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Nitric oxide and oxygen metabolism in inflammatory conditions: sepsis and exposition to polluted ambients

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1. ABSTRACT

Nitric oxide and cytokines constitute the molecular markers and the intracellular messengers of inflammatory conditions which are derived from the activation of the NF-kappaB pathway and the transcription of proinflammatory genes. Sepsis occurs with an exacerbated inflammatory response that damages tissue mitochondria and impairs bioenergetic processes. One of the current hypotheses for the molecular mechanisms underlying the complex condition of sepsis is that enhanced NO production by mtNOS leads to excessive peroxynitrite production and protein nitration in the mitochondrial matrix, causing mitochondrial dysfunction and organ failure. The mechanism of particulate matter-health effects are believed to involve inflammation and oxidative stress. Components in particles that elicit inflammation are poorly investigated, although recent research points out to the contribution of compositional elements and particle size. Nitric oxide and reactive oxygen species appears to be involved in the inflammatory conditions associated to particulate matter inhalation.

2. INTRODUCTION: THE INFLAMMATORY CONDITION

Inflammation was classically described as a combination of three clinical signs: vasodilation, hyperthermia, and edema; and it is considered as either an acute or a chronic process, according to its time course. The phenomenon is associated to cellular mobilization and migration of neutrophils and macrophages to the inflammatory focus. More recently, the recognition of the role of nitric oxide (NO) and cytokines in the intercellular communication led to a new operational concept, this time at the molecular level, in which inflammation is defined by increased concentrations of NO and of the inflammatory cytokines, mainly interleukin 1-beta (IL-1) and tumor necrosis factor-alpha (TNF-alpha) in the involved biological fluids (1). Nitric oxide influences many aspects of the inflammatory cascade ranging from its own production by immunocompetent cells to the recruitment of leukocytes. Cytokines constitute a heterogeneous group of proteins produced by different cell types that modulate the functions of proximate cells, including the secretion of

other cytokines with synergistic or inhibitory effects. In inflammation, proinflammatory cytokines and lipid mediators play a key role in triggering the expression of the inducible isoform of NO synthase (iNOS or NOS-2) in various cell types.

When considering the multifaceted roles of NO, it is not surprising that the pharmacological inhibition of the production of NO in inflammation may have either detrimental or protective effects. In general, NO produced by the constitutive endothelial NOS (eNOS) is considered protective. This primary NO production is essential in mantaining vascular function. Induction of the inducible NOS isoform (iNOS) has been demonstrated in almost all forms of inflammation; in these conditions the overproduction of NO is found to be cytotoxic (2).

An extensively studied inflammatory condition with respect to the mechanism of iNOS expression, is the systemic inflammation induced by the bacterial lipopolisaccharide (LPS). The primary initiator of the inflammatory response in gram-negative septic shock is bacterial LPS, a component of the cell wall (3,4). This component is extensively used for producing experimental endotoxic shock. The binding of LPS to the surface of endothelial cells results in endothelial activation, as demonstrated by the expression of proinflammatory cytokines and adhesion molecules. LPS also activates monocytes and macrophages to stimulate the production of proinflammatory mediators, which in turn modulate endothelial function. Collectively, this initiates a parallel cascade of events that contribute to the clinical manifestations of sepsis (5). One of the current hypothesis for the molecular mechanism underlying the complex condition of septic shock is that the enhanced NO production by mtNOS and cytosolic iNOS leads to excessive peroxynitrite production and protein nitration in the mitochondrial matrix, causing mitochondrial dysfunction and contractile failure.

Another inflammatory condition is observed during the exposition to particulate matter polluted ambients. There is strong evidence from cell culture and animal models that exposure to particles is associated with inflammation (6). Components in particles that elicit inflammation are poorly investigated, although recent research points out to the contribution of compositional elements and particle size. Small (ultrafine) particles can cause inflammation by surface-mediated effects, whereas large (coarse) authentic particles cause inflammation through the presence of endotoxins.

3. SEPSIS

Sepsis constitutes a major cause of death following trauma and a persistent problem in surgical patients. Three related syndromes, with the common background of sepsis, are recognized in increasing order of severity: systemic inflammatory response syndrome (SIRS), septic shock, and multiple organ failure (MOF). The onset of sepsis is characterized by fever, usually accompanied by shivering or hypothermia, tachycardia and tachypnea. These symptoms characterize SIRS, and evolve to septic shock when hypotension and loss of conscience appears, and to MOF when organ dysfunction develops (7). The prevalent hypothesis regarding the mechanism of the three related syndromes is that they are caused by an excessive defensive and inflammatory response. Sepsis is a paradigm of acute whole body inflammation, with massive increases of NO and inflammatory cytokines in the biological fluids, with systemic damage to vascular endothelium, and with impaired tissue and whole body respiration despite adequate oxygen supply (8-10).

