owing to the creation of new redox and cytokine status in injured tissues and owing to the presence of glucocorticoids (GCs), which commonly exert the effects on the transcription of genes involved in metabolic axes and participate in the maintenance of systemic immunologic homeostasis (Sato et al., 1996; Besedovsky and del Rey, 2006).

Not surprisingly, we also found that gasoline components and stress conditions induced a profound imbalance of biometal homeostasis in organs that are related with the processes of absorption, distribution, biotransformation, and excretion of toxic metabolites. The mechanisms are unknown, but the data imply that MTs through binding and redisplacement of essential divalent cations may induce a significant deprivation of

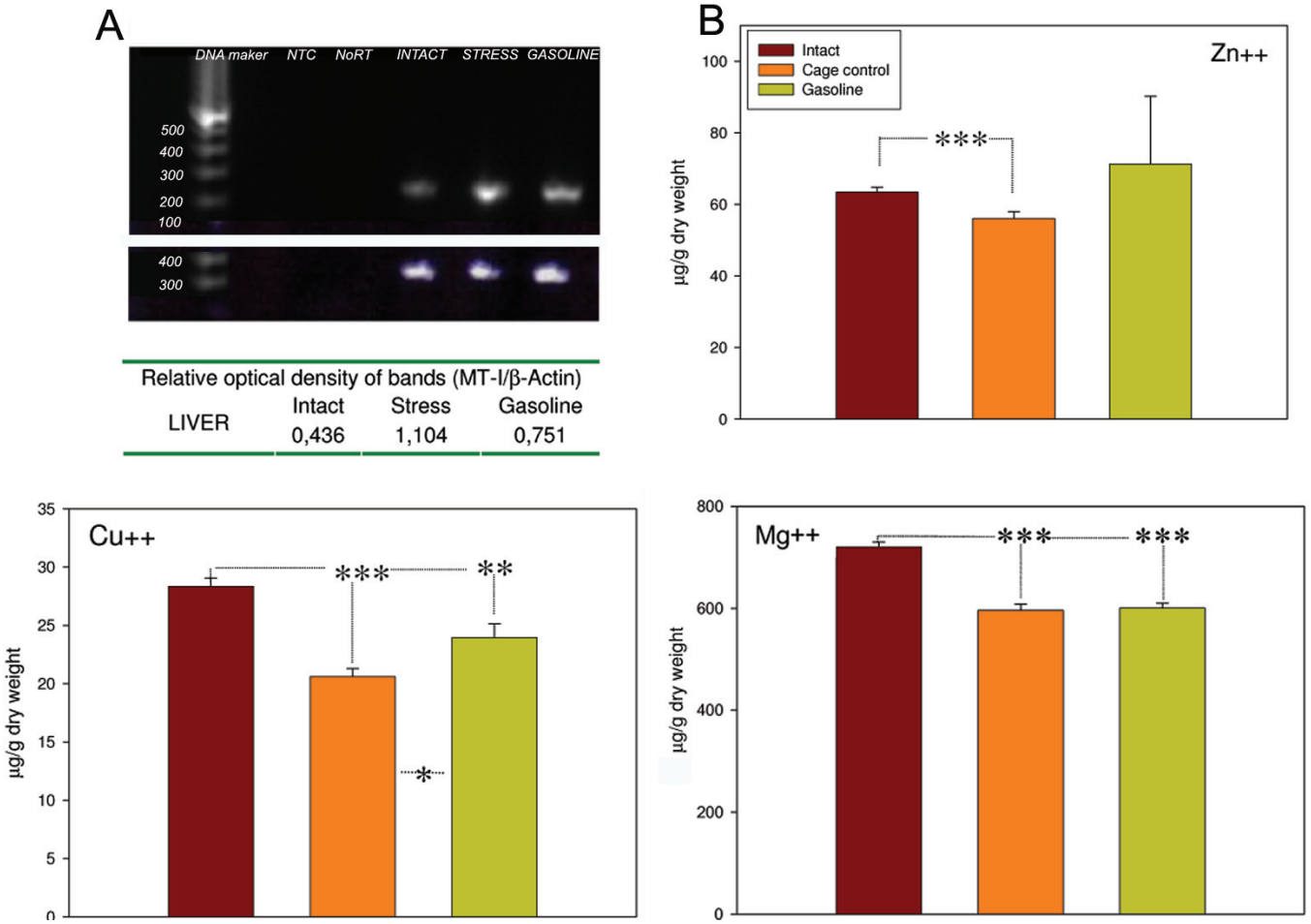


Fig. 5. Expression of MT-I gene **(A)** and metal tissue content **(B)** in the LIVER of three groups of mice: 1) untreated mice ("intact"), 2) mice closed in the metabolic chamber, ventilated with fresh air ("cage control") and 3) mice closed in the metabolic chamber, ventilated with gasoline vapor ("gasoline"). In RT-PCR **(A)** the data obtained with all additives but without cDNA (NTC) and those without the reverse transcription (NoRT) are also shown. The ß-actin mRNA was used as a loading control. The Table shows the ratio of MT-I/ß-actin mRNA, calculated from the optical density of bands on the Northern blot membranes. Analyses were done with tissue samples, prepared as a pool from 3 mice. In **B)** the metal concentrations are expressed as micrograms per gram on dry organ weight. The values represent the mean ± SE of four to six mice. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Ca and Mg, which subsequently may adversely affect several other aspects of cellular functions. In this sense, owing to the higher Cu-MT binding affinity relative to Zn (Ba et al., 2009) the exchange of Zn for Cu in MTs should be taken into account, since this may occur in conditions of Cu overload after structural or functional