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Mieczyslaw Pokorski *Editor*

Oxidative Stress and Cardiorespiratory Function

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Mieczyslaw Pokorski
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Oxidative Stress and Cardiorespiratory Function

Editor
Mieczyslaw Pokorski
Institute of Psychology
University of Opole
Poland

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Preface

This is a new book series entitled Neuroscience and Respiration, a subseries of Springer's renowned Advances in Experimental Medicine and Biology. The book volumes present contributions by expert researchers and clinicians in the field of pulmonary disorders. The chapters provide timely overviews of contentious issues or recent advances in the diagnosis, classification, and treatment of the entire range of pulmonary disorders, both acute and chronic. The texts are thought as a merger of basic and clinical research dealing with respiratory medicine, neural and chemical regulation of respiration, and the interactive relationship between respiration and other neurobiological systems such as cardiovascular function or the mind-to-body connection. In detail, topics include lung function, hypoxic lung pathologies, epidemiology of respiratory ailments, sleep-disordered breathing, imaging, and biomarkers. Other needful areas of interest are acute respiratory infections or chronic inflammatory conditions of the respiratory tract, exemplified by asthma and chronic obstructive pulmonary disease (COPD), or those underlain by still unknown factors, such as sarcoidosis, respiratory allergies, lung cancer, and autoimmune disorders involving the respiratory system.

The prominent experts will focus their presentations on the leading-edge therapeutic concepts, methodologies, and innovative treatments. Pharmacotherapy is always in the focus of respiratory research. The action and pharmacology of existing drugs and the development and evaluation of new agents are the heady area of research. Practical, data-driven options to manage patients will be considered. The chapters will present new research regarding older drugs, performed from a modern perspective or from a different pharmacotherapeutic angle. The introduction of new drugs and treatment approaches in both adults and children will be discussed. The problem of drug resistance, its spread, and deleterious consequences will be dealt with as well.

Lung ventilation is ultimately driven by the brain. However, neuropsychological aspects of respiratory disorders are still mostly a matter of conjecture. After decades of misunderstanding and neglect, emotions have been rediscovered as a powerful modifier or even the probable cause of various somatic disorders. Today, the link between stress and respiratory health is undeniable. Scientists accept a powerful psychological connection that can directly affect our quality of life and health span. Psychological approaches,

by decreasing stress, can play a major role in the development and course of respiratory disease, and the mind-body techniques can aid in their treatment.

Neuromolecular aspects relating to gene polymorphism and epigenesis, involving both heritable changes in the nucleotide sequence and functionally relevant changes to the genome that do not involve a change in the nucleotide sequence, leading to respiratory disorders will also be tackled. Clinical advances stemming from basic molecular and biochemical research are but possible if the research findings are "translated" into diagnostic tools, therapeutic procedures, and education, effectively reaching physicians and patients. All that cannot be achieved without a multidisciplinary, collaborative, "bench-to-bedside" approach involving both researchers and clinicians, which is the essence of the book series Neuroscience and Respiration.

The societal and economic burden of respiratory ailments has been on the rise worldwide leading to disabilities and shortening of life span. COPD alone causes more than three million deaths globally each year. Concerted efforts are required to improve this situation, and part of those efforts are gaining insights into the underlying mechanisms of disease and staying abreast with the latest developments in diagnosis and treatment regimens. It is hoped that the books published in this series will fulfill such a role by assuming a leading role in the field of respiratory medicine and research and will become a source of reference and inspiration for future research ideas.

Titles appearing in Neuroscience and Respiration will be assembled in a novel way in that chapters will first be published online to enhance their speedy visibility. Once there are enough chapters to form a book, the chapters will be assembled into complete volumes. At the end, I would like to express my deep gratitude to Mr. Martijn Roelandse and Ms. Tanja Koppejan from Springer's Life Sciences Department for their genuine interest in making this scientific endeavor come through and in the expert management of the production of this novel book series.

Opole, Poland Mieczyslaw Pokorski

Volume 1: Oxidative Stress and Cardiorespiratory Function

Cardiorespiratory function is prominently affected by oxidative stress. Cigarette smoking is the archetype of oxidative and nitrative stress and free radical formation. New adverse effects of smoking keep on propping up in research. The chapters provide a comprehensive view of new developments in this area regarding cardiovascular and lung function and muscle catabolism. Alterations in inflammatory cytokines and proteins as well as degradation of muscle proteins due to smoking, by far unrecognized, caused by oxidative stress are also presented. Much less is known about the effect of cognitive stress on vagally mediated cardiorespiratory function and, surprisingly, on vagal immune pathway. The experimental studies also show that clinically important meconium aspiration syndrome contains an oxidative trait, which is amenable to antioxidative treatment. This volume creates a source of information on the damaging role of oxidative stress in cardiorespiratory function that has by far not been available.

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Peroxynitrite Induces Degradation of Myosin Heavy Chain via p38 MAPK and Muscle-Specific E3 Ubiquitin Ligases in C2 Skeletal Myotubes

O. Rom, S. Kaisari, A.Z. Reznick, and D. Aizenbud

Abstract

Oxidative stress and inflammation play an important role in the catabolism of skeletal muscles. Recently, cigarette smoke (CS) was shown to stimulate muscle catabolism by activation of p38 MAPK and up-regulation of the muscle-specific E3 ubiquitin ligases (E3s) atrogin-1 and MuRF1 which are over-expressed during muscle atrophy. Peroxynitrite ($ONOO^-$), an oxidative ingredient of CS, also produced during oxidative stress and inflammation, was previously shown to induce ubiquitination and degradation of muscle proteins. To investigate the involvement of p38 MAPK and the muscle-specific E3s in $ONOO^-$-induced muscle catabolism, C2 myotubes, differentiated from a myoblast cell line, were exposed to $ONOO^-$ (25 μM) in a time-dependent manner. Following exposure, degradation of myosin heavy chain (MyHC) and actin, activation of p38 MAPK, and levels of atrogin-1 and MuRF1 were studied by Western blotting. Peak phosphorylation of p38 MAPK was observed at 1 h of $ONOO^-$ exposure. $ONOO^-$ caused a significant increase in the levels of atrogin-1 and MuRF1. In accordance, a significant decrease in MyHC levels was observed in a time-dependent manner. These findings support previous studies in which the catabolic effects of $ONOO^-$ were shown. In addition, $ONOO^-$ was demonstrated to induce degradation of muscle proteins by activation of p38 MAPK and up-regulation of the muscle-specific E3s atrogin-1 and MuRF1.

Keywords

Atrogin-1 • Cigarette smoke • Inflammation • Muscle catabolism • MuRF1 • Oxidative stress • p38 MAPK • Reactive nitrogen species

O. Rom, S. Kaisari, and A.Z. Reznick (✉)
Department of Anatomy and Cell Biology, Rappaport Faculty of Medicine, Technion – Israel Institute of Technology, Efron St., P.O. Box: 9649, Bat Galim, Haifa 31096, Israel
e-mail: reznick@tx.technion.ac.il

D. Aizenbud
Department of Anatomy and Cell Biology, Rappaport Faculty of Medicine, Technion – Israel Institute of Technology, Efron St., P.O. Box: 9649, Bat Galim, Haifa 31096, Israel

Department of Orthodontic and Craniofacial Anomalies, Rambam Health Care Campus, Haifa, Israel

1 Introduction

The effects of reactive oxygen species (ROS) and reactive nitrogen species (RNS) on atrophy of skeletal muscle have been studied extensively. Oxidative and nitrative stress, in which high levels of ROS and RNS are produced, were suggested to promote muscle atrophy by regulation of muscle proteolysis (Rom et al. 2012a; Sukhanov et al. 2011; Meng and Yu 2010; Supinski and Callahan 2007). Degradation of skeletal muscle proteins is mainly regulated by the ubiquitin-proteasome system (UPS). The E3 ubiquitin-ligating enzymes (E3s) of the UPS are responsible for determining which proteins are targeted for degradation by the proteasome (Rom et al. 2012b; Meng and Yu 2010). Muscle atrophy F-box protein (atrogin-1) and muscle RING finger-1 protein (MuRF1) are muscle-specific E3s that are over-expressed in various conditions of muscle atrophy. These E3s target specific muscle proteins for ubiquitination and subsequent degradation by the proteasome during skeletal muscle atrophy (Foletta et al. 2011).

ROS and RNS can induce cellular damage through oxidation and nitration of biomolecules, including DNA and proteins which can affect signal transduction pathways and cellular processes (Yeo et al. 2008; Bar-Shai and Reznick 2006a, b). ROS were previously shown to promote muscle catabolism by affecting the UPS. Li et al. (2003) found that exposure of skeletal myotubes to hydrogen peroxide (H_2O_2) stimulated ubiquitin conjugation to muscle proteins and up-regulation of the muscle-specific E3s atrogin-1 and MuRF1. In accordance, increased catabolism of muscle proteins and decreased expression of myosin heavy chain (MyHC) were found in C2C12 myotubes treated with H_2O_2 (Gomes-Marcondes and Tisdale 2002).

Peroxynitrite ($ONOO^-$) is a potent oxidizing and nitrating RNS that promotes the development of various pathologies (Bar-Shai and Reznick 2006a, b). Increased production of $ONOO^-$ occurs during conditions of inflammation in which excess nitric oxide (NO) is generated by neutrophils and phagocytes. Excess NO reacts with superoxide and forms various RNS, including $ONOO^-$ (Yeo et al. 2008; Hasnis et al. 2007). The role of $ONOO^-$ in muscle proteolysis was previously studied by Bar-Shai and Reznick (2006a, b). It was found that exposure of L6 skeletal myotubes to $ONOO^-$ caused degradation of muscle-specific proteins that was mediated by ubiquitination of muscle proteins. In addition, $ONOO^-$ exposure resulted in a non-transient activation of nuclear factor-κB (NF-κB) in skeletal muscle cells.

Another major source of exposure to $ONOO^-$ is cigarette smoke (CS). CS contains numerous of ROS and RNS, including H_2O_2, superoxide, and NO. These ROS and RNS can enter the bloodstream and cause macromolecular damage (Rom et al. 2012a; Csiszar et al. 2009). CS is considered the main source of human exposure to NO. Superoxide and NO from CS can react and produce $ONOO^-$ (Hasnis et al. 2007). Recently, we have shown that exposure of C2 skeletal myotubes to CS stimulated muscle catabolism by increased oxidative stress, phosphorylation of p38 mitogen-activated protein kinase (MAPK), activation of the NF-κB pathway and up-regulation of the muscle-specific E3s atrogin-1 and MuRF1 (Kaisari et al. 2013; Rom et al. 2013a). These findings led us to propose a cellular model of CS-induced catabolism of skeletal muscle. According to this model, components of CS enter the bloodstream and reach skeletal muscle of smokers. In skeletal muscle, CS components lead to increased oxidative stress that induces the activation of intracellular signaling pathways including the p38 MAPK and the NF-κB pathways. Activation of these signaling pathways results in up-regulation of muscle-specific E3s leading to increased breakdown of muscle proteins (Rom et al. 2012b).

Various component of CS such as aldehydes, ROS, and RNS have the potential to promote catabolism of skeletal muscle (Rom et al. 2012a). For instance, it was recently found that exposure of C2 myotubes to acrolein, a toxic unsaturated aldehyde present in high levels in CS, stimulated muscle catabolism by activation

of the p38 MAPK pathway and up-regulation of muscle-specific E3s (Rom et al. 2013b). H_2O_2, also present in CS, was shown to induce breakdown of muscle proteins by activation of the UPS and over-expression of atrogin-1 and MuRF1 (Li et al. 2003; Gomes-Marcondes and Tisdale 2002). The RNS $ONOO^-$ is also present in CS and was previously shown to stimulate degradation of muscle proteins (Bar-Shai and Reznick 2006a, b). However, the effects of $ONOO^-$ on the p38 MAPK pathway and muscle-specific E3s during muscle catabolism have yet to be studied. Therefore, the present study aims to investigate the effects of $ONOO^-$ on muscle catabolism as mediated by p38 MAPK and the muscle-specific E3s atrogin-1 and MuRF1.

2 Methods

The study was approved by an institutional Ethics Committee.

2.1 Cell Culture

The C2 cell line of mouse myoblasts was a generous gift from Prof. Eyal Bengal (Rappaport Faculty of Medicine, Technion, Israel). C2 myoblasts were grown in 24 wells and 100 mm plates at 37 °C in humidified 95 % air and 5 % CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % heat-inactivated fetal bovine serum, 1 % penicillin/streptomycin and 1 % L-glutamine (Biological Industries, Bet HaEmek, Israel). For differentiation into myotubes, myoblasts were plated in 0.1 % gelatin-coated plates and were grown to 90 % confluence. Then, medium was replaced by DMEM, supplemented with 2 % heat-inactivated horse serum, 1 % penicillin/streptomycin, and 1 % L-glutamine (Biological Industries, Bet HaEmek, Israel), which was replaced every 48 h for 6 days until cell fusion and formation of multi-nucleated myotubes was achieved. A successful cell differentiation was determined by expression of MyHC as measured by Western blotting.

2.2 Cell Treatments

Experiments were held on Day 7 of differentiation when the cells had completed their differentiation into myotubes. The culture medium was replaced by fresh medium at the beginning of each experiment. Stock solutions of $ONOO^-$ (Cayman Chemical Company, Ann Arbor, MI) were prepared in 0.3 M NaOH, due to its stability in alkaline pH. $ONOO^-$ was added to the culture medium at a final concentration of 25 μM followed by incubation for increasing periods at 37 °C. Control myotubes were treated with fresh medium without $ONOO^-$.

2.3 Cell Viability

To assess the effects of $ONOO^-$ on the viability of myotubes, 2×10^5 cells were seeded in 24-well plates and grown to 90 % confluence for differentiation into myotubes. On Day 7 of differentiation, myotubes were treated with 25 μM $ONOO^-$ and incubated for increasing periods. Following incubation with $ONOO^-$, viability of myotubes was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich, St. Louis, MO, USA) as previously described (Rom et al. 2013a, b). For each experiment, OD was measured in triplicate at 570 nm using ELISA reader (Biochrom Anthos Zenyth, Cambridge, UK). The viability was expressed as a percentage of the values of control myotubes (corresponding to 100 %).

2.4 Western Blot Analysis

Following $ONOO^-$ exposure, myotubes were lysed for cytosolic proteins as previously described (Kaisari et al. 2013; Rom et al. 2013a, b). Then, 20 μg of cytosolic proteins were loaded in each lane, separated by SDS-PAGE and later transferred to nitrocellulose membranes. Membranes were blocked with 5 % non-fat milk powder in TBS-T (0.125 % Tween) (Sigma-Aldrich,

St. Louis, MO, USA) for 1 h and exposed to primary antibodies overnight at 4 °C. Primary antibodies were MyHC (1:1,000), MAFbx/atrogin-1 (1:1,000), MuRF1 (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), actin (1:4,000) (Millipore, Billerica, MA, USA), p38 MAPK (1:1,000), and phosphorylated p38 MAPK (1:1,000) (R&D Systems, Minneapolis, MN, USA). The next day, membranes were washed with TBS-T followed by 1 h incubation at ambient temperature with the appropriate secondary antibodies (Jackson Immuno-Research, West Grove, PA, USA). Detection was performed by enzyme-linked chemiluminescence (ECL) (Biological Industries, Bet HaEmek, Israel) using Image-Quant LAS 4000 digital imager system (GE Healthcare, Chalfont St. Giles, Bucks, UK). Protein quantities were determined by densitometry and analyzed using Total Lab Software V2006C (Nonlinear Dynamics, Newcastle, UK).

2.5 Protein Loading Control

Since the effects of $ONOO^-$ on the main contractile muscle proteins, MyHC and actin, were investigated in the present study, actin could not be used as a housekeeping protein for loading control. Alternatively, quantification of total proteins by reversible Ponceau staining was used for protein loading control as previously described (Kaisari et al. 2013; Rom et al. 2013a, b; Romero-Calvo et al. 2010). Briefly, membranes were rinsed in Ponceau S solution (Bio-Rad, Hercules, CA, USA) for 10 min before antibody probing, followed by a brief rinse in DDW until bands were clearly visible. In each lane, total protein quantities were determined by densitometry and used for normalization of ECL detected proteins. This method was validated as an alternative to actin blotting (Romero-Calvo et al. 2010).

2.6 Statistical Analysis

Results are expressed as means ± SE of 3 independent experiments. A *t*-test and one-way ANOVA followed by Tukey's or Dunnett tests were used for statistical analysis. $p < 0.05$ was considered statistically significant. Statistical analysis was performed by SPSS 17 software (SPSS Inc., Chicago, USA).

3 Results

3.1 Effects of $ONOO^-$ on Viability of Myotubes

Differentiated myotubes were exposed to 25 μM $ONOO^-$ and incubated for 1, 2, and 6 h. Following incubation, viability of myotubes was assessed by the MTT assay as described earlier in the Methods section. Exposure of myotubes to 25 μM $ONOO^-$ caused a time-dependent decrease in cell viability. However, compared with control, viability of myotubes remained higher than 80 % and the change in viability was not significant in all time points examined (Fig. 1). Therefore, experiments were held with $ONOO^-$ at the concentration of 25 μM for up to 6 h, the time in which $ONOO^-$ was found to be non-cytotoxic.

3.2 $ONOO^-$ Activates p38 MAPK

p38 MAPK is a stress-activated kinase that responds to various stimuli including oxidative stress. In addition, p38 MAPK is a key mediator of catabolic signaling in skeletal muscles and was previously shown to mediate the

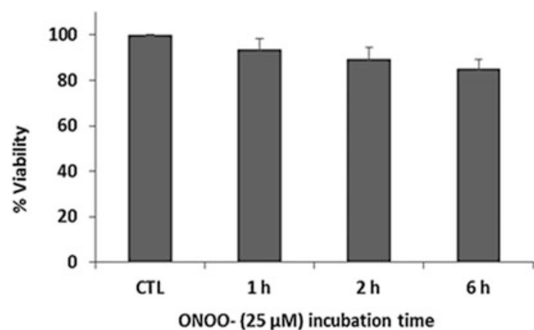

Fig. 1 Effects of $ONOO^-$ on viability of myotubes. Myotubes were exposed to 25 μM $ONOO^-$ for 1, 2, and 6 h. Untreated myotubes served as control (CTL). Following incubation, viability of myotubes was assessed by the MTT assay. Results are means ± SE of 3 independent experiments.

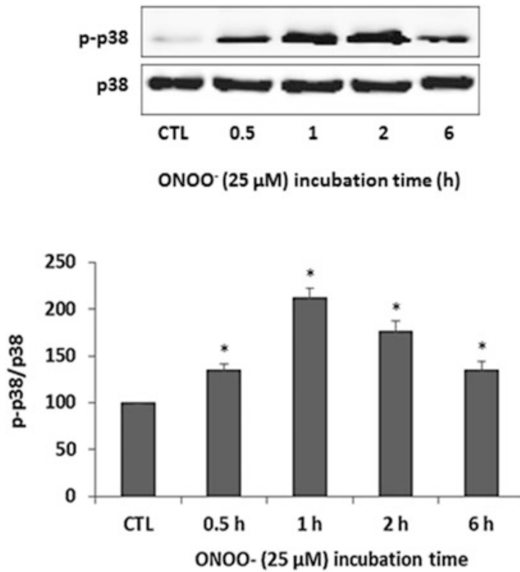

Fig. 2 ONOO$^-$ activates p38 MAPK. Myotubes were exposed to 25 μM ONOO$^-$ for 0.5, 1, 2, and 6 h. Untreated myotubes served as control (CTL). Following incubation, cell lysates were subjected to Western blot analysis with antibodies against p38 MAPK (p38) and phosphorylated p38 MAPK (p-p38). Protein levels of p-p38 and p38 were quantified by densitometry and the values of p-p38 were normalized to p38 and compared with CTL. Results are means ± SE of 3 independent experiments; $^*p < 0.05$ *vs*. CTL

up-regulation of the muscle-specific E3s (Rom et al. 2012b, 2013a, b; Li et al. 2005). Therefore, it was of interest to study the effects of ONOO$^-$ on p38 MAPK in skeletal myotubes. Myotubes were exposed to 25 μM ONOO$^-$ for increasing periods and activation of p38 MAPK was studied by examining the levels of phosphorylated p38 (p-p38) relative to non-phosphorylated p38. Significant phosphorylation of p38 was observed from 0.5 h until 6 h of ONOO$^-$ exposure and peak phosphorylation was evident at 1 h of exposure (Fig. 2).

3.3 ONOO$^-$ Upregulates the Muscle-Specific E3s MuRF1 and Atrogin-1

ONOO$^-$ was previously shown to cause degradation of muscle proteins which was mediated by protein ubiquitination (Bar-Shai and Reznick 2006a). These findings suggest that ONOO$^-$

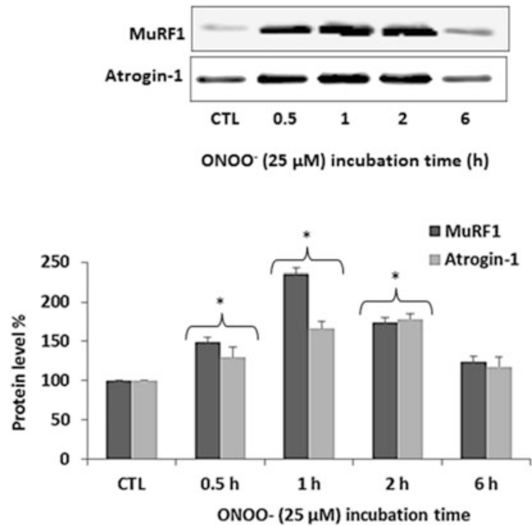

Fig. 3 ONOO$^-$ up-regulates MuRF1 and atrogin-1. Myotubes were exposed to 25 μM ONOO$^-$ for 0.5, 1, 2 and 6 h. Untreated myotubes served as control (CTL). Following incubation, cell lysates were subjected to Western blot analysis with antibodies against MuRF1 and atrogin-1. Protein levels were normalized by total protein densitometry detected by Ponceau S staining and expressed relative to the corresponding value of CTL. Results are expressed as mean ± SE of 3 independent experiments. $^*p < 0.05$ *vs*. CTL

can activate the UPS. The muscle-specific E3s MuRF1 and atrogin-1 play a major role in the UPS by determining which muscle proteins are targeted for degradation by the proteasome (Foletta et al. 2011; Meng and Yu 2010). To study the effects of ONOO$^-$ on the expression of the above muscle-specific E3s, myotubes were exposed to 25 μM ONOO$^-$ for increasing periods and protein levels of MuRF1 and atrogin-1 were examined by Western blotting as described earlier in the Methods section. Starting from 0.5 h of exposure, ONOO$^-$ caused a significant increase in protein levels of both MuRF1 and atrogin-1. By 6 h of ONOO$^-$ exposure, protein levels of MuRF1 and atrogin-1 decreased and were not significantly different from the control (Fig. 3).