Treatment of septic shock usually includes respiratory support to optimize tissue oxygenation, intravenous fluid administration, broad-spectrum antimicrobial therapy and anti-inflammatory agents (glucocorticoids and nonsteroidal anti-inflammmatory drugs), and blood pressure support; all of which are limited in their effectiveness (11-13). Competitive inhibitors of NOS activity were considered as potentially useful in the treatment of the severe hypotension of septic shock. However, clinical trials using L-NMMA (targinine) failed to show a beneficial effect in septic shock patients (1). The new inhibitors of CXCL-8 receptors, that reduce neutrophil recruitment and activation, seem at the moment the best pharmacological approach for septic shock (14).

3.1. Animal models of sepsis

When utilizing sepsis models, a number of important questions arise. What is the relevance of skeletal muscle data to "more vital" organs such as liver or kidney? Are these changes causal or epiphenomenal? Ethical and technical difficulties constrain the availability of vital human biopsy tissue, especially when repeated sampling is desirable to monitor disease progression and concurrent mitochondrial function. Studies in patients with septic shock have shown an increase in blood pressure after administration of inhibitors of NO synthesis. An association between NO overproduction, antioxidant depletion, mitochondrial dysfunction, and decreased ATP concentrations was recently related (in humans) to organ failure and eventual outcome (15). These indicators correlate with the severity of disease and outcome and support the notion that mitochondrial dysfunction resulting in bioenergetic failure is the critical factor in the pathophysiology of septic shock and MOF.

It is thus incumbent to develop representative animal models that reflect many, if not all, of the biochemical and physiological abnormalities evidenced in patients. However, laboratory models of sepsis show considerable variation in organ function and ultraestructural damage, due in no small part to the model itself. One of the animal models most extensively used is the ip injection of the *E. coli* LPS in an approximate dose of 10 mg/kg (16,17). The studies are performed after 6 h of treatment, where the peak of acute inflammation effects is observed. The LPS molecule consists of a oligosaccharide core and a highly conserved lipid A portion. The lipid A moiety is responsible for the toxic proinflammatory properties of LPS, and is also used for producing experimental endotoxic shock (dose: 10 mg/kg). LPS induces a receptor mediated

