

EARLY SYSTEMIC BIOMARKERS OF ACUTE LUNG INJURY: APPLICATION OF MULTIPLEX PROTEOMIC ARRAY TECHNOLOGY.

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1. INTRODUCTION

Many questions related to the cellular mechanisms of lung injury/toxicity from high-dose exposure to militarily relevant toxic gases, chemicals and/or irritants still remain unanswered. This is primarily due to limited interest in the toxicity of such high-level exposures in the civilian sector. Early detection and delineation of cellular changes following exposure to high-level environmental oxidants/irritants is essential for the development of much-needed preventive strategies and tools for therapeutic intervention, and also for monitoring pre- and post-deployment 'lung health', of active duty U.S. soldiers. A number of militarily relevant environmental and occupational 'insults', such as noxious irritants, air-born particulates, toxic gases, chemicals and pathogens have been shown to cause acute pulmonary inflammation/injury, which often lead to acute respiratory distress syndrome (ARDS), multi-organ dysfunctions and/or failures. The same is applicable to blast overpressure (BOP) shockwave - induced lung and/or multi-organ injury, which is currently a major concern in today's Force Health Protection and Military Operational Medicine.

Because lung is the major entry portal and target of toxic environmental or air-born agents, delineation of early cellular changes and specific cellular and/or molecular targets of acute lung injury and/or toxicity, have been one of the major objectives of our research mission on lung injury and/or toxicology. Historically, the Department of Respiratory Research within the Division of Military Casualty Research, at WRAIR, has been engaged in studies in various animal models related to inhalation injury/toxicology and its associated patho-physiology, and also in lung

injury models associated with BOP shockwave-mediated blunt trauma and/or injury. These studies were primarily supported by the USAMRMC/RADIII's research programs under Military Operational Medicine. Both *in vitro* and *in vivo* experimental models of acute lung injury have been used for these studies, results of which have been published (Ayyagari et al. , 2004, 2007; Gorbounov et al. , 2002, 2003, 2004). More recent research efforts in our laboratory have focused on delineation of early cellular and biochemical changes in the lung, following exposure to toxic fire gases and/or BOP-mediated shockwaves, in an attempt to identify early biomarkers of acute lung injury/toxicity and to develop effective strategies and tools for therapeutic intervention. Our *in vitro* studies along these lines have clearly documented an early pro-inflammatory response in normal human bronchial epithelial (NHBE) cells, when the cells were exposed to a brief high-dose of nitrogen dioxide (NO₂), a toxic fire gas (Ayyagari et al. , 2004, 2007). Of particular relevance to our research mission, an early pro - inflammatory response was also observed in a BOP shockwave-mediated rat lung injury model (Gorbounov et al. , 2004), which further corroborated our results obtained from our *in vitro* studies. Taken together, our *in vitro* and *in vivo* studies have documented an early systemic inflammatory response, oxidative/nitrosative stress, expression of redox - sensitive stress proteins, cell adhesion molecules and resultant increase in cell-cell interactions, infiltration of neutrophils and cell death via apoptosis and necrosis, when pulmonary cells, or the lung, were subjected to acute oxidative stress. The results of these studies prompted us to further characterize the nature of the early pro- inflammatory response(s) in a rat model of 'inhalation injury', to identify early

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'biomarkers' of acute lung injury/toxicity and to further characterize the nature of the systemic inflammatory response, which may have real-life translational potential in health-protection and development of effective treatment strategies for active-duty U.S. soldiers in a more clinical setting.