3.4 ONOO$^-$ Induces a Time-Dependent Degradation of MyHC

To study the effects of ONOO$^-$ on the main contractile muscle proteins, myotubes were

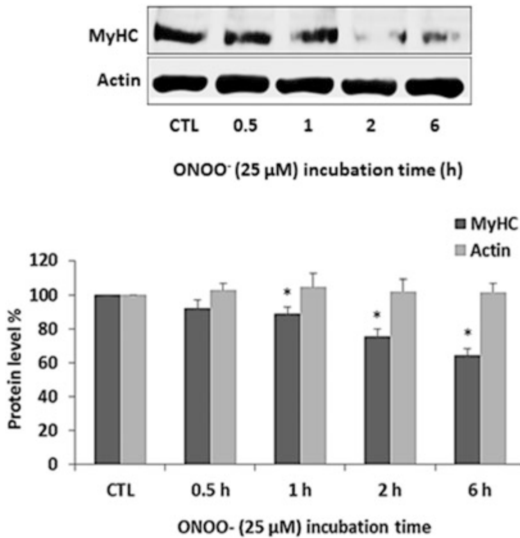

Fig. 4 $ONOO^-$ induces degradation of MyHC. Myotubes were exposed to 25 μM $ONOO^-$ for 0.5, 1, 2, and 6 h. Untreated myotubes served as control (CTL). Following incubation, cell lysates were subjected to Western blot analysis with antibodies against MyHC and actin. Protein levels were normalized to total protein and expressed relative to the corresponding value of CTL. Results are means ± SE of 3 independent experiments. $^*p < 0.05$ *vs.* CTL

exposed to 25 μM $ONOO^-$ for increasing periods and protein levels of MyHC and actin were examined by Western blotting as described earlier in the Methods section. A significant decrease in protein level of MyHC was evident from 1 h of $ONOO^-$ exposure. The level of MyHC decreased as the time of exposure to $ONOO^-$ increased (Fig. 4). No significant change in the level of actin was found.

4 Discussion

The present study investigated the effects of $ONOO^-$ on muscle catabolism as mediated by p38 MAPK and the muscle-specific E3s atrogin-1 and MuRF1. It was found that exposure of C2 skeletal myotubes to $ONOO^-$ at the concentration of 25 μM stimulated a time-dependent degradation of MyHC that was mediated by phosphorylation of p38 MAPK and up-regulation of the muscle-specific E3s atrogin-1 and MuRF1.

It was previously shown that exposure of skeletal muscle cells to $ONOO^-$ resulted in a loss of the muscle-specific proteins MyHC and telethonin, which was mediated by a constitutive activation of NF-κB and ubiquitination of muscle proteins (Bar-Shai and Reznick 2006a, b). Moreover, exposure of rat muscle fibers to $ONOO^-$ resulted in a reduced maximum force of slow-twitch fibers and cross-linking of MyHC1 appearing as larger protein complexes (Dutka et al. 2011). Also, exposure of skeletal muscle S1-myosin ATPase (S1) to SIN-1 (3-morpholinosydnonimine), which mimics the effects of chronic exposure to $ONOO^-$, caused inhibition, oxidation, and a partial unfolding of S1 (Tiago et al. 2006). In addition, treatment of various proteins with $ONOO^-$ resulted in enhanced proteolytic susceptibility toward degradation by the proteasome, suggesting that $ONOO^-$ can react with proteins and lead to their recognition and degradation by proteasome (Grune et al. 1998). The findings from the present study suggest that additional signaling pathways of muscle catabolism are activated in skeletal muscle in response to $ONOO^-$ exposure.

p38 MAPK is a stress-activated protein kinase that is known to be phosphorylated in response to oxidative stress. In addition, activation of p38 MAPK was demonstrated in various pro-catabolic conditions such as limb immobilization, type 2 diabetes, aging, and exposure to CS (Kaisari et al. 2013; Rom et al. 2012b, 2013a; Li et al. 2003). Therefore, it was of great interest to study the effects of $ONOO^-$ on p38 MAPK in skeletal myotubes. Indeed, a significant phosphorylation of p38 MAPK was observed shortly after exposing myotubes to $ONOO^-$ (Fig. 2). It was previously shown that exposure of C2C12 myotubes to tumor necrosis factor-alpha (TNF-α) or H_2O_2 resulted in up-regulation of atrogin-1, which was blunted by SB203580, a specific inhibitor of p38 MAPK. Therefore, the muscle-specific E3 atrogin-1 was suggested to be a downstream target of p38 MAPK signaling (Li et al. 2003).

The main mechanism of protein degradation during conditions of muscle atrophy is the UPS. Addition of ubiquitin molecules to a protein

substrate requires the action of three enzymes: E1 ubiquitin activating enzyme, E2 ubiquitin-conjugating enzyme, and E3s which play the important role of determining which proteins will be targeted for proteasomal degradation. MuRF1 and atrogin-1 are two muscle-specific E3s that were shown to be up-regulated in various states of muscle catabolism including diabetes, cancer, renal failure, denervation, and exposure to CS and cytokine (Rom et al. 2013a; Foletta et al. 2011; Meng and Yu 2010). Due to the key role of these muscle-specific E3s in muscle catabolism, it was of great importance to study the effects of $ONOO^-$ on their expression. Interestingly, it was found that exposure of skeletal myotubes to $ONOO^-$ up-regulated both MuRF1 and atrogin-1, which was accompanied by degradation of MyHC, the main contractile muscle protein.

A major source of human exposure to $ONOO^-$ is CS (Rom et al. 2012a; Hasnis et al. 2007). $ONOO^-$ from CS can penetrate into the blood circulation and CS can stimulate increased generation of $ONOO^-$ within the cells (Csiszar et al. 2009). Yamaguchi et al. (2007) showed that sodium peroxynitrite and CS extract, which contains stable ROS and $ONOO^-$-like reactants, can penetrate into the blood through the lung alveolar wall and cause oxidative vascular injury. Also, exposure of rats to gas phase CS resulted in increased serum levels of 3-nitrotyrosine, which may indicate protein nitration by $ONOO^-$ (Yamaguchi et al. 2007; Bar-Shai and Reznick 2006a, b; Tiago et al. 2006). In addition, Barreiro et al. (2012) found that chronic exposure of mice to CS caused oxidative modifications to muscle proteins, including increased levels of 3-nitrotyrosine in the diaphragm and gastrocnemius muscles. The above studies suggest that $ONOO^-$ from CS is capable of reaching skeletal muscles and induce oxidative damage to proteins.

In a similar manner to $ONOO^-$ exposure, CS exposure to C2 skeletal myotubes was recently shown to stimulate muscle catabolism by activation of p38 MAPK and up-regulation of the muscle-specific E3s atrogin-1 and MuRF1 (Rom et al. 2013a). Various ROS and aldehydes which are present in CS can promote catabolism of skeletal muscle. For instance, H_2O_2, one of the ROS found in CS, was found to up-regulate atrogin-1 and MuRF1 and induce muscle breakdown in skeletal myotubes (Li et al. 2003; Gomes-Marcondes and Tisdale 2002). Also, we have recently shown that acrolein, a toxic unsaturated aldehyde present in CS, stimulated muscle catabolism by activation of the p38 MAPK pathway and up-regulation of the muscle-specific E3s in C2 myotubes (Rom et al. 2013b). Thus, our findings suggest that RNS from CS such as $ONOO^-$ may also have a catabolic effect on skeletal muscles.

In conclusion, $ONOO^-$ was found to induce a time-dependent degradation of MyHC that was mediated by activation of the p38 MAPK pathway and up-regulation of the muscle-specific E3s atrogin-1 and MuRF1 in skeletal myotubes. These findings are in line with previous studies that demonstrated the deleterious effects of $ONOO^-$ on skeletal muscle. This study provides additional cellular mechanisms that may explain the catabolic effects of $ONOO^-$ on skeletal muscle during condition of increased exposure to this RNS including inflammation, oxidative stress, and exposure to CS.

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Alterations in the Coagulation System of Active Smokers from the Ludwigshafen Risk and Cardiovascular Health (LURIC) Study

G. Delgado, R. Siekmeier, T.B. Grammer, B.O. Boehm, W. März, and M.E. Kleber

Abstract

Smoking is an important and preventable risk factor of cardiovascular diseases with effects on blood coagulation. Our aim was to analyze the influence of smoking on coagulation parameters. Concentrations or activities of blood coagulation factors were compared in 777 active smokers and 1,178 lifetime non-smokers of the Ludwigshafen Risk and Cardiovascular Health (LURIC) study. The association with mortality was examined using Cox regression. The findings show that AS had a tendency toward thrombosis. They displayed significantly higher values for fibrinogen, soluble fibrinogen, factor XIII, and tissue factor pathway inhibitor; whereas FVII, FVIII, FXII, von Willebrand factor (vWF), and thrombomodulin were decreased. The Cox regression analysis showed fibrinogen, FVIII, vWF, thrombomodulin, and tissue factor pathway inhibitor to be independent risk factors for mortality in active smokers with hazard ratios of 1.16 (95 % CI: 1.02–1.31), 1.40 (1.22–1.59), 1.37 (1.22–1.56), 1.19 (1.07–1.31), and 1.22 (1.06–1.40) per increase of one standard deviation. We conclude that active smokers have an increased

G. Delgado, T.B. Grammer, and M.E. Kleber (✉)
Fifth Department of Medicine (Nephrology, Hypertensiology, Endocrinology, Diabetology, Rheumatology), Medical Faculty of Mannheim, Heidelberg University, Theodor-Kutzer-Ufer 1, 68167 Mannheim, Germany
e-mail: marcus.kleber@medma.uni-heidelberg.de

R. Siekmeier
Drug Regulatory Affairs, University of Bonn, Bonn, Germany

B.O. Boehm
Division of Endocrinology, Department of Medicine, University Hospital, Ulm, Germany

LKC School of Medicine, Imperial College London and Nanyang Technological University, Singapore, Singapore

W. März
Fifth Department of Medicine (Nephrology, Hypertensiology, Endocrinology, Diabetology, Rheumatology), Medical Faculty of Mannheim, Heidelberg University, Theodor-Kutzer-Ufer 1, 68167 Mannheim, Germany

Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University Graz, Graz, Austria

Synlab Academy, Synlab Services GmbH, Mannheim, Germany

thrombogenic potential associated with significant changes in the coagulation system. Individual parameters of the coagulation system are independent predictors of mortality. Therefore, parameters of the coagulation system, apart from other risk factors for cardiovascular disease (e.g., lipids or life-style) should be determined for risk prediction in active smokers.

Keywords
Cardiovascular disease • Hemostasis • Mortality • Smoking • Thrombosis

1 Introduction

Smoking is an important and preventable risk factor of cardiovascular diseases, as it is associated with increased inflammation, oxidative stress, thrombosis and atherosclerosis (Danaei et al. 2009). Cigarette smoke exposure seems to interfere with hemostasis through multiple pathways by affecting the functions of endothelial cells, platelets, and coagulation factors (Barua and Ambrose 2013). While a number of different hypotheses has been put forward to explain the harmful effect of tobacco smoke on the coagulation system, e.g., altered clot structure (Pretorius et al. 2010), decreased NO availability (Barua et al. 2001), increased oxidative stress or the generation of procoagulant microvesicles (Li et al. 2010), the underlying pathological mechanisms are still far from being fully understood. Therefore, the aim of our study was to analyze the influence of smoking on the subtle balance of antithrombotic and prothrombotic factors in a cohort with moderate to high risk for coronary heart disease.

2 Methods

2.1 Study Population

The study was approved by the Ethics Committee at the Ärztekammer Rheinland-Pfalz in Germany. All patients signed informed written consent at study onset. The LUdwigshafen RIsk and Cardiovascular Health (LURIC) study is an ongoing prospective study of 3,316 patients of German ancestry who had an indication for coronary angiography and were recruited between June 1997 and May 2001 at the Ludwigshafen Cardiac Center (Winkelmann et al. 2001). All patients were clinically stable (except for acute coronary syndromes). The information on vital status was obtained from local registries. Death certificates were obtained in 97 % of dead participants. Of the persons studied, 523 deaths (26.8 %) occurred during a median follow-up of 10 years. Cardiovascular death included the following categories: sudden death, fatal myocardial infarction, death due to congestive heart failure, death immediately after intervention to treat CHD, fatal stroke, and other causes of death due to CHD. Smoking status was assessed based on a questionnaire and verified by measurement of serum cotinine concentration.

2.2 Laboratory Procedures

Fasting blood samples were taken by venipuncture in the early morning prior to angiography. Aliquots were frozen at −80 °C. Coagulation factors were analyzed at the hemostaseology laboratory of the Ludwigshafen Heart Center at the same day. Endogenous thrombin potential (ETP) was determined from frozen aliquots of baseline samples using Innovance ETP on a BCS coagulation analyzer (Siemens Healthcare Diagnostics Inc., Munich, Germany).

2.3 Statistical Analysis

All continuous variables were checked for normality and the variables showing a skewed

distribution were logarithmically transformed to get a normal distribution. Continuous variables were compared between groups by Student's *t*-test. Associations between categorical variables were examined by chi-square testing. To examine the relationship of coagulation factors with mortality, we calculated hazard ratios (HR) and 95 % confidence intervals (95 % CI) using the Cox proportional hazards model. Multivariable adjustment was carried out as indicated. IBM SPSS Statistics v. 20.0 (IBM Corporation, USA) was used for all analyses.

3 Results

Active smokers (AS) were significantly younger, predominantly male, had a higher concentration of triglycerides, lower concentrations of LDL-C and HDL-C, and showed a higher percentage of coronary artery disease and hypertension compared with lifetime nonsmokers (NS) (Table 1). Their international normalized ratio (INR) of prothrombin time was significantly lower and endogenous thrombin potential (ETP) was higher, which speaks for a higher thrombogenic potential. We therefore investigated the differences in the concentration of coagulation factors in AS and NS.

In AS we observed significantly higher values for fibrinogen, soluble fibrinogen, factor XIII, and tissue factor pathway inhibitor; whereas the concentration of the factors VII, FXII, von Willebrand (vWF), and thrombomodulin were decreased (Table 2). No significant differences were found for the factors, activated prothrombin fragments 1 and 2, and antithrombin III (AT3).

We next examined whether parameters showing different values in AS and NS were associated with mortality by Cox regression analysis adjusted for other cardiovascular risk factors and found fibrinogen, factor VIII, vWF, thrombomodulin, and TFPI to be independent risk factors for mortality in AS with HRs of 1.16 (95 % CI: 1.02–1.31), 1.40 (1.22–1.59), 1.37 (1.22–1.56), 1.19 (1.07–1.31), and 1.22 (1.06–1.40) per increase of one standard deviation, respectively (Table 3). The HRs were similar in NS.

Table 1 Selected anthropometric data of study patients at study onset

	Never-smokers	Active smokers	p
Number	1,178	777	
Smoking (pack years)	0	30.0 (15.0–43.2)	
Age	65.3 ± 10.1	56.2 ± 10.3	<0.001[a]
Male Gender (%)	45.4	77.9	<0.001[b]
BMI	27.4 ± 4.2	27.0 ± 4.2	0.833[a]
LDL-C (mg/dl)	119.1 ± 36.4	117.5 ± 32.1	0.012[a]
HDL-C (mg/dl)	41.2 ± 11.1	36.2 ± 10.2	0.002[a]
Triglycerides (mg/dl)	136 (102–192)	154 (112–218)	<0.001[a]
Coronary artery disease (%)	68.1	80.1	<0.001[b]
Diabetes mellitus (%)	38.3	36	0.314[b]
Hypertension (%)	76.6	63.3	<0.001[b]
Lipid lowering drugs (%)	42.4	52.8	<0.001[b]
INR	1.05 (1.00–1.10)	1.03 (0.98–1.08)	<0.001[a]
ETP (%)	94.4 ± 28.5	99.1 ± 24.5	<0.001[a]

Data are means ± SD or median and 25th + 75th percentiles
BMI body mass index, *LDL-C* low density lipoprotein cholesterol, *HDL-C* high density lipoprotein cholesterol, *INR* International Normalized Ratio, *ETP* endogenous thrombin potential
[a]*t*-test
[b]chi-square test

Table 2 Concentration or activity of coagulation factors in never-smokers (NS) and active smoker (AS)

	Never-smokers		Active smokers		
	n		n		p[a]
Fibrinogen (mg/dl)	1,178	384.1 ± 99.7	775	417.0 ± 113.9	<0.001
Soluble fibrin (u/ml)	1,006	55.8 (34.6–83.9)	673	63.3 (41.5–95.8)	<0.001
Factor II (u/dl)	962	107 (93–122)	611	109 (95–122)	0.195
Factor V (u/dl)	680	111 (98–126)	435	115 (99–131)	0.172
Factor VII (u/dl)	1,173	125 (108–139)	775	120 (106–135)	0.023
Activated factor VII (u/l)	1,174	34.0 (21.8–51.0)	773	35.0 (22.0–52.0)	0.341
Factor VIII (u/dl)	1,175	172 (132–220)	775	154 (116–204)	<0.001
Factor XII (u/dl)	960	119 (87–145)	611	94 (80–131)	<0.001
Activated factor XII (μg/l)	1,176	2.64 ± 1.11	776	2.71 ± 1.24	0.214
Factor XIII (u/dl)	967	115 (100–131)	619	124 (109–141)	<0.001
Prothrombin fragments 1 + 2 (nmol/l)	1,176	0.63 (0.41–0.99)	777	0.64 (0.44–1.01)	0.182
Thrombomodulin (μg/l)	1,139	46.0 (35.0–59.0)	750	43.0 (33.0–56.0)	0.006
Antithrombin III (%)	1,169	97.7 ± 13.6	768	97.4 ± 13.3	0.575
Tissue factor pathway inhibitor (μg/l)	1,172	1.23 ± 0.37	773	1.30 ± 0.37	<0.001
Von Willebrand factor antigen (u/dl)	1,174	156 (122–202)	773	150 (112–196)	0.040

Data are means ± SD or median and 25th + 75th percentiles
[a]*t*-test for normally distributed variables, *t*-test of log transformed values for non-normally distributes variables

Table 3 Cox regression analysis of all-cause mortality per 1-SD increase adjusted for age, sex, LDL-C, HDL-C, BMI, diabetes, and hypertension

	Never-smokers		Active smokers	
	HR (95 % CI)	p	HR (95 % CI)	p
von Willebrand factor	1.37 (1.24–1.51)	<0.001	1.37 (1.22–1.56)	<0.001
Factor VIII	1.21 (1.11–1.32)	<0.001	1.40 (1.22–1.59)	<0.001
Tissue factor pathway inhibitor	1.20 (1.08–1.34)	0.001	1.22 (1.06–1.40)	0.005
Thrombomodulin	1.15 (1.03–1.29)	0.012	1.19 (1.07–1.31)	0.001
Fibrinogen	1.24 (1.10–1.39)	<0.001	1.16 (1.02–1.31)	0.026

4 Discussion

In participants of the LURIC study we found significant changes in individual parameters of the coagulation system in active smokers compared with lifetime nonsmokers. Elevated concentrations of fibrinogen in smokers have been reported before (Dotevall et al. 1994) as well as elevated levels of the A-subunit of factor XIII (Ariens et al. 1999) which covalently cross-links fibrin clots. Both parameters are also elevated in AS in the LURIC study. The concentration of soluble fibrin, which is created through the cleavage of fibrinogen by thrombin, also was increased in AS. These results point toward increased fibrin formation in AS. This is supported by the observed decrease in thrombomodulin levels in AS, which is a potent inhibitor of coagulation (Anastasiou et al. 2012). While there was a decrease in factor XII concentration (which initiates the intrinsic coagulation cascade) and factor VII (involved in the initiation of extrinsic coagulation cascade), we did not detect any differences in the activated factor XII or activated factor VII concentrations. In contrast to previous studies that reported an increase in vWF concentration through smoking (Price et al. 1999), we found a slight decrease in it, which was marginally significant. In cell culture assays, the serum from smokers induced a decrease in tissue factor pathway inhibitor (TFPI) secretion by endothelial cells accompanied

by a relative increase in tissue factor (TF) to TFPI ratio (Barua et al. 2002), while we observed a highly significant increase of TFPI in AS. TFPI is the most important inhibitor of the TF-mediated coagulation pathway, but TF itself was unfortunately not available in the LURIC study. However, there have been multiple reports of increased TFPI concentration in diseases like atherosclerosis and coronary artery disease, which has recently been discussed by Winckers et al. (2013). This might explain the observed increase of TFPI in AS of the LURIC study, since the majority of LURIC participants suffer from coronary artery disease.

Several prothrombotic factors, namely fibrinogen, vWF, and factor VIII were independent predictors of all-cause mortality in the LURIC study. Regarding TFPI, there also was a positive association with mortality, which might result from the association of this factor with coronary artery disease. We found that thrombomodulin also was associated with increased mortality in AS. Thrombomodulin, beside its antithrombotic functions, has antifibrinolytic activity (Anastasiou et al. 2012), which could be responsible for the increased risk of death in this study which comprises coronary artery disease patients.

5 Conclusions

The present study shows significant changes in individual parameters of the coagulation system in active smokers. These alterations point toward an increased thrombogenic potential. Individual parameters of the coagulation system were independent predictors of mortality in the LURIC study. Therefore, beside other risk factors for cardiovascular disease (e.g., lipids or life-style) parameters of the coagulation system should additionally be determined for risk prediction in active smokers.

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Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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C-Reactive Protein and Lipoprotein-Associated Phospholipase A_2 in Smokers and Nonsmokers of the Ludwigshafen Risk and Cardiovascular Health Study

M.E. Kleber, R. Siekmeier, G. Delgado, T.B. Grammer, B.R. Winkelmann, H. Scharnagl, B.O. Boehm, and W. März

Abstract

Measurement of high sensitivity CRP (hsCRP) and lipoprotein-associated phospholipase A_2 ($LpPLA_2$) provides information on systemic inflammation and stability of atherosclerotic plaques. Data analyzing the effect of smoking on these parameters are sparse. The aim of our study was the analysis of these parameters in active smokers and never-smokers. The study included 777 smokers and 1,178 never-smokers, of whom 221 and 302 died during a follow-up, respectively. The values of $LpPLA_2$ and hsCRP were significantly higher in smokers than in never-smokers. Mortality was highest in smokers and never-smokers with elevation of both biomarkers. Multivariate adjusted hazard ratios for patients in the highest tertile of both hsCRP and $LpPLA_2$ compared with patients in the lowest tertile of both markers were 1.85 (1.04–3.28) in never-smokers and 1.94 (1.10–3.45) in smokers. Our data confirmed the predictive value of hsCRP and $LpPLA_2$. However, there were a relevant number of patients with an increase of only one of these parameters. Therefore, beside other risk factors for cardiovascular disease, both parameters should be determined at least in high risk patients.