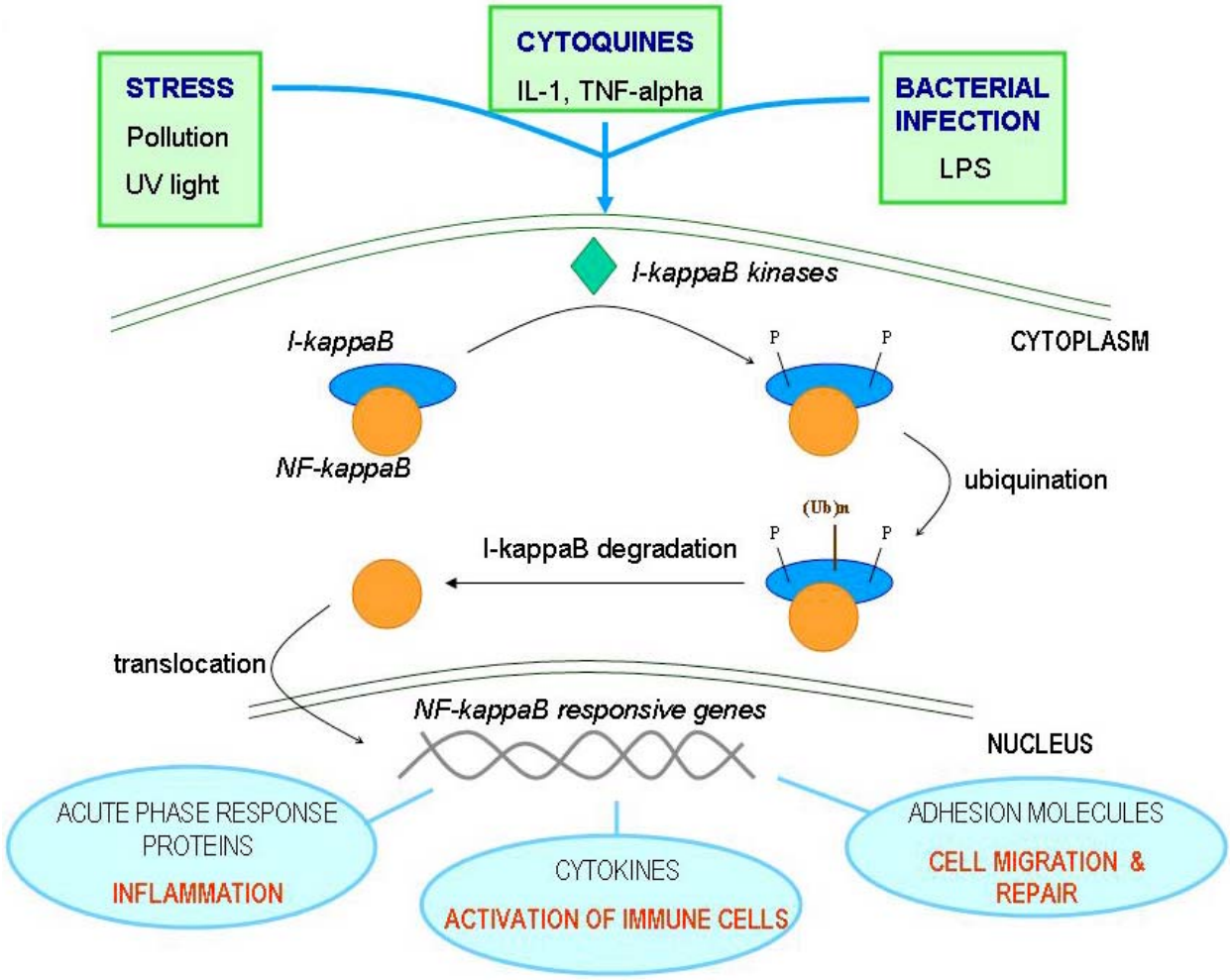

Figure 1. LPS and PM signalling pathway. The signalling cascade leads to NF-kappa B activation and translocation to the nucleus, where it activates the transcription of mediators (acute phase proteins, cytokines and adhesion molecules), which in turn leads to inflammation, cell migration and activation of immune cells.

signaling cascade that leads to nuclear factor-kappaB (NF-kappaB) activation and the transcription and subsequent release of cytokines and other proinflammatory mediators by monocytes and macrophages (see Figure 1). The intracellular events that lead to the activation of NF-kappaB are complex and seem to be controlled by the redox status of the cell (18). The generation of peritonitis on rats better simulates the human disease process; two procedures are utilized. The cecal ligature and puncture procedure is performed in anesthetized animals (e.g. 100 mg/kg ketamine plus 0.2 mg/kg xylazine) (19,20). This model of sepsis results in animals with septic shock, lactic acidosis, and hypothermia 12 h after the surgery; mortality is about 13 %. Another procedure to generate peritonitis is the ip injection of fecal slurry (dose: 6.25 ml/kg). This is prepared from the bowel contents of rats, suspended in saline solution and filtered to remove fibrous material (21). The animals can be studied at 24, 48 and 72 h after the induction of sepsis. By 24 h, 17% of rats can be classified as moderate septic; at 48 hs, 41% of rats are in the same level of sepsis; at 72 h, 73% of the rats still alive can be classified as mild and 27% as moderate. This model of sepsis results in animals with a fall in mean blood pressure and a rise in serum urea, creatinine, and alkaline phosphatase.

Ideally, it is advisable to generate a long-term septic model closely resembling the human disease process, combining physiological and biochemical markers of mitochondrial function. Such model would enable monitoring the temporal changes, comparison of vital and less vital organs, and serve as a potentially useful test bed for therapies. When evaluating the role of NO or elucidating the effects of NOS inhibitors in animals models of shock, one needs to consider that: (a) rodent production of NO in sepsis is much greater than in humans, (b) many of the models used are acute, non-resucitated, hypodynamic models of shock, (c) any observed effect will obviously depend on the chosen dose regimen and timing of the intervention, and (d) the effects (and side effects) of non selective inhibitors of NOS activity will greatly vary depending on the degree of iNOS induction.

3.2. NO metabolism in muscle during sepsis

During the last five years significant and novel findings have been described for NO metabolism in muscle mitochondria during sepsis. The main findings for this syndrome are: (a) a marked increase in mtNOS activity and NO production in heart and diaphragm (22,23); (b) an increased mitochondrial production of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and peoxynitrite ($ONOO^-$) (23); (c) increases of the steady-state concentrations of O_2^-, H_2O_2 and $ONOO^-$ secondary to an increase in NO steady state level, (16,24); (d) mtNOS and Mn-SOD show a parallel increase in activity in sepsis (15, 23); (e) contractile failure follows mitochondrial impairment (17,23); f) a mechanism of translocation of iNOS (determined as a Ca^{2+}-independent activity) to mitochondria was described (25).