2. BACKGROUND

Oxidative stress and inflammation are common biological denominators in most acute pulmonary inflammation, hypersensitive conditions and its patho-physiology, which are often linked to multi-organ dysfunction and/or failure (Zhang et al. , 2000) . Our recent *in vitro* studies in NHBE cells have clearly documented an acute oxidative/nitrosative stress, changes in cell-cell interactions, expression of cell adhesion molecules (ICAM-1) and redox-sensitive stress proteins (HO-1) and significant increases in levels of a number of pro-inflammatory cytokines and chemokines, as early as 1- 2 hours, when the cells were exposed to a brief high-dose of nitrogen dioxide (NO₂), a toxic fire gas and an environmental oxidant of concern (Ayyagari et al. , 2004, 2007) . In related studies, we have also documented similar oxidative stress-mediated changes in a BOP shockwave -mediated rat lung injury model (Gorbounov et al. , 2004). In view of the results obtained from our earlier studies, the present study were undertaken to further delineate and identify early systemic biomarkers of acute lung inflammation and/or toxicity, which may have real-life translational potential in pre- and post-deployment health protection and/or injury assessment of active-duty U.S. soldiers. A non-invasive rat model of inhalation injury/toxicity has been used for this purpose and a sensitive proteome array-based multiplex ELISA was utilized to monitor changes in various pro-inflammatory cytokines/chemokine levels in NO₂-exposed rat lung. Since infiltration of inflammatory neutrophils are known to play a central role in most acute pulmonary inflammation (Quinton et al. , 2004), we also assessed that in NO₂ - exposed rat lung, to further correlate the systemic changes with the severity of pulmonary inflammation and/or injury.

3. OBJECTIVE

The primary objectives of the present study were: 1) to set up a non-invasive animal model of 'inhalation injury', to monitor early changes in

levels of pro-inflammatory cytokines in breath condensates of live rats, to delineate early biomarkers of pulmonary inflammation; 2) to further investigate the role of specific pro-inflammatory mediator(s) in acute lung inflammation/injury following exposure to toxic fire gases and/or BOP shockwave - mediated blunt trauma ; 3) to monitor changes in various pro-inflammatory cytokines/chemokines in small volumes of biological samples (10-50 µl), by utilizing a highly sensitive Proteome Array-based Multiplex ELISA; 4) to identify early systemic 'biomarkers' of acute lung injury/toxicity, for development of much-needed strategies/tools for effective therapeutic intervention and lung-health assessment of active-duty U.S. soldiers deployed around the world. The primary goal of this study was to identify early 'biomarkers' of acute pulmonary inflammation, as an predictive indicator of acute lung toxicity and/or injury, following exposure of rats to a brief high-dose of NO₂, a militarily relevant toxic fire gas and a product of high temperature combustion. Since infiltration of inflammatory neutrophils and resultant oxidative stress-mediated cell/tissue injury are known to play a central role in pulmonary host defense (Zhang et al. , 2000) pulmonary cell histopathology were also assessed in the NO₂-exposed rats, to further correlate the systemic changes with the severity of pulmonary inflammation and/or injury.

4. MILITARY RELEVANCE

Early systemic 'biomarkers' of battlefield-related injuries are necessary for both early detection and timely intervention and treatment of the injury. The latter is particularly relevant to the injuries, which often do not have any external signs or symptoms until much later and remain undetected until much later. In the context of our present study, early biomarkers of 'inhalation injury' are much-needed requirements for better health assessment of pre-deployed 'lung health' of active-duty soldiers and also for post-deployment treatment and/or assessment of the severity of lung injury, which are afflicted by various militarily relevant toxic environmental or occupational health hazards, such as toxic gases, chemicals, and irritants. This is an essential prerequisite to maintain a healthy and effective force. The scientific rationale of the data presented here are likely to contribute toward development of

much-needed strategies/tools for effective therapeutic intervention(s) and thereby, protect the health of active duty U.S. soldiers deployed around the world.

4. METHODS

Animals Used. Male Sprague-Dawley rats (~250 gms) were purchased from Charles River Laboratories. After a 1-week stabilization period, the rats were brought to the laboratory and, over several days, the exposure cones were placed as an acclimatization procedure. (See Fig. 1B). All studies were carried out in accordance with the WRAIR IACUC - approved procedures and protocols.