M.E. Kleber (✉), G. Delgado, and T.B. Grammer
Fifth Department of Medicine (Nephrology, Hypertensiology, Endocrinology, Diabetology, Rheumatology),
Medical Faculty of Mannheim, Heidelberg University,
Theodor-Kutzer-Ufer 1, 68167 Mannheim, Germany
e-mail: marcus.kleber@medma.uni-heidelberg.de

R. Siekmeier
Drug Regulatory Affairs, University of Bonn, Bonn, Germany

B. Winkelmann
Cardiology Group, Frankfurt-Sachsenhausen, Germany

H. Scharnagl
Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University Graz, Graz, Austria

B.O. Boehm
Division of Endocrinology, Department of Medicine, University Hospital, Ulm, Germany

LKC School of Medicine, Imperial College London and Nanyang Technological University, Singapore, Singapore

W. März
Fifth Department of Medicine (Nephrology, Hypertensiology, Endocrinology, Diabetology, Rheumatology),
Medical Faculty of Mannheim, Heidelberg University,
Theodor-Kutzer-Ufer 1, 68167 Mannheim, Germany

Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University Graz, Graz, Austria

Synlab Academy, Mannheim, Germany

Keywords
Cardiovascular disease • hsCRP • Inflammation • $LpPLA_2$ • Mortality • Plaque stability • Smoking

1 Introduction

Premature atherosclerosis is a common disease worldwide and atherosclerotic vascular disease, causing e.g., myocardial infarction, ischemic stroke, and lower limb ischemia, results in immense socioeconomic burden due to mortality and morbidity. A large number of risk factors of atherosclerotic vascular disease have been identified, including changes in plasma lipoprotein pattern, factors of coagulation and fibrinolysis, changes of inflammation parameters, and individual lifestyle factors (Ross 1999). Concerning the last factor, cigarette smoking plays the most relevant role which has been identified and confirmed within the last decades in numerous epidemiological studies, as well as studies for primary and secondary prevention of myocardial infarction (Danaei et al. 2009; U.S. Department of Health and Human Services 2006; Price et al. 1999).

The characteristics of atherosclerosis as a chronic inflammatory disease can be described by changes of different plasma proteins playing a role in the inflammatory process (e.g., high sensitive C-reactive protein (hsCRP), intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), matrix metalloproteinase-1 (MMP-1)), and changes in the activity of circulating blood cells (e.g., monocytes) (Libby 2002; Ross 1999). However, beside the analysis of lipid status, hsCRP is currently the most established inflammatory parameter for the assessment of individual risk of atherosclerotic vascular disease, predicting future cardiovascular events (Ridker 2003). CRP, a pentraxin consisting of 5 unglycosylated subunits was firstly described more than 80 years ago as a marker of bacterial infection and is synthesized by hepatocytes under the control of inflammatory cytokines including interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α). Having a plasma half-life time of about 19 h, its plasma concentration is largely determined by the rate of its synthesis (Ridker 2009). The first observations of an increase of CRP plasma concentrations in patients following myocardial infarction were made 70 years ago and confirmed later on. However, it required many more years, until the middle of the 1990s, to establish the plasma concentration of CRP as an independent risk marker for future cardiovascular events in patients at risk (Ridker 2009). As plasma CRP concentrations often were far below the threshold detectable by standard CRP assays, it has been necessary to develop high sensitivity assays (hsCRP).

Lipoprotein-associated phospholipase A_2 ($LpPLA_2$) is a novel marker of systemic inflammation and plaque stability, which has recently been in the focus of multiple studies in atherosclerosis research and serves as a risk factor of cardiovascular disease (CVD) and ischemic stroke, independently from the traditional risk factors such as LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C), and hsCRP (Kleber et al. 2011; Corson et al. 2008; Sudhir 2005; Ballantyne et al. 2004). $LpPLA_2$, also known as platelet activating factor acetylhydrolase (PAF-AH), is secreted from various inflammatory cells (e.g., monocytes/macrophages, T-cells, hepatic Kupffer cells, and mast cells) (Stafforini 2009; Sudhir 2005). Its function consists of hydrolysis of the short acyl group at the Sn-2 position of phospholipids in oxidized LDL, resulting in production of the proinflammatory compounds lysophosphatidylcholine and oxidized nonesterified fatty acids. The majority of the enzyme (70–80 %) is associated with the more atherogenic dense LDL subfractions (LDL_4 and LDL_5) due to its affinity to the C-terminus of

apolipoprotein B, and therefore shows a good correlation to the plasma concentration of LDL-cholesterol (LDL-C) (Stafforini 2009; Sudhir 2005; Ballantyne et al. 2004). It has been shown that humans and rabbits with elevated $LpPLA_2$ suffer from an increased coronary artery plaque burden and there is evidence that $LpPLA_2$ enhances atherosclerosis. Furthermore, the enzyme has been found in the apoptotic macrophages of vulnerable plaques and in human coronary arteries, which suggests its role in the acute coronary syndrome (Sudhir 2005). However, beside its proatherogenic properties, it should be noted that $LpPLA_2$ has also anti-inflammatory and antiatherogenic potential (Stafforini 2009).

The aim of the present study was to characterize the diagnostic value of two independent risk factors for cardiovascular events, hsCRP and $LpPLA_2$ which provide information on inflammation and plaque stability in active smokers and never-smokers of the Ludwigshafen Risk and Cardiovascular Health (LURIC) study.

2 Methods

The study was approved by the Ethics Committee at the Ärztekammer Rheinland-Pfalz (Mainz, Germany). All patients signed informed written consent. They were recruited from the Ludwigshafen Risk and Cardiovascular Health study, for which the enrolment was between June 1997 and May 2001 (Winkelmann et al. 2001). In brief, the study group consisted of patients of German ancestry who were clinically stable (except for acute coronary syndromes) and who received a coronary angiogram.

A total of 3,316 patients were included into the study. Smoking status was assessed based on a questionnaire and verified by cotinine measurement. Seven hundred seventy seven participants were active smokers and 1,178 never-smokers. Ex-smokers were discarded from further analysis.

Blood samples for the determination of laboratory indices, including hsCRP and $LpPLA_2$, were taken by venipuncture in the early morning and frozen at −80 °C until completion of study recruitment in 2001. hsCRP was then determined by immunonephelometry on a Behring Nephelometer II (N High Sensitivity CRP, Dade Behring, Germany). Plasma concentrations of $LpPLA_2$ mass, which is highly correlated with $LpPLA_2$ activity (Sudhir 2005), were determined by means of an ELISA ($LpPLA_2$ PLAC test, diaDexus Inc., U.S.). Both parameters were available in 1,048 never-smokers and 685 smokers.

Continuous variables showing a normal distribution were compared between groups by a *t*-test and not normally distributed variables were compared by the Mann-Whitney U-test. Associations between categorical variables were examined by chi-square testing. To examine the relationship with mortality, we calculated Kaplan-Meier curves in addition to hazard ratios and 95 % confidence intervals (95 % CI) using the Cox proportional hazards model. Multivariable adjustment was carried out as indicated. $P < 0.05$ was used to define statistical significance. An SPSS commercial packet version 20.0 was used for all analyses.

3 Results

Based on the results obtained by the questionnaire, 654 out of the 3,316 patients were active smokers and 1,194 were never-smokers. However, cotinine values were above 15 μg/l in 123 patients who reported to be ex-smokers or never-smokers; these patients were added to the group of active smokers, resulting in a final count of 777 active smokers and 1,178 never-smokers (Table 1).

Smoking status in the group of active smokers showed a large variability, ranging from 0.3 up to 132 pack-years. Males were more frequent in the group of active smokers and the mean age was lower. Plasma concentrations of LDL-C and HDL-C were significantly higher in never-smokers, whereas total triglycerides were higher in active smokers. However, results for LDL-C were likely affected by treatment with lipid lowering drugs (mainly statins), which was

Table 1 Selected anthropometric data of study patients at study begin (means ± SD or median and 25–75th percentile)

	Never-smokers (*n* = *1,178*)	Active smokers (*n* = *777*)	p
Smoking status (pack-years)	0	30.0 (15.0–43.2)	
Age (year)	65.3 ± 10.1	56.2 ± 10.3	<0.001
Male Gender (%)	45.4	77.9	<0.001
BMI	27.4 ± 4.2	27.0 ± 4.2	0.833[a]
LDL-C (mg/dl)	119.1 ± 36.4	117.5 ± 32.1	0.012[a]
HDL-C (mg/dl)	41.2 ± 11.1	36.2 ± 10.2	0.002[a]
Triglycerides (mg/dl)	136 (102–192)	154 (112–218)	<0.001[c]
Preexisting CVD (%)	68.1	80.1	<0.001[b]
Diabetes mellitus (%)	38.3	36	0.314[b]
Hypertension (%)	76.6	63.3	<0.001[b]
Lipid lowering drugs (%)	42.4	52.8	<0.001
hsCRP (ng/ml)	2.7 (1.2–7.0)	4.9 (1.8–10.3)	<0.001[c]
$LpPLA_2$ (ng/ml)	383.8 (272.3–533.5)	424.2 (293.7–630.2)	<0.001[c]

BMI body mass index, *HDL-C* high density lipoprotein cholesterol, *LDC* low density lipoprotein cholesterol, *hsCRP* high sensitivity C-reactive protein, $LpPLA_2$ lipoprotein-associated phospholipase A_2
[a]*t*-test
[b]chi-square-test
[c]Mann-Whitney-U-test

more frequent in active smokers. Preexisting CVD was also more prevalent in active smokers, whereas a higher prevalence of arterial hypertension was observed in never-smokers. However, body mass index (BMI) and prevalence of diabetes mellitus were inappreciably different between the study groups (Table 1).

Valid measurements of both hsCRP and $LpPLA_2$ were available for 1,048 never-smokers and 685 smokers. Plasma concentrations of hsCRP and $LpPLA_2$ were significantly higher in active smokers than in never-smokers (hsCRP, 4.9 (1.8–10.3) ng/ml *vs*. 2.7 (1.2–7.0) ng/ml; $LpPLA_2$, 424.2 ng/ml (293.7–630.2) ng/ml *vs*. 383.8 (272.3–533.5) ng/ml, respectively; $p < 0.001$ for both comparisons) (Table 1).

As a next step we analyzed the proportion of concentrations higher or lower than the median value of hsCRP and $LpPLA_2$ in either study group. Three hundred forty (49.6 %) of active smokers had plasma concentration of hsCRP and 343 (50.0 %) had $LpPLA_2$ above the median. Concentrations above the median for both markers were found in 190 (27.7 %) and below the median in 192 (28.0 %) of active smokers. Five hundred eighteen (49.4 %) of never-smokers had plasma concentrations of hsCRP

Table 2 Absolute and relative number of patients with concentrations of hsCRP and $LpPLA_2$ higher or lower than the median

	Never-smokers	Active smokers
	1,048	685
hsCRP low, $LpPLA_2$ low	284 (27.1 %)	192 (28.0 %)
hsCRP low, $LpPLA_2$ high	246 (23.5 %)	153 (22.3 %)
hsCRP high, $LpPLA_2$ low	241 (23.0 %)	150 (21.9 %)
hsCRP high, $LpPLA_2$ high	277 (26.4 %)	190 (27.7 %)

Chi-square $p < 0.001$

and 523 (49.9 %) had $LpPLA_2$ above the median. Concentrations above the median for both markers were found in 277 (26.4 %) and below the median in 284 (27.1 %) of never-smokers (Table 2).

Within the observation time (median of 10 years), 995 patients died. From these, 221 were active smokers (28.4 % out of the 777) and 302 never-smokers (25.6 % out of the 1,178); the difference between active smokers and never-smokers did not reach here significance (log rank test $p = 0.212$). The Kaplan-Meier curves were calculated for both groups depending on the concentrations of hsCRP and $LpPLA_2$ below or above the median as

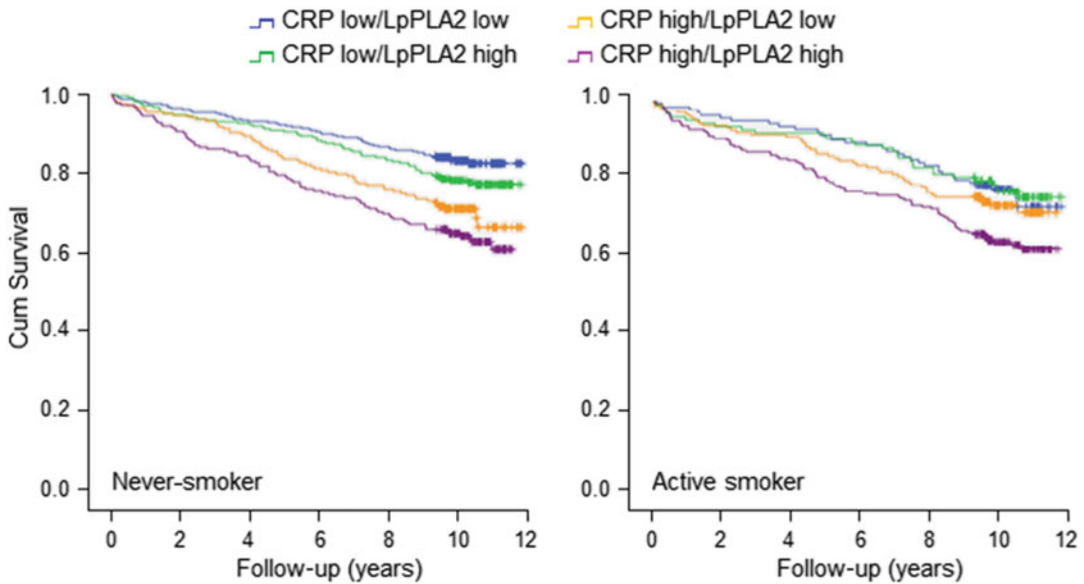

Fig. 1 **Survival of patients** with values of hsCRP and $LpPLA_2$ higher or lower than the median

Table 3 Dependence of survival on concentrations of hsCRP and $LpPLA_2$ higher or lower than the median

	Never-smokers (NS)		Active smokers (S)		p (χ^2 test)
	n_{patients}	n_{events}	n_{patients}	n_{events}	NS *vs.* S
	1,048	264 (25 %)	685	197 (29 %)	0.100
hsCRP low, $LpPLA_2$ low	284	44 (15 %)	192	48 (25 %)	0.010
hsCRP low, $LpPLA_2$ high	246	54 (22 %)	153	40 (26 %)	0.337
hsCRP high, $LpPLA_2$ low	241	68 (28 %)	150	41 (27 %)	0.850
hsCRP high, $LpPLA_2$ high	277	98 (35 %)	190	68 (36 %)	0.927
p (log-rank test)	<0.001		<0.02		

described above. In active smokers, the subgroup with the concentration of both markers above the median showed the worst survival compared with all other groups (Fig. 1). The survival difference in the different subgroups is compiled in Table 3.

In never-smokers, a stepwise decrease of survival was observed in patients with both hsCRP and $LpPLA_2$ below the median, hsCRP below and $LpPLA_2$ above the median, hsCRP above and $LpPLA_2$ below the median, and hsCRP and $LpPLA_2$ above the median; with the best survival in cases with a low concentration and the worst in those with a high concentration of both markers (Fig. 1, Table 3).

Comparison of mortality rates of active smokers and never-smokers demonstrated a large similarity in all groups, except for the group with low concentration of both markers. This group displayed a significant difference between active smokers and never-smokers, with a χ^2 p-value of 0.010 (Table 3).

To investigate whether the observed differences in survival between the smokers and never-smokers, according to the concentration of hsCRP and $LpPLA_2$, were influenced by a different distribution of risk factors between both groups, we conducted the Cox regression analysis with adjustment for age and sex (Model 1) or with an additional adjustment for diabetes, hypertension, coronary artery disease, BMI, LDL-C, HDL-C, and triglycerides (Model 2). For the analysis, we divided smokers and never-smokers according to tertiles of hsCRP and

Table 4 Cox regression of survival dependence on tertiles of hsCRP and $LpPLA_2$ concentrations adjusted for risk factors

	n	Model 1 HR (95 % CI)	p	Model 2 HR (95 % CI)	p
Never-smoker					
hsCRP low, $LpPLA_2$ low	135	$1^{reference}$		$1^{reference}$	
hsCRP low, $LpPLA_2$ high	113	1.31 (0.70–2.43)	0.401	1.60 (0.84–3.04)	0.152
hsCRP high, $LpPLA_2$ low	95	2.21 (1.25–3.89)	0.006	1.75 (0.97–3.16)	0.062
hsCRP high, $LpPLA_2$ high	118	2.16 (1.24–3.76)	0.006	1.85 (1.04–3.28)	0.036
Active smoker					
hsCRP low, $LpPLA_2$ low	94	$1^{reference}$		$1^{reference}$	
hsCRP low, $LpPLA_2$ high	60	1.01 (0.49–2.06)	0.987	0.89 (0.43–1.85)	0.749
hsCRP high, $LpPLA_2$ low	51	1.37 (0.73–2.58)	0.326	1.54 (0.80–2.97)	0.194
hsCRP high, $LpPLA_2$ high	93	2.05 (1.20–3.48)	0.008	1.94 (1.10–3.45)	0.023

Model 1: adjusted for age and sex; Model 2: additionally adjusted for LDL-C, HDL-C, triglycerides, BMI, diabetes, hypertension, and presence of coronary artery disease

$LpPLA_2$ concentrations and compared the groups with both markers in the lowest or highest tertile, and hsCRP and $LpPLA_2$ each in the highest tertile. For both smokers and never-smokers, there was a significant increase in risk for patients in whom the concentration of both hsCRP and $LpPLA_2$ was in the upper tertile. When only one marker was elevated, there was a trend toward increased risk, which became significant only in Model 2 in never-smokers (Table 4).

4 Discussion

In this study patients participating in the LURIC study were investigated. Due to the inclusion criteria study patients showed a high number of risk factors of CVD. In addition, patients frequently received lipid lowering drugs, often inhibitors of hydroxymethylglutaryl-CoA. Interestingly, concentrations of LDL-C in our active smokers were lower than in never-smokers standing in contrast to many prior publications (Craig et al. 1989). However, this might be due to an intense lipid lowering treatment in this group, especially with statins (Genser et al. 2008) and strict treatment goals in cardiovascular patients (National Cholesterol Education Program 2002).

In our study we compared active smokers and never-smokers, firstly defined according a questionnaire filled out by the patients at study entry. This method may result in an underestimation of smoking due to smoking denial (Wallner-Liebmann et al. 2013), so that we additionally verified smoking status by measuring serum cotinine and corrected the number of smokers. The active smokers showed a broad spectrum of smoking habits ranging from 0.3 pack years to 132 pack years, respectively. Ex-smokers were not subject to our analysis because this group is very different regarding their smoking habits, including individuals with mild or severe abuse and individuals who quitted smoking many years or only short time prior to study entry.

The plasma concentration of hsCRP was higher in our active smokers than in never-smokers. The observed increase in hsCRP is in accord with a number of prior studies, some of them also reporting dose-dependent increases of this acute phase protein in smokers, which were only partially reversible years after smoking cessation (Hastie et al. 2008; Wannamethee et al. 2005; Fröhlich et al. 2003). On the other hand, there are also studies showing no increase in hsCRP in smokers compared with nonsmokers (Lavi et al. 2007). The increase reported in smokers might be due to the chronic inflammation caused by components of cigarette smoke (Barua et al. 2013; Yanbaeva et al. 2007; Fröhlich et al. 2003), upregulation of CRP synthesis by nicotine (Mao et al. 2012), or a higher atherosclerotic burden often found in cigarette

smokers (U.S. Department of Health and Human Services 2006; Price et al. 1999). However, the partial reversibility of the increase reported in cigarette smokers and the *in vitro* effect of nicotine causing an increase of CRP synthesis indicate that there might be a relevant increase in plasma CRP concentration independent of atherosclerosis and individual risk of a vascular event.

Compared with never-smokers, our smokers also demonstrated a higher plasma level of $LpPLA_2$, which confirms prior studies (Tselepis et al. 2009; Lavi et al. 2007). A higher level of $LpPLA_2$ might be due to its upregulation or chronic inflammatory and atherosclerotic burden in smokers (Barua et al. 2013; U.S. Department of Health and Human Services 2006; Price et al. 1999). It was unclear whether the observed increases in hsCRP and $LpPLA_2$ were caused by various influencing factors or could be considered diagnostic markers for the estimation of individual cardiovascular risk. Therefore, we calculated the Kaplan-Maier curves dependent on the values above or below the median of hsCRP and $LpPLA_2$ plasma concentrations. In both groups, the lowest mortality was observed when the plasma concentration of both hsCRP and $LpPLA_2$ was below the median and the highest when both were above the median. In principle, this confirms prior studies investigating the predictive value of these parameters in cardiovascular and ischemic stroke studies (Corson et al. 2008; Ballantyne et al. 2004).

Interestingly, we found a difference in the Kaplan-Maier curves between never-smokers and active smokers. In the never-smokers, the Kaplan-Meier curves for individuals with only one marker above the median (hsCRP or $LpPLA_2$) were between the curves with both markers above or below the median, showing a trend for a better survival in individuals with low hsCRP and high $LpPLA_2$ compared with those with high hsCRP and low $LpPLA_2$. In contrast, in the active smokers there was no obvious difference between the curves of patients with low concentrations of both markers and those with elevated concentrations of only one marker. We also determined whether elevated hsCRP and/or $LpPLA_2$ were related to a worse outcome independent of traditional risk factors by means of adjusted Cox regression analysis and found a significant effect only if both markers were increased.

In addition to a different pattern of the Kaplan-Meier curves in active smokers compared with never-smokers we also found differences in the number of patients with hsCRP or $LpPLA_2$ below or above the median, even though there was a correlation between the results of both markers. These differences confirm the results of prior studies demonstrating the role of $LpPLA_2$ as an independent risk factor in cardiovascular disease (Kleber et al. 2011; Corson et al. 2008; Lavi et al. 2007; Sudhir 2005; Packard et al. 2000). Therefore, estimation of individual risk for an acute cardiovascular event should not be based on the determination of just hsCRP or $LpPLA_2$ alone. Beside, the evaluation of these markers should be performed together with other cardiovascular risk markers, e.g., plasma lipoproteins, parameters of coagulation or fibrinolysis.

The study has a number of limitations. Only patients referred to coronary angiography were investigated, which is an apparent bias in population selection. Although the study focuses on the effect of cigarette smoke, the specific mechanisms of smoking on the atherosclerotic process remain unclear as tobacco contains too many different compounds and their toxic effects are also affected by many other parameters (e.g., smoking habits or type of smoke). Further, both markers under study were determined only once at the study onset, so that potential short-time variations of these analytes, e.g., due to pharmacological treatment cannot be excluded.