injuries on the BBB and the blood-cerebrospinal fluid barrier (Zheng and Monnot, 2012). Moreover, since the sequestration of copper by metallothioneins in mucosal cells may account for the decrease in copper absorption, we can speculate that this mechanism contributed to the deprivation of copper in tissues of mice exposed to gasoline vapor, but since the cytoprotective actions of MTs far outweigh the side effects of their actions (Moffatt and Denizeau, 1997; Coyle et al., 2002; Cousins et al., 2006; Hidalgo et al., 2006; Mocchegiani et al., 2006a; Penkowa, 2006; Nielsen et al., 2007; Thirumoorthy et al., 2007), the effects on other copper transporters and chaperones in the complex copper-trafficking pathways (Bertinato and L'Abbe´, 2004) should be taken into account. Additionally, it must be emphasized that the response to metal ions depends on the metal concentrations and metal binding affinities, on intrinsic properties of a particular metalloregulatory proteins, on the distribution and localization of metal transporters, and on other factors that control metal specificity in these systems (Waldron et al., 2009; Reyes-Caballero et al., 2011).

Mechanisms need to be clarified, but gasoline- and stress-induced copper deficiency might have serious consequences since copper, as an essential micronutrient, affects a number of metalloenzymes, including Cu/Zn superoxide dismutase (anti-oxidant defense), cytochrome c oxidase (mitochondrial respiration), lysyl oxidase (development of connective tissue), tryrosinase (melanin biosynthesis), ceruloplasmin (iron homeostasis), hephaestin (intestinal iron efflux), dopamine ß-hydroxylase (catecholamine production), and peptidylglycine α-amidating monooxygenase (peptide hormone processing) (Bertinato and L'Abbe´, 2004). Besides, since Cu can displace Zn from its normal binding site on p53, resulting in abnormal protein folding and disruption of p53 function, the imbalances in Cu and Zn levels may lead to a higher prevalence of p53 mutations (Formigari et al., 2013).