NO₂ - Exposure of Rats. Rats were exposed using a dynamic exposure system developed at WRAIR (Inhalation Toxicology 17: 755-764, 2005). Briefly, this system consists of a 12.5 L high-density polyethylene (HDPE) gas mixing chamber connected to a .24 L 4-port, nose-only HDPE inhalation chamber. The animals were placed in polycarbonate exposure cones (Laboratory Products), where the nose cone fitted snugly in the exposure chamber, and the rear of the cone was fitted with a large rubber stopper (for access and placement of the animals) and a stainless steel tail pipe that functioned as a tail heat sink. Electrically-activated solenoid valves in the system allowed for a rapid change of inhalation chamber gas composition (from USP air to test gas and subsequently back to USP air). The T₉₉, time to reach 99% of the target gas concentration, was measured to be approximately 5.5s at a nominal flow rate of approximately 12L/min. All components of the entire system are composed of glass, Teflon, stainless steel, and HDPE. Two of the inhalation ports were capped off and rats were exposed two per session. The animals breathed USP air for a 5-min period, the solenoids were actuated, and then the animals breathed ~150 ppm nitrogen dioxide (NO₂) in air (certified, Air Products) for 5 min. The solenoids were then deactivated, allowing the animals to breathe USP air for a 5-min recovery period. Nitrogen dioxide concentrations were continuously monitored using a UV-IR analyzer (Rosemount).

Breath Condensate Collection. Live rat breath condensate was collected before exposure and at 3- and 24-hr post exposure. A novel system was developed at WRAIR to capture live rat breath

condensates. This system also utilized the same exposure cones used for the NO₂ exposures. However, for this application the nose end was equipped with a one-way non-rebreathing valve (Hans-Rudolf) so that the expired breath could be captured. The exit port of the non-rebreathing valve was connected to one side of a stainless steel "Y" connector. The bottom of the "Y" connector was positioned through a loosely capped 5-ml cryogenic tube that was immersed in an ice bath. Therefore, water in the breath could easily condense on the stainless steel tip and be collected in the collection tube. The other end of the "Y" connector was attached to a second rat exposure cone so that condensate from two rats was simultaneously pooled. Rat breath condensate was collected for approximately 45 min for each pair of rats. It was determined that in order to collect sufficient condensate for analysis (> 100 µl), condensates had to be pooled from at least 4 rats. Condensate was stored at -80 degrees until analyses could be performed.

Broncho - Alveolar Lavage. After the final sampling time (or in control non-exposed rats), the animals were humanely euthanized (Beuthanasia) and BALF collected as follows. Immediately upon death, the trachea was surgically exposed and a small access incision made below the larynx. A beveled section of PE240 tubing was inserted through the incision and tied into place. The proximal end of the tubing was connected to a 16 gauge luer stub and a 3-ml syringe. The syringe was filled with iced PBS and instilled into the lungs. The dwell time for the PBS was ~ 5 s. The fluid was aspirated from the lungs, centrifuged to collect the cells for staining and stored at -80 degree until analyses could be performed. Recovery of the instilled PBS was approximately 75%.

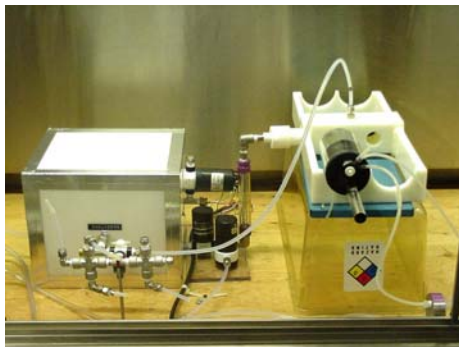
Blood Collection. Immediately at the point of death, the thorax was opened and blood was collected via cardiac puncture, using a sterile heparinized syringe. The blood was immediately centrifuged at 4 ° (at 3000 rpm x 15 min). Plasma was removed and stored at -80 degree until analyses could be performed.