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Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Green Tea Drinking Improves Erythrocytes and Saliva Oxidative Status in the Elderly

B. Narotzki, A.Z. Reznick, T. Mitki, D. Aizenbud, and Y. Levy

Abstract

We have previously shown that green tea (GT) drinking combined with vitamin E supplementation reduced plasma protein carbonyls and increased erythrocytes catalase activity in exercising healthy elderly. In the present study we set out to investigate the antioxidative effects of GT drinking in an aging population. We performed an interventional, crossover, controlled prospective trial with 35 healthy elderly subjects (mean age 67.3 ± 4.8 years), supplemented with four daily placebo maltodextrin "tea-bags" for 12 weeks, followed by four 1.5 g daily GT bags for another 12 weeks. Data were obtained at baseline, at the end of the placebo period, and at the end of the GT intervention period. We found that GT did not alter erythrocyte catalase activity. However, it provided protection against 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced oxidative hemolysis which declined by 10.2 % ($p < 0.001$). No changes were observed in saliva oral peroxidase enzymes. Nonetheless, saliva total antioxidant capacity increased by 42.0 % ($p < 0.01$). Plasma oxidative products, such as protein carbonyls, lipid peroxides and thiobarbituric acid reactive substances (TBARS) were stable throughout the intervention period. We conclude that four daily cups of GT are well tolerated in elderly free living subjects. Our results demonstrate that both erythrocyte resistances to oxidation and saliva antioxidant capacity are improved by GT drinking. The clinical implications of these oxidation modifications require further research.

Keywords

Aging • Erythrocytes • Green tea • Oral peroxidases • Oxidative hemolysis • Protein carbonyls • Saliva antioxidant capacity

B. Narotzki, A.Z. Reznick, and D. Aizenbud
Rappaport Faculty of Medicine, Technion–Israel Institute of Technology, Haifa, Israel

T. Mitki
Clalit Health Services, Haifa, Israel

Y. Levy (✉)
Rappaport Faculty of Medicine, Technion–Israel Institute of Technology, Haifa, Israel

Department of Medicine D, Rambam Health Care Campus, POB 31096, Bat Galim, Haifa, Israel
e-mail: ys_levy@rambam.health.gov.il

1 Introduction

Population aging is a global phenomenon. The elderly population has substantially increased over the past 50 years. For instance, in Israel, the elderly population percentage has more than doubled (Mashavh 2012). In some countries, the increase rate is even higher. Japan's aging rate shows more than a fourfold increase in the elderly population (Arai et al. 2012). High incidence of diseases may hamper the elderly life quality. Therefore, strategies for diseases prevention by health promotion in the older population are required.

Oxidative stress (OS) is suggested to be involved in the pathogenesis of various age related disorders, for instance, cancer, diabetes mellitus, neurodegeneration, atherosclerosis and cardiovascular diseases (Lagouge and Larsson 2013). The OS hypothesis suggests that reactive oxygen species (ROS) lead to molecular oxidative damage and senescence associated losses in physiological functions. According to this theory, attenuation of oxidative damage/stress is crucial for delaying the rate of aging (Sohal et al. 2002). Accumulated reactive oxygen species (ROS), on top of inadequate antioxidants function, lead to further OS and oxidative damage to macromolecules and progressive decline in cell functions (Salmon et al. 2010). Hence, much interest has arisen in the role of antioxidants, especially natural food derived antioxidants for maintenance of human health and disease prevention (Niki 2010). Thus, humans can achieve elevated antioxidant potential and alleviate ROS related diseases with increased dietary intake of antioxidant compounds (Jówko et al. 2011).

Tea (*Camellia sinensis*) is a caloric free beverage and constitutes an important source of antioxidants, including carotenoids, tocopherols and vitamin C, as well as polyphenols called catechins. Tea catechins antioxidant properties have been extensively investigated *in vitro*; the main four catechins include epigallocatechin 3 gallate (EGCG), epigallocatechin (EGC), epicatechin 3 gallate (ECG) and epicatechin (EC). Tea catechins reduce oxidation directly and indirectly by chelating prooxidant metals, separation of oxidative enzymes, and induction of antioxidant enzymes. The health potentials of tea, and particularly of green tea (GT) are progressively acknowledged in the western world due to the accumulation of evidence demonstrating favorable health effects in various body compartments, including the oral cavity, heart and vessels, skin, and adipose tissue (Narotzki et al. 2012a; Erba et al. 2005).

We have previously shown that GT drinking combined with vitamin E supplementation reduced plasma protein carbonyls, elevated erythrocytes catalase activity, and tended to increase the activity of the antioxidant oral peroxidases (OPO) in exercising healthy elderly (Narotzki et al. 2013b). Moreover, we have demonstrated that GT addition to saliva or mouth rinsing resulted in a sharp rise in the activity of OPO (Narotzki et al. 2013a). Therefore, the purpose of this study was to evaluate the effects of a long term (12 weeks) GT drinking on plasma, erythrocytes, and saliva oxidative stress biomarkers and on antioxidant capacity in healthy aged men and women. We hypothesize that GT drinking would improve antioxidative mechanisms. To examine this hypothesis, plasma oxidation biomarkers such as protein carbonyls, peroxides, thiobarbituric acid reactive substances (TBARS), erythrocyte antioxidant (catalase activity), and resistance to oxidative hemolysis and saliva antioxidants [OPO activity, total antioxidant capacity (TAC)] were investigated.

2 Methods

This study was approved by the institutional Helsinki committee at Rambam Health Care Campus, Haifa, Israel. All subjects provided a written informed consent.

2.1 Green Tea/Placebo Analysis

Subjects received 1.5 g GT bags (provided by Wissotzky Tea Company-Tel Aviv, Israel) and 1.1 g placebo (PB) "tea bags" containing maltodextrin (custom made by Wissotzky Tea Company). Analyses of the GT and PB contents were conducted for total phenols and TAC. The beverages were prepared by brewing PB (5 min) or GT bags (1, 3, 5, 7, or 10 min), in 240 mL of boiling water without stirring. Total phenols content of the drinks was determined by the colorimetric method of Folin-Ciocalteau, according to the modified methodology (Singleton and Rossi 1965). Briefly, 150 μL of GT/placebo drink was added to 500 μL of ethanol (100 %), 2,500 μL of DDW, and 250 μL of Folin-Ciocalteau reagent (50 %). After 5 min, 500 μL of 5 % sodium bicarbonate was added. The mixture was left for 1 h at room temperature and absorbance of the colored product was measured at 765 nm. Gallic acid in ethanol served as standard solution, and the results for total phenols were expressed as μL/mL of gallic acid equivalents.

Tea and placebo hydrophilic and lipophilic antioxidant capacity was measured by TAC kit (ScienCell Research Laboratories, CA, USA) and expressed as mM of Trolox equivalents.

2.2 Subjects

Thirty-five healthy men and women (13 men and 22 women), aged 60–76 years participated in this study. Baseline body mass index (BMI) was 22.4–34.6 kg/m^2. Table 1 displays baseline characteristics. Recruitment took place at Kibbutz Beit HaShita community family clinic in northern Israel. Kibbutz is an Israeli collective settlement; its communities are known for a high level of health, wellbeing, and longevity (Narotzki et al. 2012b). Exclusion criteria included any active disease state or unstable chronic disease (diabetes, vascular, or renal).

Table 1 Baseline characteristics of subjects

Age, year	67 ± 4.6
BMI, kg/m^2	28.6 ± 3.0
Blood pressure systolic/diastolic (mmHg)	136 ± 23/84 ± 15
Fasting glucose (mg/dL)	97.1 ± 14.0
Creatinine (mg/dL)	0.8 ± 0.2

Data are means ± SD
BMI body mass index

2.3 Experimental Design

The study was a prospective, crossover trial, in which every individual served as its own control. The experimental design is displayed in Fig. 1. All subjects underwent a 4 weeks washout period, during which they were briefed to avoid tea drinking and antioxidant supplements until the end of the study. At the end of the washout period, blood and saliva samples were collected after 12 h of night fast. Subsequently, all subjects received the maltodextrin containing PB "tea bags". The subjects were instructed to brew the PB sachets for 5 min in 240 mL boiling water without stirring and to drink 4 cups per day for 12 weeks period (PB period). After the PB period, fasting samples were collected once more and the subjects received the GT bags. Preparation and drinking instructions were the same as those for the PB drink. At the end of the 12 weeks, GT drinking, fasting blood, and saliva samples were drawn for the third time. During the study, every 4 weeks the subjects were given additional PB or GT bags. For compliance purposes, leftover tea bags were counted and actual consumption was assessed. Nutritional consumption of macronutrients and antioxidants was assessed using a food diary at the beginning, middle, and end of the study. The mean intakes were analyzed using Tzameret (version 2) dietary analysis program (Department of Nutrition, Ministry of Health, Jerusalem, Israel).

2.4 Blood Analysis

Blood was collected into disposable vials containing EDTA and separated for plasma

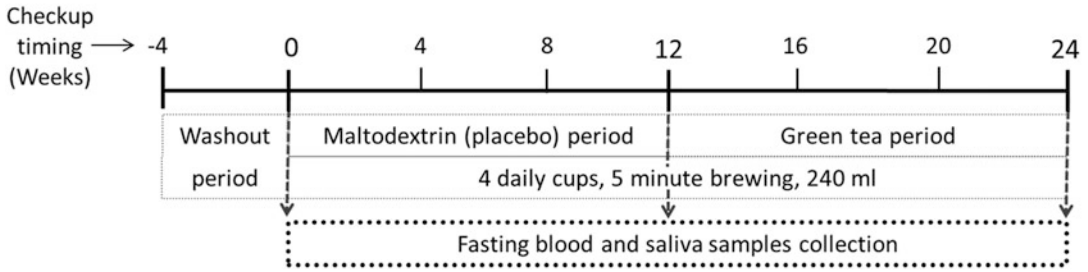

Fig. 1 Time plot of experimental design

and erythrocytes. Plasma was stored at −80 °C for subsequent analyses of oxidized proteins and lipids. Plasma protein carbonyls assay was performed according to the Reznick and Packer (1994) procedure and is presented in nmol per mg of plasma proteins. Plasma lipid peroxidation TBARS assays and plasma peroxides are described elsewhere and expressed as nmol per ml (Lavie et al. 2004). Oxidized lipid results was also adjusted for total plasma lipids [total lipids = 1.28(total cholesterol + triglycerides) + 96 (mg/dL)] (Thuresson et al. 2005).

Erythrocytes were washed 3 times with isotonic saline, kept for 6 days in 4 °C for catalase assay and analyzed immediately for 2,2′-azo-bis (2-amidinopropane) dihydrochloride (AAPH)-induced hemolysis test (HT). The test was based on previously reported protocols (Costa et al. 2009; Verma et al. 2006) and modified as follows: 0.4 ml washed erythrocytes was added to 9.5 ml saline. Two ml of diluted erythrocytes was added to DDW, 100 mM AAPH or saline to a final tube volume of 4 ml. All tubes were incubated at 37 °C for 2.5 h with intermittent shaking. After centrifuging the incubated tubes at 4,000 rpm for 10 min, absorbance was read spectrophotometrically at 540 nm. The HT percentage was calculated by dividing absorbance of AAPH-induced hemolysis from DDW-induced hemolysis. Saline tubes served as a negative control. Catalase activity was determined in erythrocytes hemolysates according to Aebi's method (1984) and expressed as mU per mg of hemoglobin protein.

2.5 Saliva Analysis

Non-stimulated whole saliva was collected between 7:00 and 8:00 a.m. to avoid circadian variations and stored in 4 °C until the formation of solid sediment containing squamous cells and cell debris. The supernatant was used for the following assays. The activity of OPO was measured according to the 5,5′-dithiobis, 2-nitrobenzoic acid thiocyanate (NBS-SCN) assay as described previously (Narotzki et al. 2013a) and expressed as mU per mg of saliva supernatant protein. Saliva was diluted 1:2 with DDW and used for TAC analysis as described above. Saliva TAC expressed as mM Trolox equivalents per mg of saliva supernatant protein.

2.6 Statistical Analysis

Results are expressed as means ± SD. SPSS Statistics 17 (SPSS Software, Chicago, IL, USA) was used for GT and placebo drinks total phenols and TAC analysis (one way ANOVA followed by post hoc Tukey's test).

SAS software (version 9.2, SAS Institute Inc, Cary, NC, USA) was used for The MIXED model analyzes of the time changes. Time was specified as categorical. The covariance structure was unspecified and was estimated on the basis of the data. In the comparisons of the means that correspond to different time points, Tukey's procedure was used to allow for the multiple comparisons. All statistical tests were 2-tailed with a significance level set at $P < 0.05$.

3 Results

Total phenols content of PB bags brewing in boiling water, was close to zero. GT phenols content rose substantially from 1 to 5 min brewing in boiling water, and marginally increased from 5 to 10 min (Fig. 2). Similar GT different brewing time trends were observed with TAC analysis, while PB capacity was lower than the TAC assay buffer (Fig. 3).

Compliance with PB and GT drinking was 94.0 ± 5.9 % and 95.9 ± 3.4 %, respectively. According to the nutritional food diary, the consumption of total calories, fat, carbohydrates, dietary fibers, vitamin C, vitamin E, and carotenes remained stable during the study.

Plasma, erythrocytes, and saliva oxidative stress biomarkers as well as antioxidant capacity are summarized in Table 2. Plasma oxidative stress biomarkers: protein carbonyls, TBARS, and peroxides remained unchanged by GT drinking. However, peroxides concentrations slightly increased after PB drinking (4.4 % increase at Week 12, compared with baseline, $p = 0.006$). Oxidized lipids, adjusted for the total plasma lipid, did not make any difference compared with crude data.

Erythrocytes catalase activity was not different at any time point. Nonetheless, the extent of AAPH-induced hemolysis was significantly lower after GT drinking (10.2 % decline at Week 24, compared with Week 12, $p = 0.001$), but not after PB drinking. Saliva OPO activity was not affected by either drink. However, saliva TAC measurements revealed that GT, but not PB drinking, increased its antioxidant capacity (41.7 % increase at Week 24, compared with Week 12, $p = 0.0063$). The effects of GT *vs.* PB drinking in relation to baseline oxidative status are illustrated in Fig. 4.

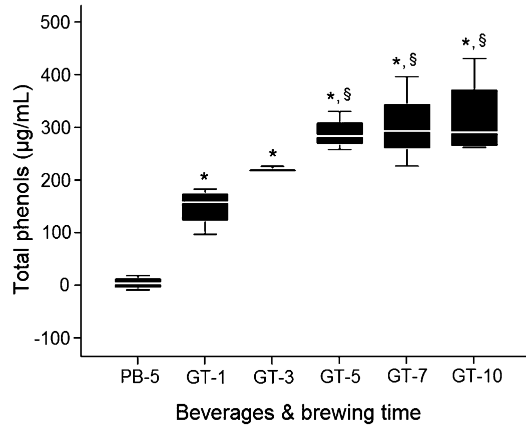

Fig. 2 Brewing time effects on placebo (PB)/green tea (GT) total phenols content. PB or GT bags were added to 240 ml of boiling DDW for different brewing time points (min). Changes in total phenols expressed as µg/mL of gallic acid equivalents. *Difference from placebo, $p < 0.05$; §difference from 1 min brewing, $p < 0.05$. Data are means ± SD (n = 4)

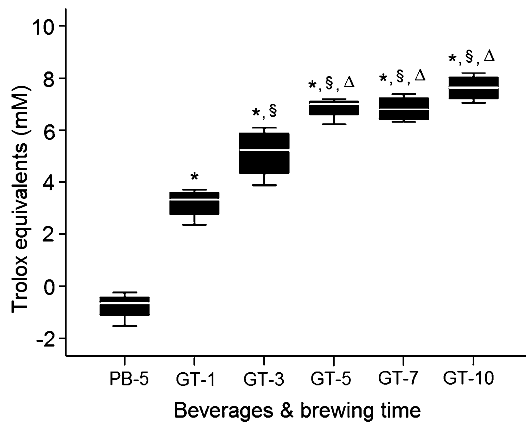

Fig. 3 Brewing time effects on placebo (PB)/green tea (GT) total antioxidant capacity (TAC). PB or GT bags were added to 240 ml of boiling DDW for different brewing time points (min). Changes in TAC expressed as mM of Trolox equivalents. *Difference from placebo, $p < 0.05$, §difference from 1 min brewing, $p < 0.05$; Δdifference from 3 min brewing, $p < 0.05$. Data are means ± SD (n = 4)

4 Discussion

The results of this study show that regular consumption of four daily cups of GT for 12 weeks may improve erythrocytes and saliva antioxidant capacities and contribute to the antioxidative mechanisms in healthy elderly men and women. On the basis of the obtained concentration curves regarding the content of total phenols and the

Table 2 Plasma, erythrocytes, and saliva oxidative stress biomarkers as well as antioxidant capacities

	Baseline	Week 12	Week 24	% change (Week 24 *vs*. Week 12)
Plasma				
Protein carbonyls, nmol/mg	0.65 ± 0.27	0.62 ± 0.20	0.66 ± 0.17	+6.5
TBARS, nmol/mL	11.27 ± 1.53	11.51 ± 1.80	11.50 ± 1.87	−0.1
Peroxides, nmol/mL	487.43 ± 41.38	508.83 ± 42.81[a]	519.77 ± 48.22	+2.2
Erythrocytes				
Catalase activity, mU/mg	32.95 ± 3.34	31.42 ± 3.72	32.99 ± 4.18	+5.0
HT, percentage	69.55 ± 15.39	71.27 ± 9.89	64.01 ± 12.48[b]	−10.2
Saliva				
OPO activity, mU/mg	347.51 ± 286.14	313.18 ± 299.85	328.20 ± 189.44	+4.8
TAC, mM/mg	7.17 ± 5.53	9.08 ± 4.04	12.87 ± 5.13[b]	+41.7

Data are least squares means ± SD
TBARS Thiobarbituric acid reactive substance, *HT* erythrocytes AAPH-induced hemolysis test, *OPO* oral peroxidases, *TAC* total antioxidant capacity
[a]Significant difference of Week 12 *vs*. Week 0 (placebo effect), $p < 0.01$
[b]Significant difference of Week 24 *vs*. Week 12 (green tea effect), $p < 0.001$

level of TAC, a protocol of 5 min' brewing time was selected.

In vitro antioxidant activity of GT's catechins and their bioavailability after tea drinking are generally agreed upon; yet there are conflicting results there regarding the *in vivo* antioxidant activity (Ellinger et al. 2011; Erba et al. 2005). Markers of protein oxidation are decreased after GT consumption (Ellinger et al. 2011). In an animal study of GT extract administration (500 mg/kg) to the aged rats, the percentage of cardiac, hepatic, and renal protein carbonyls was reduced by 30–40 %, compared with the age-matched control group (Wang 2013). In a human study we have shown that GT drinking combined with vitamin E supplementation reduced plasma protein carbonyls in exercising healthy elderly (Narotzki et al. 2013b). However, in our current study the administration of GT alone did not attenuate plasma protein carbonyls. It is possible that a reduction of protein carbonyls has occurred in tissues other than plasma, which was not monitored. Secondly, the combination of daily exercise with aging might have enhanced oxidative stress in our previous protocol (Narotzki et al. 2013b). We suggest that a lesser extent of ROS production was expected in our current study, in which the subjects were not exposed to exercise-induced OS. Consequently, GT administration alone was not advantageous, compared with the combination of GT, exercise, and vitamin E regarding the protection against plasma protein carbonyl production.

Other studies which investigated GT have not demonstrated any affects on lipid peroxidation. Ellinger et al. (2011) suggested that regular GT drinking may be effective when the antioxidative/oxidative balance is impaired due to harmful environmental exposure conditions and non-healthy life style. Such interventional studies, which failed to show GT effects on lipid peroxidation, included only nonsmokers. In agreement with this, our current study included non-smoking elderly and, no GT effects on plasma TBARS and peroxides were demonstrated. Adjusting oxidative biomarkers to total plasma lipids may provide additional information. Higher plasma lipids concentrations can result in higher lipid peroxides. Nonetheless, such adjustment did not make any difference compared with the crude data in our study (data not shown).

Erythrocytes can be used to study oxidative defense systems at the cellular level. Animal studies have shown that green tea was able to increase the activity of catalase in different tissues (Lin et al. 1998). GT and vitamin E supplementation with exercise resulted in a 10 % increase in erythrocytes catalase activity (Narotzki et al. 2013b). However, this increase was not followed in the current study.

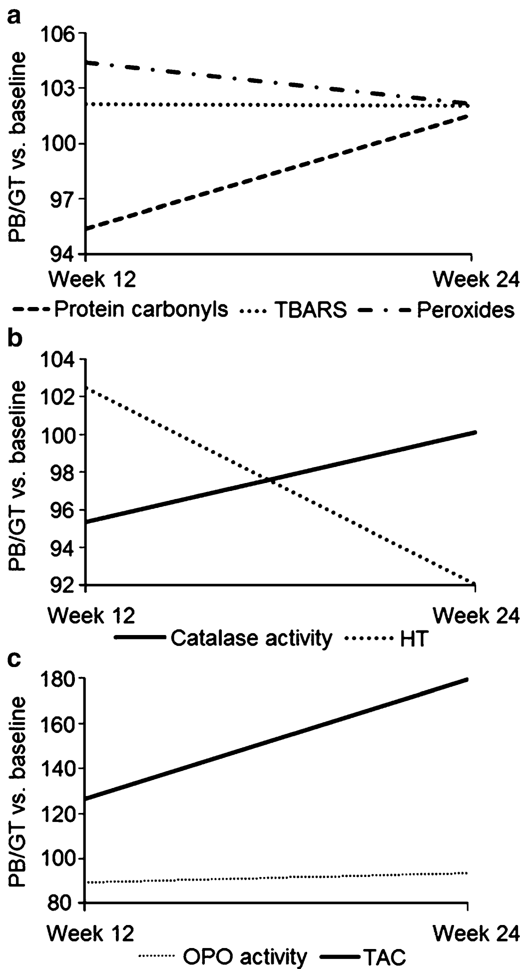

Fig. 4 Oxidation biomarkers and antioxidative mechanisms. The change from baseline levels is presented as Week 12 (Placebo (PB) effect) and Week 24 (green tea (GT) effect). Baseline levels are expressed as 100 %. Full line represents antioxidants activity, broken line represents oxidation. (**a**) **Plasma oxidation biomarkers**. The effects of PB/GT drinking on protein carbonyls, thiobarbituric acid reactive substances (TBARS) and peroxides; (**b**) **Erythrocyte antioxidative mechanisms**. The effects of PB/GT drinking on catalase activity and AAPH-induced hemolysis test (HT); (**c**) **Saliva antioxidative mechanisms.** The effects of PB/GT drinking on oral peroxidases (OPO) activity and total antioxidant capacity (TAC)

By adding AAPH to the erythrocytes, an initial lag time due to erythrocyte endogenous antioxidants, such as glutathione, tocopherols, and catalase is followed by hemolysis due to peroxyl radicals. Polyphenols are known for their ability to reduce membrane lipid peroxidation. They may interact with the membrane bilayer; decrease its fluidity and the diffusion of free radicals. Addition of GT and GT extracts to human and rat erythrocytes *ex vivo*, before performing oxidant induced HT, decreased the extent of hemolysis (Costa et al. 2009; Zhang et al. 1997). Moreover, jasmine GT, given 20 min before blood collection in rats, resulted in hemolysis amelioration (Zhang et al. 1997). Our results demonstrate that regular GT consumption can considerably enhance erythrocytes resistance to oxidative hemolysis. GT affected HT results 12 h after drinking the last GT, which may suggest an interaction of GT polyphenols with erythrocyte membranes. However, this was not investigated in the current study and verification of this hypothesis requires further research.