Similarly, our data point to the possibility that the deprivation of magnesium contributed to the gasoline intoxication and adverse effects of stress, since it is involved in a wide range of biochemical reactions that are crucial to cell proliferation, differentiation, angiogenesis, and apoptosis (Wolf et al., 2003). Moreover, in cooperation with Zn^{2+} it stabilizes the structure of nucleic acids and serves as a co-factor of the enzymes of nucleic acid metabolism, assisting in DNA replication and repair, in gene expression, and in maintenance of the genomic stability, ensuring also the prevention against carcinogenesis (Hartwig, 2001; Wolf et al., 2007). It should be also emphasized that low concentrations of Mg contribute to the creation of a pro-inflammatory environment, stimulating the production of nitric oxide (NO) and the synthesis of the pro-inflammatory cytokines IL-1 and IL-6 which enhance vascular permeability, endothelial migration and proliferation and the expression of matrix metalloproteases (MMP)-2 and -9 in the vasculature (Wolf et al., 2007). Therefore, the magnesium deficit in humans is often associated with several acute and chronic illnesses, such as myocardial infarction, dyslipidemia, hypertension, metabolic syndrome, congestive heart failure, type II diabetes mellitus, tumor growth and progression (Wolf et al., 2003), as well as with the increased inflammatory burden and accelerated aging (Innerarity, 2000; Weglicki, 2012). Besides, providing some support to our data (Fig. 2) it was also reported that hypomagnesemia might particularly affect the activity of hippocampal neurons (Furukawa et al., 2009), where after chronic stress exposure the synaptic excitation of dentate granule cells was also observed (Karst and Joels, 2003). Similarly, in acute stressor states, a dyshomeostasis of several other trace elements was described, provoked by the coordinated translocation of cations to injured tissues, showing that homeostatic activation of the hypothalamic-pituitary-adrenal axis (Weber et al., 2010; Whitted et al., 2010) and other endocrine glands, such as parathyroid, thyroid and pancreas (Durlach, and Durlach, 1984) are crucial for the maintenance of biometal balance.

In this sense, our data imply that systemic Cu and Mg deprivation may be an important and under-recognized sign of intoxication with volatile pollutants, which in industrialized countries probably contributes to the development of neurological and cognitive dysfunctions (Cairney et al., 2004) and other forms of neurogenic inflammation and disorders, such as central nervous system autoimmunity, Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, prion disease, occipital horn syndrome or genetic disorders (Kramer et al., 2009; Skjorringe et al., 2012; Massilamany et al., 2013). Additionally, our findings in the liver (Fig. 5A,B) suggest that these changes probably contributed to the induction of adverse health effects of toxic pollutants and stress, since it is well known that particularly the hepatic MT I/II and cytokines participate in maintenance of zinc ion homeostasis and in the creation of specific cytokine microenvironment that governs the local balance between tolerance and immunity (Knolle and Gerken, 2000) and regulate the immune-neuroendocrine interactions both at central and peripheral levels (Mocchegiani et al., 2006b). In this context it was repeatedly shown that hepatic Zn- and Cu-bound MT were involved in the protection of animals from hepatotoxicity of several chemicals, such as ethanol, carbon tetrachloride, acetaminophen, cadmium and mercury, as well as from other types of injuries, including inflammatory, allergic, and oxidative damage (Nordberg and Nordberg, 2000; Stankovic et al., 2003; Tapiero and Tew, 2003; Weber et al., 2010), which have been shown to induce the upregulation of MT gene

transcription and synthesis of MT-I/II proteins after activation of distinct or overlapping transduction pathways (Sato et al., 1996; Hernandez et al., 2000; Nishimura et al., 2001; Kotani et al., 2012).

In conclusion, enlarging our previously reported data, the present study demonstrates that repeated exposure of mice to gasoline vapor enhances not only the expression of MT I/II proteins (Grebic et al., 2007), but also the MT-I mRNA and induces a marked copper and magnesium deprivation in tissues that receive a strong respiratory and olfactory input (lungs and brain) or participate in gasoline degradation and elimination (kidney and liver). Furthermore, showing that the repeated stress, provoked by the closure of mice into the metabolic cage has similar consequences we confirm that the phylogenetically ancient mechanisms associated with MT-induced cytoprotection may be activated by multiple factors and similar transcriptional pathways.

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Conflict of interest statement. The authors declare that there are no conflicts of interest.

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