Cytokine Analysis. A sensitive plate-based Multiplex ELISA, which can detect low levels of analytes (1-5 pgm/ml l), was used to monitor changes in levels of various pro-inflammatory cytokines/chemokines in breath condensates collected from live rats and also in broncho-alveolar lavage fluids (BALFs) and plasma

samples obtained from the control and NO₂-exposed rats. Figs. 1A and 1B show the details of the NO₂-exposure chamber that was used in our present study. Rat breath condensates (100-200 µl) from control (i.e., air-exposed) and NO₂-exposed (100 ppm) animals were collected as described above and immediately frozen at -80 degree until further analyses. The control and NO₂-exposed BALFs collected from the control and experimental animals (1-2 ml) were centrifuged at 4° C to remove and collect the cell pellets for histo-chemical examination and cell morphology. The respective cell pellets from

control and NO₂-exposed BALFs were smeared on slides, air-dried and stained for morphological examination. The breath condensates and BALFs were kept frozen at -80 degree, until further analyses of their pro-inflammatory cytokines and chemokine levels by Pierce Biotechnology's SearchLight™ Assay. The latter is a highly sensitive Proteome Array - based Multiplex ELISA, capable of measuring (at 1-5 pgm/ml) up to 16 different analytes in small volumes of biological samples (10-50 µl).

1A: NO₂-Exposure Chamber



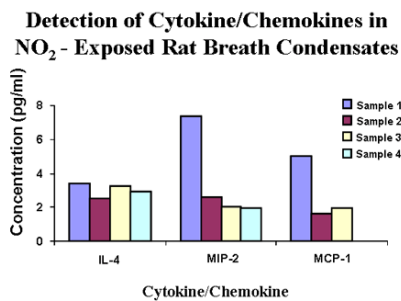
1B: Nose-Only NO₂ Exposure in Rats



5. RESULTS

As shown in Fig. 2, low levels of pro-inflammatory cytokines/chemokines such as IL-4 and MIP-2 and MCP-1 were detected in NO₂-exposed rat breath condensates at 3 hr. post-exposure. Each sample represents pooled breath condensates from 4 rats. Control rats, i.e., air-exposed, did not have any detectable amounts of cytokines/chemokines in their breath condensates (data not shown).

Figure 2

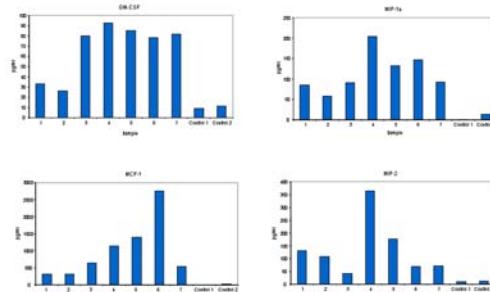


Levels of some well-characterized pro-inflammatory cytokines and chemokines, such as GM-CSF, MIP-1a, MCP-1 and MIP-2 in control

and NO₂-exposed rat BALs are shown in Fig. 3. Data from 7 different NO₂-exposed rats and 2 control rats are shown, to document significant variability in the pro-inflammatory responses of individual rats. This is consistent with the known variations in respiratory rates and lung function of individual rats in response to various toxic inhalants or 'insults'.

Figure 3

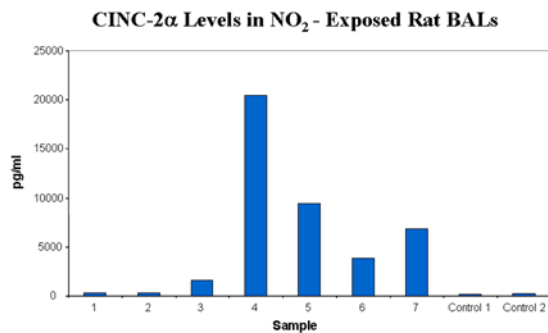
Effect of NO₂-Inhalation on Inflammatory Cytokine/Chemokine Levels in Rat BALs



Of particular relevance to our present study and as shown in Fig. 4, marked increases (>1000-fold)

in levels of CINC-2 were observed in NO₂-exposed BALF samples, which is a cytokine-induced neutrophil chemoattractant, a potent rat chemokine. CINC-2 is generated in the airway cells of the rat lung and is known to play a central role in early recruitment of inflammatory neutrophils into the inflamed or injured lung (Quinton et al., 2004). In Fig. 3, samples 1-3 shows 3 hr. post-NO₂ levels and samples 4-7 shows 24 hr. post-NO₂ levels. Interestingly, consistent with the data shown in Fig. 4, immunofluorescence staining of BALF collected from NO₂-exposed rat at 24 hour showed marked increase in expression of CINC-2 levels (Fig. 5, Panels C and D). Fig. 5 also includes the Nomarski images of the same control and NO₂-exposed BAL samples (Panels A and B) and shows epithelial cell damage in NO₂-exposed BALF as well.