The majority of studies that have followed bolus ingestion of GT have demonstrated an *ex vivo* increase in plasma antioxidant capacity (assays include the ferric reducing ability of plasma (FRAP) and total radical antioxidant power (TRAP)) (Ellinger et al. 2011). However, regular consumption was less conclusive. Unlike plasma, information on saliva antioxidant capacity is limited. We have observed a substantial and significant increase in saliva TAC after GT drinking, which occurred 12 h after last tea drink. The effects of GT on saliva TAC were described in another study on chemical laboratory workers. In that study, a single daily cup of 300 mL of GT for a month, was followed by an elevation of TAC. Nonetheless, contrary to our study, TAC was measured short time after GT drinking (Tavakol et al. 2013).

Oral defense mechanisms against free radicals are particularly dependent on the antioxidant enzymes OPO (Narotzki et al. 2013b). The addition of black tea, EGCG, and particularly GT to saliva or mouth rinsing resulted in an increase in the activity of OPO (Narotzki et al. 2013a). Nevertheless, no changes in OPO activity were seen after drinking, despite the sharp elevation in the saliva TAC in our current study. Our former study suggested that GT catechins may be responsible for the increase in OPO activity.

Some of GT components may be present in saliva, even after 12 h fast, as evidenced by TAC modification. However, it is possible that saliva concentration is too low for a change in OPO activity. Since GT catechins undergo methylation, glucuronidation, and sulfation in the human body (Feng 2006), it is also conceivable that GT metabolites have different effects on OPO activity.

Limitations of the study include the selection of subject from a community with unique healthy life style, not representative of similar age groups in Israel. Increased study size would be favorable for some of our marginal results. Quantification of blood and saliva polyphenols would have shown availability of GT catechins. Consumption of more than 4 daily GT cups might have achieved stronger biological effects. However, regarding Israeli GT drinking habits, compliance is suspected to be low. Advantages of this study include the crossover-controlled experimental design with high compliance. Administration of maltodextrin placebo "tea bags" enabled a placebo controlled study.

In conclusion, drinking four daily cups for 12 weeks increased erythrocytes and saliva antioxidative mechanisms in healthy elderly, as demonstrated by a reduction in AAPH-induced hemolysis and elevation in saliva TAC. Despite our subjects' age, they were generally healthy with minimal oxidative stress exposure. Future studies in this age-group should investigate GT antioxidative effects in individuals prone to oxidative damage such as smoking, diseases states (diabetes), and exposure to environmental pollution.

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Conflicts of Interest Statement Wissotzky Tea Company supported the study and provided placebo and GT bags. The company was not involved in any phase of the study including design and data analysis. The authors declare no other potential conflicts of interest in relation to this article.

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Cardiovascular Effects of N-acetylcysteine in Meconium-Induced Acute Lung Injury

D. Mokra, I. Tonhajzerova, H. Pistekova, Z. Visnovcova, A. Drgova, J. Mokry, and A. Calkovska

Abstract

Anti-inflammatory drugs are increasingly used for treatment of neonatal meconium aspiration syndrome (MAS), but their adverse effects are poorly known. Therefore, the aim of this study was to evaluate the effects of the antioxidant N-acetylcysteine on cardiovascular parameters in an animal model of MAS. Oxygen-ventilated rabbits were intratracheally instilled 4 mL/kg of meconium suspension (25 mg/mL) or saline. Thirty minutes later, meconium-instilled animals were given N-acetylcysteine (10 mg/kg, i.v.) or the same volume of saline. Changes in cardiovascular parameters (blood pressure, heart rate, and heart rate variability) were recorded over a 5-min course of solution administration, over 5 min after its end, and then hourly for 5 h. Oxidation markers (thiobarbituric acid-reactive substances (TBARS) and total antioxidant status) and aldosterone, as a non-specific marker of cardiovascular injury, were determined in plasma. Meconium instillation did not evoke any significant cardiovascular changes, but induced oxidative stress and elevated plasma aldosterone. N-acetylcysteine significantly reduced the mentioned markers of injury. However, its administration was associated with short-term increases in blood pressure and in several parameters of heart rate variability. Considering these effects of N-acetylcysteine, its intravenous administration in newborns with MAS should be carefully monitored.

Keywords

Blood pressure • Heart rate • Heart rate variability • Meconium aspiration • N-acetylcysteine

D. Mokra (✉), I. Tonhajzerova, H. Pistekova, Z. Visnovcova, and A. Calkovska
Department of Physiology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, 4 Mala Hora St., SK-03601 Martin, Slovakia
e-mail: mokra@jfmed.uniba.sk

A. Drgova
Department of Medical Biochemistry, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovakia

J. Mokry
Department of Pharmacology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovakia

1 Introduction

Meconium aspiration syndrome (MAS) is a serious disease, which occurs in the term and post-term newborns. When the fetus or the newborn aspirates the meconium-stained amniotic fluid, particulate meconium content obstructs the airways. Within several hours after the aspiration, surfactant dysfunction, inflammation, pulmonary vasoconstriction, and lung edema develop. Because the pathomechanism of MAS is multi-pronged, treatment is usually multiagent and includes ventilatory support, oxygen delivery, inhalation of nitric oxide, and administration of exogenous surfactant. Recently, various anti-inflammatory agents, such as glucocorticoids, phosphodiesterase (PDE) inhibitors, or antioxidants have been successfully used (Mokra et al. 2013).

The rationale for the delivery of antioxidants in MAS is based on the finding of significant lipid and protein oxidation in the meconium-injured lungs (Mokra et al. 2007, 2012). Antioxidants, for instance recombinant human superoxide dismutase (SOD), decreased several markers of oxidation, reduced lung injury score, and improved oxygenation in animal models of MAS (Lakshminrusimha et al. 2006; Lu et al. 2005). Another antioxidant, N-acetylcysteine (NAC), also possesses a great potential to alleviate inflammation and oxidative changes (Gillissen and Nowak 1998). However, little is known about potential cardiovascular side effects of the mentioned medicaments. Administration of recombinant SOD in animals decreased mean arterial blood pressure, but had no significant effect on the heart rate (Chu et al. 2005). In isolated pulmonary arteries, recombinant SOD mitigated norepinephrine-induced and KCl-induced increases in the contraction response in an animal model of neonatal pulmonary hypertension (Lakshminrusimha et al. 2006). Similarly, NAC decreased pulmonary vascular resistance in ARDS-like injury (Wagner et al. 1989). In general, due to low bioavailability, the side effects of NAC, e.g., anaphylactoid reactions, tachycardia, or hypotension are extremely rare and seen usually at very high concentrations (Flanagan and Meredith 1991).

As there is no information on short-term changes in cardiovascular parameters in relation to NAC administration in MAS, the purpose of this study was to evaluate the blood pressure and heart rate during and immediately after administration, and then hourly for 5 h after NAC. In addition, fluctuations of the heart rate around its average value, i.e., heart rate variability (HRV), representing a sensitive marker of cardiac sympathovagal control mechanisms (von Borell et al. 2007), were analyzed. To extend our understanding of the mechanisms involved in the side effects of antioxidants, the plasma level of aldosterone, a key cardiovascular hormone, were also measured. Furthermore, two markers of oxidative stress – thiobarbituric acid-related substances (TBARS) and total antioxidant status (TAS) were assessed in the plasma to elucidate a relationship between the short-term cardiovascular changes and oxidative stress (Thayer et al. 2011).

2 Methods

2.1 General Design of Experiments

The study was approved by a local Ethics Committee of Jessenius Faculty of Medicine in Martin and the National Veterinary Board in Slovakia. Meconium was collected from healthy term neonates; it was lyophilized and stored at −20 °C. Before use, meconium was suspended in 0.9 % NaCl at a concentration of 25 mg/mL.

Adult chinchilla rabbits of 2.5 ± 0.3 kg body weight were anesthetized with intramuscular ketamine (20 mg/kg; Narketan, Vétoquinol, UK) and xylazine (5 mg/kg; Xylariem, Riemser, Germany), followed by infusion of ketamine (20 mg/kg/h). Tracheotomy was performed and catheters were inserted into a femoral artery and right atrium for blood sampling, and into a femoral vein to administer anesthetics. The animals were then paralyzed with pipecuronium

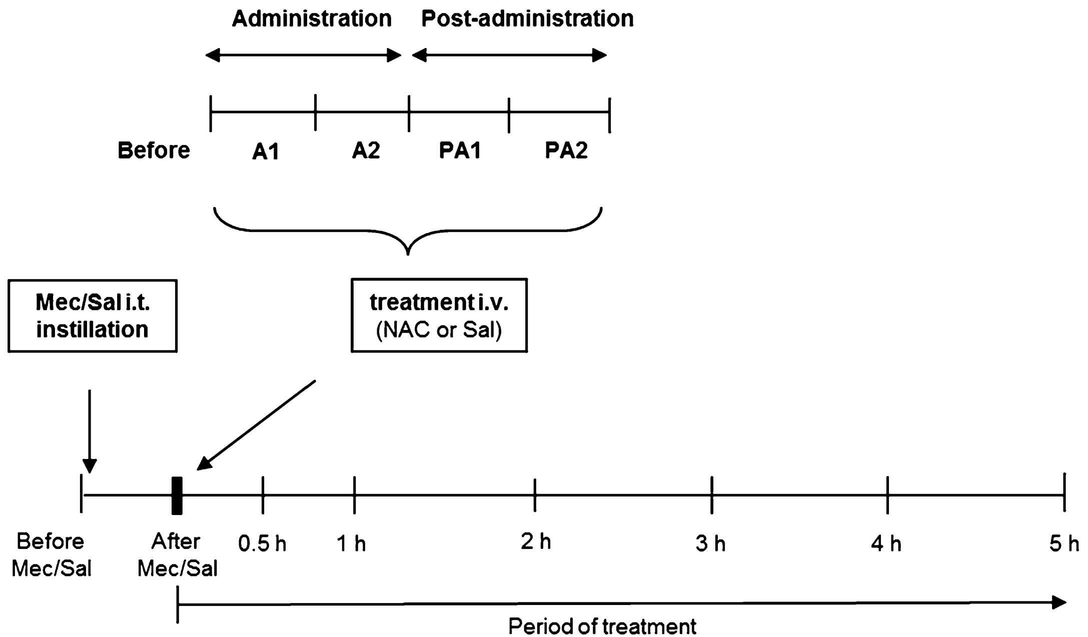

Fig. 1 Experimental paradigm. *A* treatment administration, *PA* post-administration, *Mec* meconium, *NAC* N-acetylcysteine, *SAL* physiological saline, *i.i.* intratracheal, *i.v.* intravenous

bromide (0.3 mg/kg/30 min; Arduan, Gedeon Richter, Hungary) and subjected to a pressure-controlled ventilator (Beat-2, Chirana, Slovakia). All animals were ventilated with a frequency of 30/min, fraction of inspired oxygen (FiO_2) of 0.21, inspiration time Ti 50 %, peak inspiratory pressure (PIP) to keep a tidal volume (V_T) between 7 and 9 mL/kg body weight, and no positive end-expiratory pressure (PEEP) at this stage of experiment.

After 15 min of stabilization, cardiopulmonary parameters were recorded and blood gases were analyzed (RapidLab 348, Siemens, Germany). Then, 4 mL/kg of meconium suspension or saline (served as controls) was instilled into the trachea. From this moment on, animals were ventilated with FiO_2 1.0 and PEEP 0.3 kPa. In the meconium-instilled animals, respiratory failure developed within 30 min, defined as >30 % decrease in dynamic lung-thorax compliance (Cdyn) and $PaO_2 < 10$ kPa at FiO_2 1.0. After the recordings were taken, animals obtained N-acetylcysteine (NAC) (10 mg/kg, i.v.; Injekt, Salutas Pharma GmbH, Germany) 30 min after intratracheal meconium instillation (Mec + NAC group, n = 6). Since NAC (0.1 mL/kg) was diluted in a normal saline up to a total volume of 1 mL, animals received the same volume of saline (1 mL) at the time points corresponding to NAC-treated ones (referred to as sham-treated animals; Mec + Sal group, n = 6). Finally, there was a control group, receiving saline instead of meconium (Sal + Sal group, n = 5). Treatment was delivered slowly over a period of 5 min. Immediate cardiovascular changes associated with administration (A) of treatment were evaluated over two 2.5 min intervals (A1 and A2) of NAC or saline (5 min in total) and over two similar time intervals post-administration (PA1 and PA2) (Fig. 1).

All animals were oxygen-ventilated for additional 5 h after the treatment. Cardiorespiratory parameters were recorded at 0.5, 1, 2, 3, 4, and 5 h to investigate early effects of the treatment. At the end of experiments, animals were sacrificed by an overdose of anesthetics.

2.2 Measurement and Evaluation of Cardiopulmonary Parameters

Tracheal airflow and V_T were measured by a heated Fleisch head connected to a pneumotachograph. Airway pressure was registered *via* a pneumatic catheter placed in the tracheal tube and connected to an electromanometer. Cdyn was calculated as a ratio between V_T (adjusted per kg body weight) and airway pressure gradient (PIP-PEEP). Systolic (SBP) and diastolic (DBP) blood pressures were measured *via* a catheter in the femoral artery connected to an electromanometer, and the mean arterial blood pressure (MABP) was calculated as MABP = DBP + 1/3 (SBP − DBP).

Heart rate (HR) was obtained from ECG recorded by subcutaneous electrodes. HRV was evaluated using a computer system (VariaPulse TF3, Sima Media, Czech Republic). Parameters of time analysis, mean duration of R-R interval (RR) and mean squared successive difference (MSSD), and parameters of spectral analysis, i.e., spectral powers in low frequency (LF: 0.05–0.15 Hz) and high frequency (HF: 0.15–2.0 Hz) bands were analyzed. From the mentioned parameters, MSSD and HF have been established as markers of parasympathetic (vagal) activity, while LF band represents the activity of both branches of the autonomic cardiac control and that of baroreceptors (Thayer et al. 2011).

2.3 Biochemical Analysis of Blood Plasma

Quantification of total antioxidant status (TAS) in the blood plasma at the end of experiment was carried out using ABTS (2,2′-azino-di-[3-ethylbenzothiazoline sulphonate]) radical formation kinetics (Randox TAS kit, Randox laboratories Ltd., UK) and expressed in mmol/L. A concentration of thiobarbituric-acid reactive substances (TBARS) was determined from the absorbance at 532 nm and expressed in nmol/mg protein. A concentration of aldosterone was measured by Aldosterone ELISA kit (BioVendor, Czech Republic) and was expressed in pg/mL.

2.4 Statistical Analysis

Data were expressed as means ± SE. Normality of data distribution was assessed by the Kolmogorov-Smirnov test. Since the distribution of some HRV variables (spectral powers) was extremely skewed, a logarithmic transformation of these data was used to improve normality before a statistical analysis was performed. Then, between-group differences were analyzed by one-way ANOVA with a *post-hoc* LSD test. Within-group differences were evaluated by Wilcoxon test. Strength of association between biochemical and cardiovascular markers were expressed by Pearson's correlation coefficient (r) and Bonferroni probability (p). A value of $p < 0.05$ was considered statistically significant.

3 Results

Body weight and initial values of all cardiopulmonary parameters were comparable between the groups before the intratracheal instillation of meconium or saline.

3.1 Cardiovascular Parameters

Before administration of treatment, no significant between-group differences were found. During 5 min of treatment (two intervals of 2.5 min, A1 and A2), a significant increase in MABP was found in Mec + NAC group *vs*. both sham-treated control groups (Sal + Sal and Mec + Sal). The increase in MABP was accompanied by elevation in HRV parameters, i.e., a significant increase in MSSD and insignificant one in

Table 1 Cardiovascular parameters in saline-instilled sham-treated animals (Sal + Sal group), in meconium-instilled sham-treated animals (Mec + Sal group), and in meconium-instilled NAC-treated animals (Mec + NAC group) before treatment, during 5 min of treatment administration (intervals A1 and A2, each of 2.5 min), and immediately post-treatment administration (intervals PA1 and PA2, each of 2.5 min)

	Before	A1	A2	PA1	PA2
MABP (kPa)					
Sal + Sal	8.2 ± 0.8	7.8 ± 0.8	8.5 ± 0.9	8.8 ± 0.9	9.0 ± 1.0
Mec + Sal	8.5 ± 0.7	8.2 ± 0.7	8.3 ± 0.7	8.4 ± 0.7	8.3 ± 0.7
Mec + NAC	9.9 ± 0.7	11.9 ± 0.5[cf]	12.1 ± 0.6[be]	12.3 ± 0.8[be]	12.0 ± 0.8[bd]
HR (bpm)					
Sal + Sal	211 ± 11	212 ± 12	215 ± 10	213 ± 9	210 ± 8
Mec + Sal	213 ± 14	217 ± 14	216 ± 14	207 ± 10	208 ± 10
Mec + NAC	212 ± 10	217 ± 7	216 ± 13	215 ± 10	213 ± 14
MSSD (ms^2)					
Sal + Sal	1.9 ± 0.4	1.6 ± 0.5	1.7 ± 0.5	1.7 ± 0.5	1.6 ± 0.4
Mec + Sal	2.4 ± 0.5	3.3 ± 0.7	3.8 ± 0.8	3.7 ± 0.7	2.9 ± 0.7
Mec + NAC	1.7 ± 0.3	9.9 ± 5.7	20.2 ± 12.7	10.1 ± 3.6[d]	7.1 ± 2.7
logLF					
Sal + Sal	−1.1 ± 0.6	−0.4 ± 0.4	−0.2 ± 0.3	−0.4 ± 0.6	−0.8 ± 0.6
Mec + Sal	−1.9 ± 0.6	0.0 ± 0.5	−1.1 ± 0.6	−0.3 ± 0.6	−0.2 ± 0.4
Mec + NAC	−1.4 ± 0.8	0.0 ± 1.2	0.7 ± 1.3	1.2 ± 0.9	0.7 ± 0.8
logHF					
Sal + Sal	0.5 ± 0.1	0.4 ± 0.2	0.5 ± 0.2	0.6 ± 0.1	0.6 ± 0.1
Mec + Sal	0.7 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.9 ± 0.4	0.4 ± 0.3
Mec + NAC	0.3 ± 0.4	0.9 ± 0.8	1.6 ± 1.0	1.8 ± 0.8	1.3 ± 0.8

See Methods for the explanation of cardiac acronyms. For between-group comparisons: Mec + NAC *vs*. Mec + Sal: [b]$p < 0.01$, [c]$p < 0.001$; Mec + NAC *vs*. Sal + Sal: [d]$p < 0.05$, [e]$p < 0.01$, [f]$p < 0.001$

both LF and HF spectral powers, whereas no changes were observed in the mean heart rate (Table 1).

Along the further course of experiment, most of the changes in the treated group gradually adjusted to the values comparable with the sham-treated groups. However, parameters of HRV, particularly MSSD, remained higher till the end of experiment (Table 2).

3.2 Biochemical Markers of Meconium-Induced Injury

In the meconium-instilled sham-treated animals (Mec + Sal group), higher TBARS ($p < 0.001$ *vs*. Sal + Sal) and lower TAS ($p < 0.05$ *vs*. Sal + Sal) were detected in the plasma. NAC reduced oxidation ($p < 0.001$ *vs*. Mec + Sal) and prevented a decline in TAS ($p < 0.05$ *vs*. Mec + Sal) (Table 3). Concentrations of aldosterone, a non-specific marker of stress and injury, were higher in Mec + Sal *vs*. Sal + Sal ($p < 0.01$), while NAC decreased plasma aldosterone *vs*. Mec + Sal group ($p < 0.01$, Table 3).

3.3 Correlations Between Biochemical Markers and Cardiovascular Parameters

The evaluation of associations between the biochemical markers showed negative correlations between TAS *vs*. TBARS ($r = -0.638$, $p = 0.006$), and TAS *vs*. aldosterone ($r = -0.689$, $p = 0.005$). Positive correlations were found between TBARS *vs*. aldosterone ($r = 0.729$, $p = 0.001$) and between aldosterone and logHF at 5 h of the treatment ($r = 0.517$, $p = 0.040$). However, no other significant correlations were found between the biochemical markers of injury and cardiovascular parameters.

Table 2 Cardiovascular parameters in saline-instilled sham-treated animals (Sal + Sal group), in meconium-instilled sham-treated animals (Mec + Sal group), and in meconium-instilled N-acetylcysteine-treated animals (Mec + NAC group) before and after intratracheal meconium/saline (Before/After M/S) instillation and during 5 h after treatment administration

	Before M/S	After M/S	30 min	1 h	2 h	3 h	4 h	5 h
MABP (kPa)								
Sal + Sal	9.6 ± 0.7	9.5 ± 0.7	9.6 ± 0.9	8.4 ± 0.8	10.0 ± 0.6	9.8 ± 0.3	10.0 ± 0.8	10.3 ± 0.8
Mec + Sal	9.0 ± 0.2	8.7 ± 0.7	8.6 ± 0.4	8.6 ± 0.4	9.3 ± 0.6	8.6 ± 0.4	9.1 ± 0.3	9.0 ± 0.4
Mec + NAC	9.5 ± 0.5	10.3 ± 0.3	10.7 ± 0.8	10.3 ± 0.7	10.4 ± 0.6^{a}	10.0 ± 0.9	10.1 ± 1.0	9.9 ± 0.9
HR (bpm)								
Sal + Sal	206 ± 14	208 ± 10	213 ± 9	221 ± 5	226 ± 5	227 ± 8	230 ± 9	235 ± 9
Mec + Sal	200 ± 11	206 ± 3	206 ± 3	205 ± 5	213 ± 9	220 ± 9	232 ± 16	234 ± 14
Mec + NAC	213 ± 13	226 ± 7	221 ± 7	219 ± 9	227 ± 9	221 ± 10	232 ± 19	212 ± 17
MSSD (ms^2)								
Sal + Sal	2.9 ± 0.8	1.4 ± 0.3	0.8 ± 0.2	1.4 ± 0.5	1.3 ± 0.3	1.5 ± 0.3	0.9 ± 0.2	1.5 ± 0.3
Mec + Sal	2.8 ± 0.4	1.9 ± 0.4	1.8 ± 0.4	1.8 ± 0.4	1.1 ± 0.3	1.1 ± 0.2	1.4 ± 0.3	1.9 ± 0.6
Mec + NAC	3.0 ± 0.7	2.7 ± 0.8	4.2 ± 1.4^{ad}	4.4 ± 1.0^{be}	3.9 ± 0.7^{ce}	5.0 ± 1.0^{cf}	6.8 ± 1.1^{cf}	5.0 ± 1.4^{ad}
logLF								
Sal + Sal	−0.5 ± 0.6	−0.7 ± 0.5	−0.6 ± 0.6	−0.3 ± 0.3	0.2 ± 0.5	0.1 ± 0.4	−0.3 ± 0.4	−0.5 ± 0.4
Mec + Sal	−1.7 ± 0.4	−1.1 ± 0.7	−0.9 ± 0.5	−0.8 ± 0.5	−0.3 ± 0.4	−0.2 ± 0.4	−0.5 ± 0.5	−0.3 ± 0.3
Mec + NAC	−0.7 ± 0.7	0.7 ± 0.8	0.4 ± 0.4	0.4 ± 0.6	0.2 ± 0.4	0.3 ± 0.4	0.0 ± 0.9	0.1 ± 0.3
logHF								
Sal + Sal	0.5 ± 0.2	−0.2 ± 0.4	−0.5 ± 0.3	−0.4 ± 0.7	0.2 ± 0.6	0.1 ± 0.1	−0.3 ± 0.3	−0.5 ± 0.4
Mec + Sal	−0.3 ± 0.2	−0.1 ± 0.2	0.0 ± 0.3	0.0 ± 0.3	0.1 ± 0.3	0.0 ± 0.3	−0.2 ± 0.4	0.2 ± 0.4
Mec + NAC	0.5 ± 0.5	0.7 ± 0.5	1.2 ± 0.5^{ad}	0.9 ± 0.6	0.6 ± 0.5	0.9 ± 0.6	0.7 ± 0.7	0.9 ± 0.4

See Methods for the explanation of cardiac acronyms. For between-group comparisons: Mec + NAC *vs.* Mec + Sal: $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$; Mec + NAC *vs.* Sal + Sal: $^{d}p < 0.05$, $^{e}p < 0.01$, $^{f}p < 0.001$

Table 3 Biochemical markers in plasma in saline-instilled sham-treated animals (Sal + Sal group), in meconium-instilled sham-treated animals (Mec + Sal group), and meconium-instilled N-acetylcysteine-treated animals (Mec + NAC group) at the end of experiment

	Sal + Sal	Mec + Sal	Mec + NAC
TBARS (nmol/mg protein)	0.17 ± 0.02	0.27 ± 0.02[c]	0.17 ± 0.01[f]
TAS (mmol/L)	0.80 ± 0.05	0.70 ± 0.03[a]	0.80 ± 0.03[d]
Aldosterone (pg/mL)	213 ± 48	432 ± 56[b]	250 ± 18[e]

TAS total antioxidant status, *TBARS* thiobarbituric-acid reactive substances. For between-group comparisons: Mec + Sal *vs*. Sal + Sal: [a]$p < 0.05$, [b]$p < 0.01$, [c]$p < 0.001$; Mec + NAC *vs*. Mec + Sal: [d]$p < 0.05$, [e]$p < 0.01$, [f]$p < 0.001$

4 Discussion

The future use of anti-inflammatory and antioxidant medicines in MAS can be limited by their eventual adverse effects, e.g., on cardiovascular parameters. As newborns with MAS may suffer from hemodynamic instability, any significant changes in blood pressure or heart rate might be life-threatening.

In the present study, slow intravenous administration of N-acetylcysteine caused short-term changes in cardiovascular parameters of meconium-instilled rabbits. The increases in blood pressure and heart rate variability were observed already during and immediately after NAC administration, and some HRV parameters remained enhanced for several hours till the end of experiments. The finding of increased MABP and simultaneous parasympathetic excitation may seem to be controversial. However, cardiac activity is an integrated signal, which is under the influence of both branches of autonomic nervous system, other underlying physiological mechanisms and various extrinsic factors (von Borell et al. 2007). Thus, heart rate variability cannot be explained only by peculiarities in the sympathovagal balance, but it is determined by more universal mechanisms including intrinsic heart rate (Nadareishvili et al. 2002). The mechanisms of short-term cardiovascular changes following NAC delivery are complex and not completely understood, and we may just speculate on their participation in the observed changes. For example, the barorereflex represents an important mechanism for a short-term blood pressure regulation. From this point of view, intravenous administration of a given volume could evoke baroreflex-linked regulatory alterations resulting in immediate heart rate changes, or these changes might reflect also the mechanical stretching of the sinoatrial node evoked by increased distension of atria. However, no significant increase in blood pressure followed by a decrease in heart rate mediated through the baroreceptor mechanism was observed in the sham-treated animals. Further research is necessary to elucidate the contribution of other, at present unknown mechanisms, to the cardiovascular effects of NAC.

During and shortly after the delivery, NAC increased MABP and HRV. Changes in blood pressure were only transient and disappeared within several minutes. On the other hand, HRV remained enhanced till the end of experiment. This finding confirms the general observation that HRV (especially MSSD) is a highly sensitive marker of cardiovascular autonomic dysregulation, which might be increased also in situations when the other markers are in the normal range.

The observation that the blood pressure and mean heart rate stabilized within several minutes, but changes in heart rate variability persisted till the end of experiment is particularly interesting from the clinical standpoint, as it may influence administration of other medicines. Similarly to our findings, intravenous NAC has a tendency to increase blood pressure and heart rate in piglets during hypoxia-reoxygenation, but it takes several hours for these parameters to return to the level comparable with that in sham-treated animals (Liu et al. 2010). Improved function of the left ventricle after NAC has been found also in patients with myocardial infarction (Yesilbursa et al. 2006).

In the meconium-instilled animals of the present study we found a higher plasma aldosterone level compared with that in the saline-instilled animals. An increase in aldosterone indicates activation of renin-angiotensin-aldosterone axis, playing a key role in the regulation of blood pressure. Aldosterone, through a non-genomic action, elevates vascular resistance, and changes heart rate and baroreflex sensitivity within several minutes (Yee et al. 2001; Weber and Purdy 1982). High aldosterone affects the autonomic nervous system, as it modulates sympathovagal balance toward the sympathetic activity, influences baroreflex responses, potentiates effects of catecholamines, and contributes to arrhythmias (Yee et al. 2001). However, as there were no significant differences between Mec + Sal and Sal + Sal groups in the blood pressure and heart rate, an acute increase in aldosterone may be an indicator of non-specific injury. Higher levels of aldosterone are also present in cardiovascular diseases (Ohtani et al. 2012).

Together with elevated aldosterone, we observed higher production of reactive oxygen species expressed by TBARS. Intensive oxidative stress was associated with a reciprocal decrease in total plasma antioxidant status. A connection of oxidative stress with aldosterone was also demonstrated by a positive correlation between aldosterone and TBARS and a negative one with TAS. These results correspond well with a finding that secretion of aldosterone may be stimulated by oxidized fatty acids (Goodfriend et al. 2004). Thus, higher aldosterone in the meconium-instilled animals could be partially explained by lipid peroxidation indicated by TBARS, by-products of lipid oxidation, and by decreased antioxidant status. On the other hand, attenuation of inflammation after NAC treatment diminished production of TBARS and prevented a decrease in plasma antioxidant systems. An improved clinical status of the treated animals was then underlined by a lower level of plasma aldosterone.

As recently found, there is a strong association between inflammation and autonomic nervous system, particularly reflected in the anti-inflammatory vagal pathway. In a number of disorders, higher production of ROS and pro-inflammatory cytokines is associated with a reduced vagal tone (Papaioannou et al. 2013; Thayer et al. 2011; Danson and Paterson 2006). Many a clinical study reports inverse associations between inflammatory markers and HRV indexes of parasympathetic function (i.e., MSSD and power HF). However, some researchers have found significant correlations between inflammatory markers and indexes of sympathetic or both sympathetic and parasympathetic function, such as power LF and VLF, or total power (Haensel et al. 2008). These findings may reflect the complex response of the sympathetic nervous system to the injury, as this system also modulates the production of cytokines (Nance and Sanders 2007). In the present study, a tendency for increases in HRV parameters (logLF, logHF, and MSSD) was found in the period corresponding with NAC administration, but these parameters remained enhanced for several hours till the end of observation. Chronic changes might be distinct from the acute and subacute ones. Despite on-going inflammation, we did not find any significant differences in the markers of vagal activity (MSSD, logHF) between Mec + Sal and Sal + Sal animals. In addition, except for a positive correlation between aldosterone and logHF at 5 h, no clear relation between the cardiovascular changes and biochemical markers of injury were found.

This study has limitations. Firstly, there are inter-species differences between humans and rabbits in the autonomic cardiac regulation (von Borell et al. 2007; Nadareishvili et al. 2002), which might influence the response to NAC administration. Secondly, the model of MAS used, although generally accepted, may not fully express the hemodynamic changes occurring in neonates within several hours after birth. Thus, a randomized clinical trial is needed to critically evaluate the effects of NAC treatment in the neonatal patients with MAS.

We conclude that NAC effectively reduced health harm caused by the meconium aspiration syndrome in rabbits, as demonstrated by a decrease in TBARS, an increase in TAS, and

a lowering of plasma aldosterone. On the other hand, intravenous administration of NAC led to short-term changes in blood pressure and HRV variables. Thus, systemic administration of NAC should be further evaluated in experimental and clinical studies, and pros and cons of this therapy should be carefully considered.

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Conflicts of Interest The authors declare no conflict of interest in relation to this article.

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Alterations in Vagal-Immune Pathway in Long-Lasting Mental Stress

Z. Visnovcova, D. Mokra, P. Mikolka, M. Mestanik, A. Jurko, M. Javorka, A. Calkovska, and I. Tonhajzerova

Abstract

We studied a potential impact of chronic psychosocial load on the allostatic biomarkers – cardiac vagal activity, inflammation, and oxidative stress in healthy undergraduate students. Continuous resting ECG signals were monitored in a group of 16 female healthy students (age: 23.2 ± 0.2 years, BMI: 20.9 ± 0.5 kg/m^2) at two time periods: right after holiday (rest period) and a day before final exams (stress period). Vagal activity was quantified by spectral analysis of heart rate variability at high frequency band (HF-HRV). The immune response was assessed from the level of tumor necrosis factor-alpha (TNF-α) in plasma. In addition, mean RR intervals were evaluated. We found that HF-HRV was significantly reduced and the TNF-α was increased in the stress period compared with the rest period. No significant changes were found in the RR interval. In conclusion, allostatic load induced by stress and the accompanying greater immune response decreased cardiovagal regulation in healthy young subjects. These findings may help understand the pathway by which stress can influence health and disease.

Keywords

Autonomic nervous system • Heart rate variability • Inflammation • Stress • Vagal activity

1 Introduction

The autonomic nervous system (ANS) plays a key role in health and disease. Normally, the activity of the sympathetic and parasympathetic branches is in dynamic balance indicating adaptability and flexibility of the organism under different challenges. The autonomic imbalance, in particular a decreased cardiac vagal function, may be associated with the

Z. Visnovcova, D. Mokra, P. Mikolka, M. Mestanik, M. Javorka, A. Calkovska, and I. Tonhajzerova (✉)
Department of Physiology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, 4 Mala Hora St., 03 601 Martin, Slovakia
e-mail: tonhajzerova@jfmed.uniba.sk; ingridtonhajzerova@gmail.com

A. Jurko
Pediatric Cardiology, Martin, Slovakia

development of risk factors for cardiovascular morbidity (Thayer and Lane 2007). Importantly, an increased inflammatory activity is now thought to play a major role in cardiovascular diseases. Thus, the function of the parasympathetic (vagal) tone associated with regulation of immune response has gained more attention (Thayer and Sternberg 2006).

Animal models suggest that low levels of parasympathetic activity can increase the magnitude of the inflammatory response, particularly tumor necrosis factor (TNF)-alpha (Borovikova et al. 2000). Therefore, the vagus nerve inhibitory function is critical in the regulation of the immune response *via* cholinergic anti-inflammatory pathway, in which efferent vagal activity tonically inhibits release of pro-inflammatory cytokines mediated by the interaction of acetylcholine with the alpha-7 nicotinic receptor on tissue macrophages (Huston and Tracey 2011). However, a major unanswered question in clinical application is whether it is possible to record neural activity in the vagus nerve as a surrogate marker of activity in the inflammatory reflex to determine the sensitivity of the immune response. From this aspect of view, the efferent vagal modulation can be quantified by the heart rate variability analysis.

Heart rate variability (HRV) – complex beat-to-beat oscillations in the heart rate around its mean value – is a noninvasive tool to measure autonomic regulatory inputs of the heart. The short-term HRV originates predominantly from the activity of the cardiac preganglionic vagal neurons of the nucleus ambiguus, which is crucial for cardio-respiratory coupling – respiratory sinus arrhythmia (RSA). This physiological phenomenon is characterized by a rhythmic increase in heart rate associated with inspiration when respiratory mechanisms in the brainstem attenuate the vagal efferent action on the heart and by a decrease in heart rate during expiratory phase when the vagal efferent influence on the heart is reinstated (Yasuma and Hayano 2004; Korpas and Jakus 2000). Thus, the RSA can be quantified by the HRV spectral analysis at respiratory-linked high frequency (HF-HRV) neuronal oscillations, providing important information about neurocardiac vagal function. It is important to note that recent studies provided human evidence that vagal activity indexed by HF-HRV is inversely related to inflammatory cytokines and, therefore, activation of efferent vagal pathway plays a role in the tonic inhibitory control of the release of proinflammatory mediators including TNF-α (Tonhajzerova et al. 2013; Jan et al. 2010; Marsland et al. 2007).

Furthermore, both autonomic and immune systems represent important allostatic systems regulating adaptive response of the organism to chronic load (McEwen 1998). In particular, TNF-α as a proinflammatory marker produced by macrophages and B lymphocytes has been shown to be associated with psychosocial stress. For example, higher TNF-α levels were found in burnout syndrome (von Känel et al. 2008), and a reduction of TNF-α after a 10-week relaxation program was reported in tinnitus sufferers; thus, the TNF-α has been postulated to be a stress-sensitive immunological marker (Weber et al. 2002). Importantly, the mechanistic role of autonomic dysregulation in the context of stress has been explored in a variety of animal or laboratory models. However, relatively few studies have addressed the association between autonomic dysfunction and chronic real-life stress in humans. Lucini et al. (2005) demonstrated that cardiac vagal control quantified by the HF-HRV is impaired in humans with symptoms of chronic psychosocial stress. In this regard, the vagally-mediated heart rate beat-to-beat oscillations could reflect the central-peripheral nervous system integration, in particular as a potential marker of stress (Porges 2009; Thayer and Lane 2007). Nonetheless, the vagal-immune interaction during chronic allostatic load on the body is still unclear.

In the present study we addressed the hypothesis that vagal-immune interaction is altered in response to the long-lasting mental load. The aim of the study was to evaluate the cardiac vagal function, indexed by HF-HRV, and immune activity, expressed by TNF-α, in response to mental stress evoked by day-to-day

intensive study accompanying the exam period in healthy students.

2 Methods

This study was approved by the Ethics Committee of Jessenius Faculty of Medicine in Martin, Slovakia and was performed in accordance with the Declaration of Helsinki for Human Research. All subjects were carefully instructed about the study protocol and they gave their informed consent to participate prior to examination.

2.1 Subjects

We examined 16 young, female, healthy medical students (mean age 23.2 ± 0.5 years). The exclusion criteria were the following: history of respiratory, endocrinologic, cardiovascular, infectious, mental, or other diseases potentially influencing HRV (including obesity, underweight, overweight, alcohol, or drug abuse). Smokers also were excluded from this study. All subjects were instructed not to use substances which affect the cardiovascular system (caffeine, alcohol) for at least 12 h before the recording. Importantly, because hormonal changes during menstrual cycle can affect the cardiac autonomic regulation (Hirshoren et al. 2002), the females were included being in the proliferative menstrual phase. Anthropometric characteristics of the participants were examined using an InBody J10 (Biospace; Seoul, South Korea), with the technology of direct segmental multi-frequency bioimpedance analysis (DSM-BIA), and they are presented in Table 1.

Table 1 Anthropometric characteristics ($n = 16$)

Age (year)	23.2 ± 0.2
Body mass index (kg/m^2)	20.9 ± 0.5
Waist-to-hip ratio	0.80 ± 0.01
Percentage fat (%)	23.6 ± 1.6

Data are means ± SE

2.2 Protocol

All subjects were examined under standard conditions: a quiet room in a light and temperature-controlled laboratory, in the morning between 9.00 a.m.–12.30 p.m., and after normal breakfast 2 h prior to the examination. The subjects were instructed to sit comfortably in a special armchair and not to speak or move unless necessary. A thoracic belt with ECG telemetric electrodes for R-R intervals recording with sampling frequency of 1,000 Hz (VarCorPF8, Olomouc, Czech Republic) was applied after initial 15 min of the rest period required for heart rate stabilization. Then, the subjects remained in the sitting position for a continuous ECG recording. All subjects were examined twice: at the winter term beginning after holidays (rest period) and at the exam period ending the day before the final exam (stress period).

2.3 Data Analysis

Spontaneous short-term heart rate variability: 300 R-R intervals segments were analyzed between the first and fifth minute of the recording. Slower oscillations and trends were eliminated using the detrending procedure of Tarvainen et al. (2002) and time series were resampled (resampling frequency of 2 Hz) to obtain equidistant time series using cubic spline interpolation. Subsequently, mean power spectrum of the analyzed segment was computed by fast Fourier transform (using window length of 256 samples) and spectral power in the high frequency band (HF: 0.15–0.4 Hz) was obtained by integration. We focused on the high frequency spectral power of the HRV (HF-HRV) reflecting mainly respiratory sinus arrhythmia indicating cardiovagal regulatory inputs. In addition, mean R-R interval was calculated.

2.4 Inflammatory Marker Assay

Blood samples were collected to EDTA tubes in the fasting state at least 3 h before the examination in both (rest and stress) periods. The blood

was centrifuged immediately and kept frozen at −80 °C until analysis. The concentration of the proinflammatory marker – tumor necrosis factor-alpha (TNF-α) was assessed using commercially available human ultra-sensitive ELISA kits (Invitrogen; Camarillo, Canada).

2.5 Statistical Analysis

Data were expressed as means ± SE. The nongaussian/gaussian distribution was ascertained by the Lilliefors test. Because HF-HRV index had a skewed distribution, the values were logarithmically transformed to be able to use a *t*-paired test for normal distribution. $P < 0.05$ was considered as statistically significant. Statistical analysis was performed using a commercial software package SYSTAT ver. 10 for Windows (SSI, Richmond, CA).

3 Results

3.1 Heart Rate Variability

The high frequency oscillations of the heart rate variability (HF-HRV) were significantly reduced in the stress period compared with the rest period ($p = 0.045$). No significant changes were found in the mean RR intervals (Table 2).

Table 2 Heart rate variability and TNF-α in the rest period (P1) and stress period (P2)

	Rest period	Stress period	p
Heart rate variability			
RR interval (ms)	858 ± 29	836 ± 25	0.504
logHF-HRV (ms^2)	6.46 ± 0.15	5.99 ± 0.21	0.045
Immune response			
TNF-α (pg/ml)	2.09 ± 0.10	2.41 ± 0.09	0.025

Values are means ± SE
logHF-HRV logarithmic values of spectral activity in the high-frequency band of heart rate variability, *TNF- α* tumor necrosis factor-alpha

3.2 Inflammatory Marker

The concentration of the inflammatory marker TNF-α was significantly higher in the stress period compared with the rest period ($p = 0.025$; Table 2).

4 Discussion

Chronic allostatic load, a burden of chronic stress and accompanying changes in personal behaviors, may lead to a disease in the long-run, mediated *via* autonomic, neuroendocrine, or immune system activity (McEwen 1998). Sympathetic predominance, vagal withdrawal, and baroreflex impairment represent the autonomic counterpart of the complex psychophysiological changes underlying the increase in cardiovascular risk associated with long-lasting stress (Rosengren et al. 2004). As previously noted, chronic psychological stress and the inflammatory response have been implicated in the etiology and pathogenesis of certain cardiovascular diseases, such as atherosclerosis, and of other diseases, e.g., obesity (Hamer et al. 2012). However, the response of vagal-immune pathway to allostatic load evoked by chronic stress is still unclear. In acute stress, Weber et al. (2010) demonstrated that healthy subjects with low HRV had delayed recovery of TNF-α up to an hour after the stressor had ended and suggest that vagal function is coordinated with the regulation of both acute as well as chronic inflammation in healthy humans. Our results support this hypothesis. Decreased cardiovagal regulation associated with a greater immune response as a result of allostatic load evoked by a long-lasting stress-related exam period could represent the pathomechanism by which dysregulated vagal-immune homeostasis increases the risk of inflammatory conditions associated with chronic real-life stress.

Several explanations are assumed for these findings. Firstly, the common central neural areas regulating both immune and cardiovagal functions were found in recent studies. For example, the neuroimaging research showed

associations between vagally mediated HRV and activity in specific brain areas, such as prefrontal, anterior cingulate cortex, insula, or amygdala (Thayer et al. 2012). These brain areas are also associated with the regulation of immune responses (Rosenkranz et al. 2005). Thus, cortical structures are involved in immunomodulation at least partially *via* the neural concomitants of the cholinergic anti-inflammatory pathway (Thayer et al. 2011). Taken together, the altered vagal-immune communication can be explained by discrete stress-linked control abnormalities in the common neurobiological regulatory basis for both autonomic and immune systems. Furthermore, the neurovisceral theory emphasizes the tonic inhibitory control of the prefrontal cortex on the subcortical regions, including amygdala, linked to the regulation of the immune system *via* the vagus nerve (Thayer and Sternberg 2006). Consequently, lack of inhibitory input by the vagus nerve might lead to perturbations in the neuroimmune pathway involved with the allostatic overload as observed in our studied group.

Secondly, psychological processes linked to stress (e.g., chronic emotional state, exhaustion, anxiety/depressive symptoms) or life-style modifications (e.g., physical inactivity accompanying day-to-day intensive study) are associated with a decrease in vagal activity (Thayer et al. 2012; Tonhajzerova et al. 2010). Therefore, in agreement to the neurovisceral theory, disruption of vagal inhibitory function may contribute to emotion-related activation of proinflammatory pathways (Marsland et al. 2007). It seems that the question of whether the altered vagal-immune pathway associated with chronic real-life stress in healthy subjects is a pure result of disrupted neurobiological integrity linked to the cholinergic anti-inflammatory pathway or it is only a reflection of stress-related psychological expression and behavioral inflexibility remains unclear.