Fig. 4



Consistent with the data shown in Figs. 3 and 4, we also documented presence of infiltrated neutrophils in NO₂-exposed rat BALs at 24 hour. As shown in Fig. 6, both epithelial cell damage and presence of neutrophils were noted in the NO₂-exposed rat lung.

Fig. 5

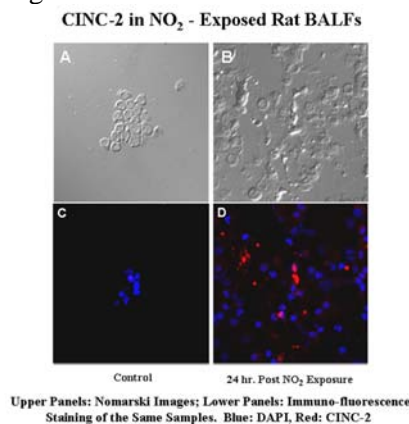


Fig. 6

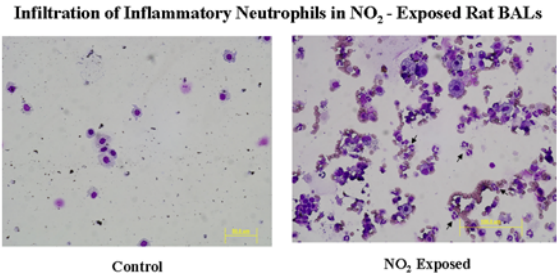
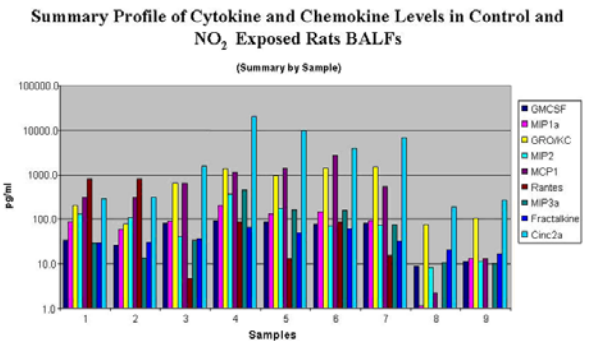


Figure 7 shows a representative summary profile of various pro-inflammatory cytokine/chemokine levels in control (samples 8-9) and NO₂-exposed (samples 1-7) rat BALFs. The BALF samples used to generate this composite profile are the same as those used for the data presented in Figs. 3 and 4.

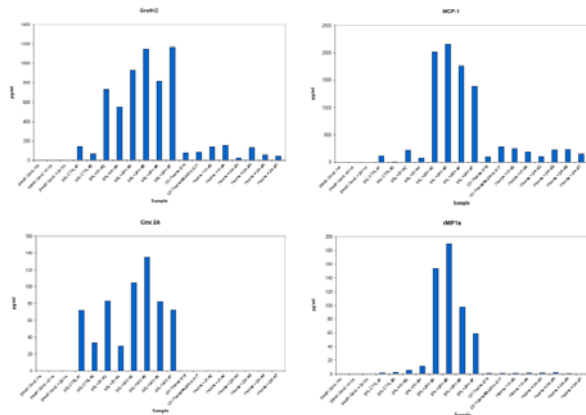
Fig. 7



In view of our experimental evidence which clearly documented an early pro-inflammatory response in the NO₂-exposed rat lung, we wanted to further characterize the nature of the systemic inflammatory response and to quantify the changes in levels of various pro-inflammatory cytokines/chemokines in breath condensates (samples 1-3), BALFs and plasma samples collected from control and NO₂-exposed rats, in parallel. In our limited studies thus far, most significant increases have been observed in BALF samples collected at 24 hr post-NO₂, although modest increases were observed as early as 3 hour post-NO₂ exposure. In figure 8, comparative levels of MIP-2α, CINC-2α, MIP-1α and GRO/KC, four well-characterized rat pro-inflammatory cytokines/chemokines are shown.