Our findings support the hypothesis that the interventions related to the regulation of both vagal control and inflammation may be of particular importance (Huston and Tracey 2011). In particular, psychological methods may be effective in parasympathetic activity increase. Initial findings show that hypnosis and meditation increase vagal nerve output and inhibit immune responses (Aubert et al. 2009). The possibility that these and other interventions might influence immunity through activation of the parasympathetic activity warrants further investigation and may have valuable clinical implications for the treatment of inflammatory and cardiovascular disorders (Marsland et al. 2007).

5 Conclusion

We conclude that long-lasting mental load is associated with an alteration in vagal-immune communication shaping the allostatic complex system: decreased vagal activity could lead to disruption of vagal inhibitory function on the inflammatory response resulting in an excess of proinflammatory cytokines. The illumination of the vagal-immune pathway in studies on chronic stress can help understand health and disease.

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Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Nocturnal Parasympathetic Modulation of Heart Rate in Obesity-Hypoventilation Patients

A. Brzecka, M. Pawelec-Winiarz, A. Teplicki, P. Piesiak, and R. Jankowska

Abstract

Heart rate variation (HRV) reflects the activity of the autonomic nervous system. The aim of the study was to analyze HRV in obstructive sleep apnea (OSA) and obesity hypoventilation (OH) patients to answer the question of whether chronic alveolar hypoventilation influences autonomic heart rate regulation. In 41 patients, diagnosed with either 'pure' OSA ($n = 23$, apnea/hypopnea index – AHI: 43.8 ± 18.0 $PaCO_2 \leq 45$ mmHg) or OH syndrome ($n = 18$, AHI 58.7 ± 38.0 $PaCO_2 > 46$ mmHg), the HRV was analyzed, based on an 8 h ECG recording during sleep. In the OH patients, compared with the OSA patients, there was a globally decreased HRV, with a corresponding decrease in high frequency power in the spectral analysis of HRV and increased low frequency/high frequency ratio ($p < 0.05$), indicating a reduced parasympathetic and increased sympathetic heart rate modulation. We conclude that hypoxemia and hypercapnia of the sleep disordered breathing have an impact on the autonomic heart rate regulation. HRV indices have a potential to become prognostic factors for the development of cardiovascular complications in patients with sleep disordered breathing.

Keywords

Cardiovascular complications • Polysomnography • Respiratory insufficiency • Sinus rhythm • Sleep disordered breathing

A. Brzecka (✉), P. Piesiak, and R. Jankowska
Department of Pneumology and Lung Cancer, Wroclaw Medical University, 105 Grabiszynska St., 53-439 Wroclaw, Poland
e-mail: anna.brzecka@umed.wroc.pl

M. Pawelec-Winiarz
Iserian Center of Pneumology and Chemotherapy, Szklarska Poreba, Poland

A. Teplicki
Department of Internal Medicine, Fourth Military Clinical Hospital, Wroclaw, Poland

1 Introduction

The obesity hypoventilation (OH) is the most severe form of the obstructive sleep apnea (OSA) syndrome, with an accompanying day-time hypercarbia ($PaCO_2$) greater than 45 mmHg (Borel et al. 2012). In OSA, there appear repeated episodes of respiratory airflow cessation during sleep lasting for more than 10 s. The cessation of naso-buccal air flow during

sleep is caused by a collapse of upper airways, mostly at the level of the oropharynx, with concomitant, although ineffective, respiratory movements of the diaphragm. Termination of an episode of apnea depends on arousal that, in turn, leads to the restoration of upper airway muscle tone. In case of partial obstruction of upper airways, an episode of hypopnea develops with the same pathophysiological consequences as does a complete upper airway closure. Sleep apneic or hypopneic episodes lead to sleep fragmentation and arterial oxygen desaturation, with subsequent reoxygenation.

A consequence of sleep disordered breathing is increased sympathetic discharge with resulting hypertension, cardiac arrhythmia, myocardial infarction, and stroke (Gami et al. 2013). There is increased risk of cardiovascular complications in patients with OH syndrome compared with both general population and patients with 'pure' OSA syndrome; the incidence of circulatory/cardiovascular morbidities prior to the diagnosis of OH is almost twice as high as in patients with OSA syndrome without chronic alveolar hypoventilation: 20 % *vs.* 11 %, respectively (Jennum et al. 2013).

Heart rate is modulated by a balance between sympathetic (SNS) and parasympathetic nervous systems (PNS), both in health and disease. Thus, measuring heart rate changes may provide the information about the autonomic influence on the sino-atrial node. The PNS instantaneously mediates heart rate through changes in acetylcholine binding. Thus, changes in heart rate, following changes in respiratory rhythm, reflect changing PNS activity (Stein and Pu 2012). However, when breathing is very slow, changes in heart rate reflect both PNS and SNS influences, as SNS acts through the cascade of complex signals initiated by the binding of norepinephrine, which involves a certain time lag (Stein and Pu 2012).

The autonomic cardiovascular modulation may be explored on the basis of computed analysis of heart rate variation (HRV); the variation of the interval between heart beats (Stein and Pu 2012; Task Force of ESCNASPE 1996). As a disturbed relationship between sympathetic and parasympathetic influences may be associated with the risk of cardiovascular disorders, it seems worthwhile to explore cardiac autonomic modulation in a high risk group, such as the OH patients.

2 Methods

The study was approved by the Ethics Committee of the Medical University in Wroclaw, Poland. A total of 41 patients aged 33–67 (mean 57.3 ± 6.6 years) were enrolled into the study. There were two groups of patients – 'pure' OSA syndrome ($n = 23$) and OH syndrome ($n = 18$), with 6 and 2 women, respectively. The inclusion criteria were the following: in-hospital nocturnal respiratory polygraphy, to diagnose sleep-related breathing disorders, combined with cardiac recordings to assess HRV in time- and frequency-domains.

Sleep-related breathing disorders included moderate to severe OSA (apnea/hypopnea index – AHI > 15) and OH syndrome. The mean AHI in the whole group was 50.4 ± 20.1. The OH syndrome was diagnosed when the following three conditions were present: AHI > 10, obesity – BMI > 30 kg/m^2, and chronic alveolar hypoventilation expressed by chronically elevated daytime $PaCO_2 > 45$ mmHg, with no other obvious clinical reasons of daytime hypercarbia. The exclusion criteria were: age exceeding 70 years, cardiac arrhythmias, and coexistence of severe co-morbidities such as malignancies or asthma.

The patients underwent nocturnal polygraphic breathing studies. The following parameters were continuously monitored: oronasal airflow, pulse oximetry, respiratory chest and diaphragmatic movements, and a one-lead EKG. Recordings took 8 h; from 10 p.m. to 6 a.m. The following parameters of HRV were analyzed: SDNN – standard deviation of all NN (i.e., between consecutive R waves) intervals (ms), SDNN index – mean of the standard deviations of all NN intervals (ms),

SDANN – standard deviation of the averages of NN intervals (ms), RMSSD – square root of the mean of the sum of the squares of differences between adjacent NN intervals (ms), NN50c – number of interval differences of successive NN intervals greater than 50 ms, NN50%–the proportion derived by dividing NN50 by the total number of NN intervals, TI – triangular index, TP – total power, i.e., variance of NN intervals (ms^2), VLF – power in very low frequency range (ms^2), LF – power in low frequency range (ms^2), HF – power in high frequency range (ms^2), and LF/HF ratio.

Normally distributed data were presented as means ± SD. Non-normally distributed data were presented as median and minimal-maximal values. To compare normally distributed data a *t*-test was used, otherwise the Mann Whitney U test was used. The significance level was set at $p < 0.05$. Statistical analysis was performed using a commercial Statistica ver. 10 package.

3 Results

Basic clinical characteristics of patients are presented in Table 1. Age, weight, and BMI of the patients in the two groups were similar. The OH patients tended to be more sleepy during daytime, but the difference in the ESS score between the two groups failed to reach significance. The OH patients were significantly more hypoxic and hypercapnic, with a compensatory increase in bicarbonate ions. The ventilatory function showed a more restrictive pattern in the OH patients, consisting of lower VC and FEV_1 %predicted values. The FEV_1/FVC ratio was similar in both groups. There were 6 patients (three in the hypercapnic and three in the normocapnic group) with FEV_1/FVC ratio below 70 % (52–69 %), and the lowest value of FEV_1 in these patients was 1.7 l, indicating that bronchial obstruction was mild-to-moderate and

Table 1 Clinical features of patients with 'pure' OSA and OH syndromes

	OSA (n = 23)	OH (n = 18)
Age (yr)	57.9 ± 6.6	56.6 ± 6.6
Weight (kg)	117.7 ± 23.5	127.9 ± 18.3
Height (m)	1.70 ± 0.09	1.76 ± 0.10*
BMI (kg/m^2)	41.7 ± 6.6	41.4 ± 7.0
ESS (points)	8.0 ± 3.2	11.7 ± 6.0
$PaCO_2$ (mmHg)	41.3 ± 3.7	49.5 ± 3.7**
HCO_3 (mmol/l)	26.7 ± 1.9	29.8 ± 1.8**
PaO_2 (mmHg)	64.6 ± 6.8	58.4 ± 8.4*
SaO_2 (%)	92.3 ± 2.9	89.5 ± 3.7*
pH	7.43 ± 0.03	7.40 ± 0.02**
VC (l)	3.5 ± 1.0	3.5 ± 9.4
VC (% of predicted)	97.9 ± 13.5	78.0 ± 14.4**
FEV_1 (l)	2.7 ± 5.3	2.6 ± 8.3
FEV_1 (% of predicted)	94.0 ± 14.9	77.0 ± 17.5**
FEV_1/FVC (%)	80.5 ± 15.4	77.4 ± 11.3
AHI (episodes/hour)	43.8 ± 18.0	58.7 ± 38.0
SaO_2, mean of minimal at the end of apnea/hypopnea (%)	82.5 ± 6.4	79.2 ± 6.9
SaO_2, average nocturnal (%)	87.2 ± 4.7	83.8 ± 5.9
SaO_2, minimal nocturnal (%)	67.6 ± 11.6	66.9 ± 10.9

Data are means ± SD

OSA obstructive sleep apnea, *OH* obesity hypoventilation, *BMI* body mass index, *ESS* Epworth Sleepiness Scale, $PaCO_2$ arterial pressure of carbon dioxide, HCO_3 bicarbonate ion, PaO_2 arterial oxygen partial pressure, SaO_2 arterial oxygen saturation, *VC* vital capacity, FEV_1 forced expired volume in one second, and *AHI* apnea/hypopnea index

*$p < 0.05$; **$p < 0.01$

Table 2 Time-domain parameters of HRV in patients with 'pure' OSA and OH syndromes

	OSA (n = 23)	OH (n = 18)
SDNN	155 (80–230)	117 (70–396)*
SDNN index	151 (60–326)	100 (40–388)*
RMSSD	183 (40–453)	98 (29–605)*
NN50c	3,171 (27–9,629)	3,768 (500–11,737)
NN50%HR	19 (6–79)	18 (2–56)
SDANN	261 (31–4,582)	105 (45–577)*
TI	16 (9–29)	16 (10–26)

Data are medians (min-max)
OSA obstructive sleep apnea, *OH* obesity-hypoventilation, *SDNN* standard deviation of all NN, i.e., distances between consecutive R-R intervals (ms), *SDNN index* mean of the standard deviations of all NN intervals (ms), *SDANN* standard deviation of the averages of NN intervals (ms), *RMSSD* square root of the mean of the sum of the squares of differences between adjacent NN intervals (ms), *NN50c* number of interval differences of successive NN intervals greater than 50 ms, *NN50%* the proportion derived by dividing NN50 by the total number of NN intervals, and *TI* triangular index
*$p < 0.05$

Table 3 Frequency-domain parameters of HRV in patients with 'pure' OSA and OH syndromes

	OSA (*n = 23*)	OH (*n = 18*)
TP	17,415 (3,190–126,666)	29,231 (8,711–96,914)
VLF	5,865 (606–68,265)	12,619 (1,352–60,972)
LF	5,612 (891–34,436)	8,359 (2,357–30,980)
HF	2,783 (466–7,541)	2,546 (976–6,750)
LF/HF	1.9 (0.9–7.5)	3.0 (1.7–9.0)*

Data are medians (min-max)
OSA obstructive sleep apnea, *OH* obesity-hypoventilation, *TP* total power, i.e., variance of NN intervals (ms^2), *VLF* power in very low frequency range (ms^2), *LF* power in low frequency range (ms^2), *HF* power in high frequency range (ms^2), and LF/HF ratio
*$p < 0.05$

could not be the primary cause of chronic alveolar hypoventilation. Although the severity of sleep breathing disorders, as measured by AHI and nocturnal desaturation, was not significantly different in both groups, the patients with OH experienced somehow more breathing disorders, with a higher AHI and lower nocturnal mean and minimal SaO_2 at the end of apneas and hypopneas.

The results of time-domain parameters of HRV are presented in the Table 2. In the OH syndrome there were significantly lower values of SDNN, SDNN index, RMSSD, and SDANN, compared with OSA, with no appreciable differences in NN50c, NN50%, and TI. Concerning the frequency-domain of HRV, LF/HF ratio was significantly greater in the OH than that in OSA patients (Table 3).

To exclude the possible gender influence on the HRV differences between the OSA and OH patients, comparison of clinical features of female and male patients was made. Women were older (61.5 ± 4.0 years *vs.* 56.1 ± 6.1 years, $p < 0.05$), of shorter stature (1.62 ± 0.06 m *vs.* 1.76 ± 0.07 m, $p < 0.001$), had lower FVC (2.6 ± 0.4 l *vs.* 3.7 ± 0.9 l, $p < 0.05$ l), lower FEV_1 (2.2 ± 0.3 l *vs.* 2.8 ± 0.7 l, $p < 0.01$), and lower FEV_1/FVC (87 ± 13 *vs.* 76 ± 12 %, $p < 0.05$) than men, respectively. Despite those differences, the results of HRV studies were similar in men and women.

We also excluded the possible influence of the presence of diabetes on the inter-group HRV differences. There were 8 (35 %) diabetic patients in the OSA group and 11 (61 %) in the OH group. The only clinical difference between diabetic and non-diabetic patients concerned the FVC which was lower in the former (81.0 ± 14.6 % *vs.* 93.0 ± 17.9 % of predicted, $p < 0.05$). There were no differences regarding the HRV results between diabetic and non-diabetic patients.

4 Discussion

The results of the current study indicate that OH patients had reduced parasympathetic heart rate modulation during sleep compared with OSA patients as verified by nocturnal HVR analysis.

HRV analysis encompasses time- and frequency-domains. Time-domain parameters are the derivatives of beat-to beat measurements and enable the estimation of high frequency variations in heart rate (Task Force of ESCNASPE 1996). The time-domain includes SDNN, the most global HRV measurement, capturing the total HRV which is relatively

insensitive to small errors. Beat-to-beat changes are also reflected in pNN50 and RMSSD measured over the entire recording time. Decreased HRV reflects reduced parasympathetic activity (Stein and Pu 2012). However, it is not possible to distinguish between the central or peripheral parasympathetic influences, as there are three main mechanisms causing changes in heart rate: signals from pulmonary stretch receptors in the vagal nerves, central medullary outputs coupling respiratory and cardiac stimuli, and arterial baroreflex (Jo et al. 2005).

Frequency-domain parameters consist of VLF power, reflecting the underlying periodicities at frequencies of 0.0033–0.04 Hz, LF power corresponding to 0.04–0.15 Hz, and HF power corresponding to 0.15–0.4 Hz (Task Force of ESCNASPE 1996). VLF is modulated by sympathetic signals and LF by both parasympathetic and sympathetic signals (Kleiger et al. 2005), whereas HF is modulated by parasympathetic efferent vagal activity; LF/HF ratio providing the estimation of sympathovagal balance (Stein and Pu 2012; Task Force of ESCNASPE 1996).

The sympathetic-parasympathetic balance, using the HRV estimation, has been previously studied in OSA patients and abnormally low levels of overall HRV, with a corresponding low HF power have been found (Zhong et al. 2013). Song et al. (2012) found that LF/HF ratio was associated with the severity of OSA syndrome, as indicated by AHI, suggesting increased sympathetic tone in OSA patients. Comparison of overnight HRV studies in severe OSA (AHI 65 ± 23) and healthy subjects revealed that NN was the best index quantifying the overnight sympathovagal balance in OSA patients (Zhu et al. 2012). A study of HRV during sleep in women revealed the signs of increased sympathetic activity in patients with moderately severe, but not very severe, OSA syndrome (Kesek et al. 2009). Cardiac sympathetic and parasympathetic modulation may improve, as shown by frequency domain analysis, in OSA patients after successful upper-airway surgical treatment, confirming the relationship between impaired autonomic heart modulation and the severity of OSA (Choi et al. 2012).

Data on the relationship between chronic alveolar hypoventilation and HRV are scarce. A recent study on HRV in sleep related alveolar hypoventilation (SRAH) reports abnormal cardiac tone during sleep (Palma et al. 2013); the finding confirmed in the present study. However, those authors did not find important differences either in time- or frequency-domains of nocturnal HRV in sleep-disorder breathing with or without chronic alveolar hypoventilation. In the present study we found that OH patients, as compared with OSA patients, had decreased time-domain parameters of HRV, indicating reduced parasympathetic in relation to sympathetic activity. Although OH patients had inappreciably greater frequency of sleep apneas-hypopneas than OSA patients, they were more desaturated during sleep; the combined effect of which was greater daytime hypercarbia and hypoxemia, pointing to chronic alveolar hypoventilation.

Obesity, in general, reduces time- and frequency-domains that quantify HRV (Karason et al. 1999). Some studies suggest a shift in sympathovagal balance toward the sympathetic dominance in obese subjects (Schmid et al. 2010), while others do not support the presence of a relationship between BMI and the time-domain of HRV (Windham et al. 2012). In this study, OH and OSA patients had a similar degree of obesity (BMI of about 41 kg/m^2 for both groups). Thus, obesity was unlikely to bear on the differences found between the two groups. Nor gender appeared to have any bearing on our findings as HRV was similar in women and men. Gender, however, might be a modulator of HRV; parasympathetic activity being increased in women (Kim and Woo 2011).

The results of the present study could have been modified by the exclusion criteria employed. For instance, we excluded patients with asthma. In asthma, even asymptomatic, there is increased central vagal outflow and decreased sympathetic efferent activity (Gupta et al. 2012). Likewise, patients with malignancies were excluded due to the known autonomic dysfunction in cancer (Fadul et al.

2010). Patients with cardiac arrhythmias were not included either, as arrhythmic events influence HRV analysis (Task Force of ESCNASPE 1996). We also put an age limit of 70 on the patients' enrollment, as increasing age is associated with an overall reduction in autonomic control of the heart, accompanied by increased sympathetic and decreased parasympathetic modulation (Abhishekh et al. 2013).

This study has some limitations. Smoking status of patients was not controlled for. Smoking strongly influences HRV (Behera et al. 2013). However, there is no reason to suppose that smoking habits were different in OH and OSA patients. It should be underlined that the abnormalities in HRV notably concerned the SDNN and SDANN, both considered of the highest prognostic value among time-domain HRV indices, especially in patients with existing heart diseases (Stein et al. 1993).

We conclude that decreased HRV in OH patients may indicate the influence of hypoxemia and hypercapnia on autonomic heart rate regulation. The finding of decreased HRV in OH patients is of importance since it has been found, in both clinical and population studies, that reduced HRV may be associated with increased cardiovascular mortality (Kleiger et al. 2005), and *vice versa* increased vagal activity has protective effects, particularly in cardiac patients (Nolan et al. 1998).

Conflicts of Interest The authors declare no conflict of interest in relation to this article.

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N-acetylcysteine Alleviates the Meconium-Induced Acute Lung Injury

D. Mokra, A. Drgova, M. Petras, J. Mokry, M. Antosova, and A. Calkovska

Abstract

Meconium aspiration in newborns causes lung inflammation and injury, which may lead to meconium aspiration syndrome (MAS). In this study, the effect of the antioxidant N-acetylcysteine on respiratory and inflammatory parameters were studied in a model of MAS. Oxygen-ventilated rabbits were intratracheally given 4 mL/kg of meconium (25 mg/mL) or saline. Thirty minutes later, meconium-instilled animals were administered N-acetylcysteine (10 mg/kg; i.v.), or were left without treatment. The animals were oxygen-ventilated for additional 5 h. Ventilatory pressures, oxygenation, right-to-left pulmonary shunts, and leukocyte count were measured. At the end of experiment, trachea and lung were excised. The left lung was saline-lavaged and a total and differential count of cells in bronchoalveolar lavage fluid (BAL) was determined. Right lung tissue strips were used for detection of lung edema (expressed as wet/dry weight ratio) and peroxidation (expressed by thiobarbituric acid-reactive substances, TBARS). In lung and tracheal strips, airway reactivity to acetylcholine was measured. In addition, TBARS and total antioxidant status were determined in the plasma. Meconium instillation induced polymorphonuclear-derived inflammation and oxidative stress. N-acetylcysteine improved oxygenation, reduced lung edema, decreased polymorphonuclears in BAL fluid, and diminished peroxidation and meconium-induced airway hyperreactivity compared with untreated animals. In conclusion, N-acetylcysteine effectively improved lung functions in an animal model of MAS.

D. Mokra (✉), M. Antosova, and A. Calkovska
Department of Physiology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, 4 Mala Hora St., SK-03601 Martin, Slovakia
e-mail: mokra@jfmed.uniba.sk

A. Drgova and M. Petras
Department of Medical Biochemistry, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovakia

J. Mokry
Department of Pharmacology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovakia

Keywords
Inflammation • Lung injury • Meconium aspiration • N-acetylcysteine • Oxidative stress

1 Introduction

Meconium aspiration syndrome (MAS) is a major cause of respiratory failure in neonates at term. Shortly after meconium aspiration, signs of airway obstruction might be observed, with hypoxemia, hypercarbia, and acidosis in the laboratory investigation. When aspirated meconium moves distally to the peripheral airways and alveolar compartment, surfactant dysfunction, neutrophil-derived inflammation, pulmonary vaso- and bronchoconstriction, and lung edema gradually develop. In patients with severe MAS, mechanical ventilation with higher concentrations of oxygen, vasodilators, and exogenous surfactant are used (Mokra et al. 2013).

Due to a key role of inflammation in the pathogenesis, anti-inflammatory medicaments including antioxidants might be beneficial in MAS (Mokra et al. 2013). For instance, superoxide dismutase (SOD) enzymatically catalyzes dismutation of superoxide into oxygen and hydrogen peroxide, and it works as an antioxidant defense in oxygen-exposed cells. In a rat model of MAS, intratracheal administration of recombinant human SOD decreases myeloperoxidase activity, NO, and 8-isoprostane as well as lung injury score (Lu et al. 2005). Similarly, in newborn lambs with persistent pulmonary hypertension, SOD increases oxygenation and reduces vasoconstriction and oxidative injury (Lakshminrusimha et al. 2006).

N-acetylcysteine (NAC) is an antioxidant that contains SH groups, and thereby scavenges hydrogen peroxide, hydroxol radicals, and hypochlorous acid. NAC is deacetylated to cysteine, a precursor of glutathione in cells, and thus stimulates the glutathione system. In addition, NAC reduces generation of pro-inflammatory mediators (e.g., TNF-alpha and IL-1). Besides potent antioxidant and anti-inflammatory effects, NAC reduces viscosity and elasticity of mucus because of its ability to reduce disulphide bonds (Gillissen and Nowak 1998). NAC may promote expression of surfactant protein (SP-A), and may improve its surface activity (Fu et al. 2000). Although NAC clearly alleviates inflammation and oxidative stress, both underlying the pathomechanism of MAS, it has not yet been tried in MAS treatment. Therefore, the purpose of the present study was to evaluate whether NAC might improve lung function and mitigate the meconium-induced acute lung injury in an animal model of MAS.

2 Methods

2.1 General Design of Experiments

The experiments were approved by a local Ethics Committee of Jessenius Faculty of Medicine and National Veterinary Board in Slovakia. Meconium was collected from healthy term neonates, lyophilized, and stored at −20 °C. Before use, meconium was suspended in 0.9 % NaCl at a concentration of 25 mg/mL. Adult chinchilla rabbits, weighing 2.5 ± 0.3 kg, were anesthetized with ketamine (20 mg/kg i.m.; Narketan, Vétoquinol, UK) and xylazine (5 mg/kg; Xylariem, Riemser, Germany), followed by infusion of ketamine (20 mg/kg/h). Tracheotomy was performed and catheters were inserted into a femoral artery and right atrium for blood sampling, and into a femoral vein to administer anesthetics. The animals were then paralyzed with pipecuronium bromide (0.3 mg/kg/30 min; Arduan, Gedeon Richter, Hungary) and subjected to a pressure-controlled ventilator (Beat-2, Chirana, Slovakia). All animals were

ventilated with a frequency of 30/min, fraction of inspired oxygen (FiO_2) of 0.21, inspiration time Ti 50 %, peak inspiratory pressure (PIP) to keep a tidal volume between 7 and 9 mL/kg, and no positive end-expiratory pressure (PEEP) at this stage of experiment. After 15 min of stabilization, cardiopulmonary parameters were recorded and blood gases were analyzed (RapidLab 348, Siemens, Germany). Then, rabbits were intratracheally administered 4 mL/kg of meconium suspension or saline (Sal group, n = 5, served as controls). From this moment on, animals were ventilated with FiO_2 1.0 and PEEP 0.3 kPa. In the meconium-instilled animals, respiratory failure developed within 30 min, defined as >30 % decrease in dynamic lung-thorax compliance (C_{dyn}) and $PaO_2 < 10$ kPa at FiO_2 1.0. After recording the parameters, meconium-instilled animals were treated with N-acetylcysteine (10 mg/kg, i.v.; ACC Injekt, Salutas Pharma GmbH, Germany) 30 min after intratracheal meconium instillation (Mec + NAC group, n = 6), or were left without treatment (Mec group, n = 6). All animals were oxygen-ventilated for additional 5 h. Blood gases and respiratory parameters were recorded 30 min after Mec or Sal instilation and then at 30 min, 1, 2, 3, 4, and 5 h afterwards. At the end of experiments, animals were sacrificed by an overdose of anesthetics.

2.2 Measurement of Respiratory Parameters

Tracheal airflow and tidal volume were measured by a heated Fleisch head connected to a pneumotachograph. Airway pressure was registered *via* a pneumatic catheter placed in the tracheal tube and connected to electromanometer. The mean airway pressure was calculated as: MAP = (PIP + PEEP)/2. C_{dyn} was expressed as a ratio between tidal volume (adjusted per kg) and airway pressure gradient (PIP-PEEP). Oxygenation index (OI) was calculated as: $OI = (MAP \times FiO_2)/PaO_2$.

Right-to-left pulmonary shunts were calculated by a computer program using the Fick equation: $(CcO_2 - CaO_2)/(CcO_2 - CvO_2) \times 100$, where CcO_2, CaO_2 and CvO_2 are concentrations of oxygen in pulmonary capillaries, arterial and mixed blood. CcO_2 was calculated by using P_AO_2 (alveolar partial pressure of oxygen) from the equation: $P_AO_2 = (PB - PH_2O) \times (FiO_2 - PaCO_2) \times [FiO_2 + (1 - FiO_2)/R]$, where PB is barometric pressure and PH_2O the pressure of water vapour. Respiratory exchange ratio (R) was assumed to be 0.8 and the current value of hemoglobin necessary for calculating the oxygen concentration in the blood was measured by combined analyzer (RapidLab 348, Siemens, Germany).

2.3 Counting of Cells in BAL Fluid and in Arterial Blood

Samples of arterial blood were taken before meconium instillation and at 1, 3, and 5 h of the treatment. A total leukocyte count was determined microscopically in a counting chamber after staining by Türck and differential leukocyte count was estimated microscopically after staining by Pappenheim.

After sacrificing the animal, lungs and trachea were excised. Left lungs were lavaged by saline (0.9 % NaCl, 37 °C) 3 × 10 mL/kg, bronchoalveolar lavage (BAL) fluid was centrifuged at 1,500 rpm for 10 min. Total number of cells in BAL fluid was determined microscopically in a counting chamber. Differential count of cells in BAL fluid sediment was evaluated microscopically after staining by Pappenheim.

2.4 Lung Edema Formation (Wet/Dry Weight Ratio)

Strips of the right lung tissue were cut, weighed, and dried at 60 °C for 24 h to determine the wet/dry weight ratio.

2.5 Markers of Oxidative Stress

Quantification of total antioxidant status (TAS) in blood plasma at the end of experiment was carried out using ABTS (2,2′-azino-di-[3-ethylbenzothiazoline sulphonate]) radical formation kinetics (Randox TAS kit, Randox laboratories Ltd., UK) and expressed in mmol/L. Thiobarbituric-acid reactive substances (TBARS) were determined in the plasma from the absorbance at 532 nm and expressed in nmol/mg protein.

2.6 Measurement of In Vitro Airway Reactivity to Acetylcholine

Tracheal and lung smooth muscle reactivity was estimated by *in vitro* methods. Muscle strips were mounted between two hooks and placed into the organ chamber containing Krebs-Henseleit's buffer at 36.5 ± 0.5 °C, aerated with 95 % O_2 and 5 % CO_2 to maintain pH 7.5 ± 0.1. One of the hooks was connected to a force transducer and amplifier, and tension changes were recorded by computer software (all equipment RES Martin, Slovakia). Tissue strips were initially set to 4 g of tension for 30 min (loading phase). Then, tension was readjusted to a baseline value of 2 g for another 30 min (adaptation phase). During both periods, tissue strips were washed at 10 min intervals. Thereafter, cumulative doses of acetylcholine (10^{-8} to 10^{-3} mol/L, Sigma-Aldrich, Germany) were added and contractions were recorded. Data of airway reactivity are shown in grams of smooth muscle tension (Mokra et al. 2007).

2.7 Statistical Evaluation

Data were expressed as means ± SE. Between-group differences were analyzed by one-way ANOVA with *post-hoc* Fisher's LSD test. Within-group differences were evaluated by the Wilcoxon test. A value of $P < 0.05$ was considered statistically significant. A statistical package SYSTAT for Windows was used for the statistical data evaluation.

3 Results

3.1 Respiratory Parameters

Initial baseline values of the parameters were comparable between the groups before meconium instillation of or saline. After meconium instillation, higher ventilatory pressures were needed to maintain adequate gas exchange. PIP, PEEP, and MAP were higher in the Mec *vs*. Sal group. An improvement in lung function in the Mec + NAC group allowed to reduce MAP compared with the untreated Mec group. Oxygenation index expressing a relationship between oxygenation and ventilatory pressures dramatically elevated after meconium instillation. NAC treatment improved oxygenation and reduced ventilatory pressures, which also decreased OI (Table 1). PaO_2/FiO_2 decreased after meconium instillation and remained lower in the Mec group till the end of experiment. Right-to-left pulmonary shunts significantly increased after meconium instillation compared with the saline-instilled controls (Sal group). NAC treatment diminished shunts *vs*. untreated Mec group (Table 1).

3.2 Cells in BAL Fluid and in Arterial Blood

After meconium instillation, the total count of leukocytes decreased in arterial blood (Fig. 1) and increased in BAL fluid (Fig. 2) in the Mec group compared with the control level in the Sal group (both $p < 0.001$); NAC reversed these effects to a major extent. The percentage changes in neutrophils closely followed changes in the absolute counts of leukocytes in both compartments.

Table 1 Respiratory parameters before and 30 min after meconium or saline instillation (Before/After Mec/Sal) and then 30 min and 1–5 h afterwards

	Before Mec/Sal	After Mec/Sal 30 min	30	1 h	2 h	3 h	4 h	5 h
Mean airway pressure (MAP, kPa)								
Sal	0.26 ± 0.02	0.59 ± 0.05	0.55 ± 0.03	0.58 ± 0.03	0.55 ± 0.01	0.51 ± 0.03	0.54 ± 0.04	0.57 ± 0.03
Mec	0.30 ± 0.01	0.91 ± 0.03^{c}	0.97 ± 0.04^{c}	1.00 ± 0.03^{c}	1.03 ± 0.03^{c}	1.02 ± 0.04^{c}	1.04 ± 0.02^{c}	1.09 ± 0.02^{c}
Mec + NAC	0.28 ± 0.01	0.86 ± 0.01	0.90 ± 0.04	0.92 ± 0.04	0.92 ± 0.04^{d}	0.89 ± 0.04^{d}	0.93 ± 0.03	0.92 ± 0.03^{f}
Oxygenation index (OI)								
Sal	0.6 ± 0.1	3.0 ± 0.6	3.3 ± 0.7	2.1 ± 0.4	1.8 ± 0.3	1.7 ± 0.2	2.2 ± 0.4	1.4 ± 0.1
Mec	0.8 ± 0.1	12.5 ± 1.4^{c}	14.2 ± 0.9^{c}	14.7 ± 0.9^{c}	16.6 ± 1.1^{c}	17.1 ± 1.6^{c}	16.5 ± 1.3^{c}	18.1 ± 1.3^{c}
Mec + NAC	0.7 ± 0.1	11.1 ± 1.0	9.7 ± 1.5^{e}	8.2 ± 1.3^{f}	8.2 ± 1.1^{f}	7.8 ± 1.1^{f}	8.8 ± 1.2^{f}	9.1 ± 1.3^{f}
PaO_2/FiO_2								
Sal	9.4 ± 0.9	24.7 ± 4.7	23.1 ± 5.5	37.4 ± 7.1	34.8 ± 4.9	34.0 ± 3.7	29.2 ± 3.6	42.0 ± 3.2
Mec	8.3 ± 0.5	7.3 ± 0.6^{c}	6.9 ± 0.4^{b}	6.9 ± 0.4^{c}	6.3 ± 0.3^{c}	6.2 ± 0.4^{c}	6.4 ± 0.4^{c}	6.1 ± 0.4^{c}
Mec + NAC	8.0 ± 0.5	8.0 ± 0.7	10.0 ± 1.1	12.4 ± 1.7	12.2 ± 1.3	12.4 ± 1.5	11.5 ± 1.4	11.0 ± 1.2
Right-to-left pulmonary shunts (RLS, %)								
Sal	17.6 ± 5.0	38.2 ± 1.7	36.8 ± 3.5	31.2 ± 3.6	30.1 ± 3.2	27.0 ± 2.8	28.3 ± 2.0	23.2 ± 2.7
Mec	16.9 ± 3.1	54.8 ± 6.0	58.5 ± 6.8^{b}	57.7 ± 4.3^{c}	60.6 ± 2.4^{c}	60.0 ± 1.9^{c}	61.5 ± 1.7^{c}	63.1 ± 3.4^{c}
Mec + NAC	18.5 ± 3.4	48.3 ± 4.2	44.7 ± 3.0^{d}	39.2 ± 4.1^{e}	34.6 ± 4.2^{f}	35.0 ± 3.9^{f}	35.3 ± 4.1^{f}	33.3 ± 3.2^{f}

Sal saline-instilled group, *Mec* meconium-instilled untreated group, and *Mec + NAC* meconium-instilled N-acetylcysteine-treated; between-group differences: Mec *vs*. Sal: $^{b}P < 0.01$, $^{c}P < 0.001$; Mec + NAC *vs*. Mec: $^{d}P < 0.05$, $^{e}P < 0.01$, and $^{f}P < 0.001$

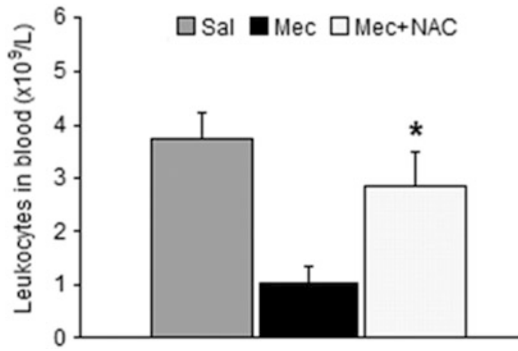

Fig. 1 Total count of leukocytes in arterial blood at the end of experiment. Between-group differences: Mec *vs.* Sal: $^{*}p < 0.001$; Mec + NAC *vs.* Mec: $^{\#}p < 0.05$

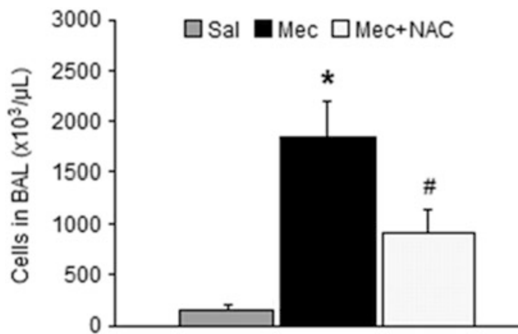

Fig. 2 Total count of cells in BAL fluid at the end of experiment. Between-group differences: Mec *vs.* Sal: $^{*}p < 0.001$; Mec + NAC *vs.* Mec: $^{\#}p < 0.05$

3.3 Lung Edema Formation and Markers of Oxidative Stress

Meconium instillation increased the accumulation of edema fluid in lung tissue in the meconium-instilled untreated animals (Mec group) compared with the Sal group; the effect was reversed by NAC treatment (Mec + NAC group) ($p < 0.001$) (Table 2).

In the Mec group, higher TBARS ($p < 0.001$ *vs.* Sal) and lower TAS ($p < 0.05$ *vs.* Sal) levels were detected in the plasma. NAC administration reduced oxidation back to that present in the control Sal group ($p < 0.001$) and prevented a decline in TAS ($p < 0.05$) (Table 2).

3.4 Airway Reactivity to Acetylcholine in Vitro

Cumulative doses of acetylcholine caused a progressive increase in the contractile response of tracheal smooth muscle in all groups. The response was significantly enhanced in the Mec *vs.* Sal group at the acetylcholine concentrations from 10^{-5} to 10^{-3} mol/L ($p < 0.05$). NAC reverted the acetylcholine-induced enhancement of tracheal reactivity back to the control level (Fig. 3). Likewise, lung tissue reactivity to acetylcholine concentrations from 10^{-7} to 10^{-3} mol/L increased in the Mec group compared with the control saline-instilled group ($p < 0.05$). NAC

Table 2 Wet-dry lung weight ratio (W/D ratio), plasma concentrations of thiobarbituric acid-reactive substances (TBARS), and total antioxidant status (TAS) at the end of experiments in saline-instilled group (Sal), meconium-instilled untreated group (Mec), and meconium-instilled N-acetylcysteine-treated group (Mec + NAC)

	Sal group	Mec group	Mec + NAC group
W/D ratio	5.73 ± 0.17	7.98 ± 0.15^{c}	5.91 ± 0.25^{f}
TBARS (nmol/mg)	0.168 ± 0.021	0.268 ± 0.016^{c}	0.172 ± 0.008^{f}
TAS (mmol/L)	0.796 ± 0.046	0.700 ± 0.021^{a}	0.798 ± 0.026^{d}

Between-group differences: Mec *vs.* Sal: $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$; Mec + NAC *vs.* Mec: $^{d}p < 0.05$, $^{e}p < 0.01$, $^{f}p < 0.001$

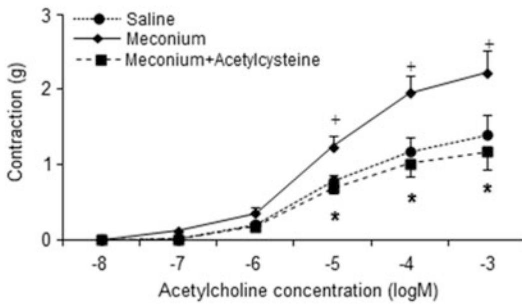

Fig. 3 Reactivity of tracheal strips to cumulative doses of acetylcholine *in vitro*. +p < 0.05 *vs.* saline, *p < 0.05 *vs.* meconium

treatment reverted the increase at the acetylcholine concentrations 10^{-4}–10^{-3} mol/L (data not shown).

4 Discussion

Aspiration of meconium causes apparent neutrophil-derived inflammation and oxidative damage of the lung tissue. In this study, NAC significantly reduced right-to-left pulmonary shunts and improved oxygenation. In addition, NAC diminished migration of neutrophils into the lungs and their activation, and decreased the production of reactive oxygen species (ROS), which prevented an increase in meconium-induced airway reactivity and lung edema formation.

NAC have properties potentially beneficial for the patient with acute lung injury: antioxidant, anti-inflammatory, and mucolytic. The SH-group of NAC can be oxidized by H_2O_2 to form H_2O and O_2. Furthermore, NAC is an excellent scavenger of ·OH radicals and HOCl. In addition to direct antioxidant properties, NAC may stimulate cells to produce antioxidants; thus, after deacetylation it provides cysteine for cellular glutathione synthesis (Gillissen and Nowak 1998). A potent antioxidant action of NAC has been shown in a number of studies. Kharazmi et al. (1988) found that NAC inhibits chemotaxis and generation of ROS by human neutrophils and monocytes *in vitro*. Favorable results of NAC have also been demonstrated in various models of ARDS-like diseases. In rats with oleic acid-induced acute lung injury, pre- and post-treatment with NAC significantly reduced the level of malondialdehyde (MDA), a marker of lipid peroxidation, and 3-nitrotyrosine (Koksel et al. 2004). In rats after acute paraquat intoxication, NAC suppressed serum MDA levels and production of superoxide anions, and augmented a total glutathione concentration (Yeh et al. 2006). In hyperoxic lung injury in mice, inhaled NAC decreased the level of 8-isoprostane, a marker of lipid peroxidation, and increased that of GSH in lung homogenates (Nagata et al. 2007). In a model of lung contusion, NAC lowered lung tissue levels of MDA and NO (Türüt et al. 2009). In a recent study, NAC decreased concentrations of TBARS in the lung tissue of lipopolysaccharide-instilled rats (Choi et al. 2012). NAC also is beneficial in patients with acute cardiac injury (Mahmoud and Ammar 2011; Yesilbursa et al. 2006), where it diminishes oxidative stress and improves hemodynamic parameters. In agreement with those results, NAC in the present study decreased plasma concentrations of TBARS and prevented a decrease in total antioxidant status, clearly showing an antioxidant potential in MAS.

It is presumed that NAC has also anti-inflammatory capabilities. In rats after acute paraquat intoxication, NAC reduces inflammatory cell infiltration in lung interstitium (Yeh et al. 2006). In hyperoxic lung injury in mice, inhaled NAC attenuated cellular infiltrations in both BAL fluid and lung tissue (Nagata et al. 2007). NAC decreases concentrations of myeloperoxidase in the lung tissue of both lipopolysaccharide-instilled (Choi et al. 2012) and oleic acid-instilled (Koksel et al. 2004) rats. On the other hand, in a model of lung contusion, NAC has no effect on neutrophils in BAL fluid (Türüt et al. 2009). In a recent study, NAC decreased the concentration of NF-kappaB in lung tissue, but did not that of TNF-alpha and -IL-1beta in BAL fluid in rats with lipopolysaccharide-induced acute lung injury (Choi et al. 2012). In addition, there were no differences in a total number of cells and percentage of neutrophils in BAL fluid. However, in

another model of lipopolysaccharide-induced acute lung injury, NAC failed to affect the level of pro-inflammatory cytokines, such as IL-1beta, IL-6, or MCP-1 (Jansson et al. 2005). Therefore, the response to NAC may differ in different models of acute lung injury, as it has been discussed by Domenighetti et al. (1999), who postulated that the mode of action of NAC may not be the same in different pathologies and clinical situations. A negative effect of NAC in healthy volunteers or patients with COPD (with normal glutathione levels in BAL fluid) and a beneficial one in ARDS (with low glutathione levels in BAL fluid, high oxidant load) have led to the hypothesis of negative feedback inhibition (Gillissen and Nowak 1998).

In addition to antioxidant and anti-inflammatory action, NAC may have a capability to reduce viscosity and thickness of meconium by breaking disulfide bonds and thereby to enhance its mobilization from the lungs. NAC also may neutralize meconium's digestive enzymes responsible for lung damage in patients with MAS (Ivanov 2006). However, these presumptions have not yet been fully verified.

As a result of the therapeutic actions of NAC above outlined, we could observe an improvement in pulmonary right-to-left shunting, and due to a reduced lung edema also in lung compliance. In accord with our results, NAC prevented an increase in pulmonary vascular resistance and delayed the development of abnormal ventilation-perfusion relationships in ARDS-like injury in dogs; the effects were associated with a decline in alveolar and interstitial edema (Wagner et al. 1989). Reduced vascular congestion and lung edema have also been found in rats after acute paraquat intoxication (Yeh et al. 2006) and in lipopolysaccharide-induced acute lung injury (Choi et al. 2012). On the other hand, no difference in a protein concentration in BAL fluid have been found in LPS-injured rats (Choi et al. 2012). Likewise, pre-treatment with NAC has not reduced the wet-dry ratio in lipopolysaccharide-induced acute lung injury (Jansson et al. 2005).

In conclusion, the present study demonstrates that N-acetylcysteine improved lung function, and alleviated neutrophil-derived inflammation and oxidative stress in meconium-instilled rabbits. Nevertheless, effects of NAC should be studied in other models of MAS and in randomized controlled studies before this treatment might be recommended for use in newborns with MAS.

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