Fig. 8

Comparison of Pro-Inflammatory Cytokine/Chemokine Levels In Rat Breath Condensates, BALs and Plasma



6. DISCUSSION

We have used an innovative rat model of ‘inhalation injury’ to delineate cellular and molecular targets of acute inflammation in the rat lung, to identify and characterize early biomarkers of lung injury/toxicity, which may have real-life application in the development of effective therapeutic strategies and/or tools for lung health assessment and/or treatment of pre- and post-deployed active-duty U.S. soldiers. Our studies clearly demonstrate an early systemic inflammation and significant up-regulation of a number of pro-inflammatory cytokines and chemokines in the rat lung following exposure to a brief high-dose of NO₂, a toxic fire gas. It is worthwhile to note here that in previously reported studies, an acute pulmonary inflammation and its associated systemic changes have also been noted in a rat model of lung injury, when inflicted by low-frequency blast overpressure-mediated shock waves (Gorbounov et al. , 2003, 2004). It is reasonable to hypothesize that early detection of pro-inflammatory cytokines and chemokines in NO₂ - exposed rat breath condensates (Fig.2) can be of particular relevance to the USAMRMC’s research missions related to Force Health Protection and/or Military Operational Medicine. Reported studies on breath condensate analyses in humans have mostly been carried out in chronic asthmatic patients, in a clinical setting of a hospital. In view of the results presented in this communication, a

more detailed systematic study is clearly warranted, to further investigate the translational potential of breath condensate analyses in assessment of ‘lung health’ of U.S. active duty soldiers and/or war veterans returning from the battlefields of Iraq. Several-fold increases in levels of GM-CSF, MIP-1a, MCP-1 and MIP-2 and CINC-2α (Figs. 3 - 4) and presence of infiltrated neutrophils and epithelial cell lining damage (Figs. 5 - 6), as observed in NO₂ - exposed rat BALFs, provide further supportive evidence for the concerted role of these rat chemokines in early recruitment of neutrophils during acute pulmonary inflammation (Quinton at al. , 2004). Documentation of increased expression of CINC-2 (equivalent to NAP-2 in humans) and its presence in NO₂ - exposed rat BALF cells after 24 hours and presence of large numbers of infiltrated neutrophils in the same BALFs, lend further credence to CINC’s active role in recruitment of neutrophils during acute pulmonary inflammation.

Successful application of the SearchLight™ Proteomic Array-based Multiplex ELISA for detection and quantification of up to 16 analytes (at 1-5 pgm/ml) in breath condensates, BALFs and/or plasma samples collected from same experimental rats, provide valuable information on the nature of the early systemic inflammatory response that ensues following inhalation of a brief high-dose of NO₂. The NO₂ - exposure doses used in our studies are attainable in a real-life battlefield scenario, such as would occur in a fiery explosion of an armored tank, or during detonation of an explosive blast device. In summary, our present study provides definitive information on early pro- inflammatory responses in the rat lung, following exposure to a high-dose of NO₂, a toxic fire gas. These studies also documented marked increases in levels of a number of well-characterized inflammatory cytokines and/or chemokines, amongst which CINC-2α, a potent rat chemoattractant known to be generated by lung airway cells during acute inflammation, showed a >100-fold increase in NO₂ - exposed BALs. The scientific information gathered from this innovative research effort may find application in the development of much-needed diagnostic tools/strategies for early detection of diverse battlefield - related health hazards and/or injuries, including blast-induced mild traumatic brain injury, which often does not

have any early signs or symptoms for timely diagnosis and treatment. The latter currently poses a major medical challenge for the U.S. Military Medicine at-large.

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