Experimental endotoxic shock produces a marked increase in the activity of rat diaphragm and heart mtNOS. The production of NO by submitochondrial membranes increased by 122% (diaphragm) and 32% (heart) after 6 h of LPS (10 mg/kg) administration (16). The mechanism of mtNOS induction in muscle by endotoxin is not fully understood; however, there is a standing hypothesis that this process involves a protein tyrosyne kinase, NF-kappaB and nuclear NOS gene activation, mRNA transcription, novel protein biosynthesis, and exportation to mitochondria (22). Significant increases in iNOS mRNA, protein and enzyme activity, have been reported in rat diaphragm and human skeletal muscle (23, 26). On the other hand, experimental endotoxic shock was observed to produce a marked increase in the activity of rat diaphragm mtNOS (22).

The mitochondrial O_2^- and H_2O_2 productions were found increased in endotoxic shock in rats, a phenomenon interpreted as caused by excess NO. Preincubation with nitroso-glutathione, as source of NO, determined an about 80% inhibition of cytochrome *b*-cytochrome *c* electron transfer in complex III with an increased rate O_2^- production. Endotoxic shock led to increased rates of mitochondrial O_2^- production: 3 times in heart mitochondria and 1.8 times in diaphragm mitochondria (16). The mitochondrial production of H_2O_2 in mitochondrial state 4 (resting mitochondria with maximal physiological H_2O_2 rates) was also markedly increased in endotoxic shock; 2.6 times in heart mitochondria and 1.8 in diaphragm mitochondria (17). High mitochondrial O_2^- and H_2O_2 production during endotoxemia seem due to the irreversible inhibition of the respiratory chain by peroxynitrite (17,22). Added NO inhibits cytochrome oxidase (complex IV) (27, 28) and complex III (29) *in vitro* and increases O_2^- and H_2O_2 production in submitochondrial particles and mitochondria, but these effects that occur in vivo, are lost during mitochondrial isolation. The effects of NO on complexes III and IV of the mitochondrial respiratory chain are likely to occur under physiological conditions and to constitute regulatory devices of the energy supply and cell signaling for mitochondrial function. At variance, the effects of peroxynitrite in complexes I and III are likely to constitute toxic actions that would be determinant of mitochondrial and cell dysfunction in inflammation and septic shock and, through cumulative effects, of mitochondria-dependent apoptosis.

Diaphragm mitochondria isolated from LPS-treated rats were reported uncoupled with a 7 and 33 % decrease in respiratory control, 3 h and 6 h after LPS inoculation (23). Western blot analysis of diaphragm mitochondrial exhibit a pattern of protein nitration with several bands, similar to the one observed after 30 min of exposure of mitochondria to 1 mM SIN-1 (26). Nitration of mitochondrial proteins and maximal impairment of mitochondrial function are associated with a significant decrease in diaphragm contraction force.

The observed increase in H_2O_2 production is in a way in agreement with the observation of higher Mn-SOD activity in endotoxic shock (16). An increased O_2^- dismutation rate to H_2O_2 catalized by Mn-SOD is expected to better compete with the O_2^- and NO reaction to yield peroxynitrite. The LPS endotoxin and TNF-alpha were reported to increase the Mn-SOD content in endothelial cells (30,31). A correlation of mtNOS and Mn-SOD activity was observed for heart and diaphragm mitochondria in endotoxic shock (Figure 2). A similar pattern was observed during rat brain development (32). These reports support the idea of a shared gene expression mechanism for mtNOS and Mn-SOD.

3.3. Oxygen metabolism and muscle mitochondrial dysfunction during sepsis

The concept of a bioenergetic failure due to muscle mitochondrial dysfunction as part of the pathogenic mechanism of septic shock was introduced about 35 years ago. This phenomenon is now considered as derived from increased NO and peroxynitrite levels in both the vascular smooth muscle and in skeletal and heart muscle. The whole syndrome of mitochondrial dysfunction is characterized by decreased rates of state 3 respiration and ATP synthesis, decreased respiratory controls and membrane potential, increased rates of state 4 respiration, and increased mitochondrial size and fragility. Some mitochondria in septic animals are observed enclosed in vacuoles and surrounded by normal mitochondria, suggesting increased mitochondrial turnover rather than necrosis (19,33).

The rates of tissue O_2 uptake (determined in tissue cubes of 1 mm^3 and suspended in Krebs medium) have to be interpreted as the result of a fast and at random oscillation of mitochondria between sates 3 and 4, driven by local energy demands and ADP availability. It must be taken into account the marked difference in O_2 uptake of mitochondrial state 3 (active respiration) and mitochondrial state 4 (resting respiration). It has been recently considered that under physiological conditions, a mitochondrial subpopulation is exposed to high ATP levels and another subpopulation is exposed to ADP levels that stimulate respiration (34). Sepsis increased tissue O_2 consumption by 40% in heart and 30% in diaphragm as shown in Table 1. At variance, mitochondria isolated from muscle in endotoxic and septic shocks exhibit impaired respiration. These dysfunctional mitochondria show a decreased active state 3 O_2 uptake. Table 2 summarizes data from a few

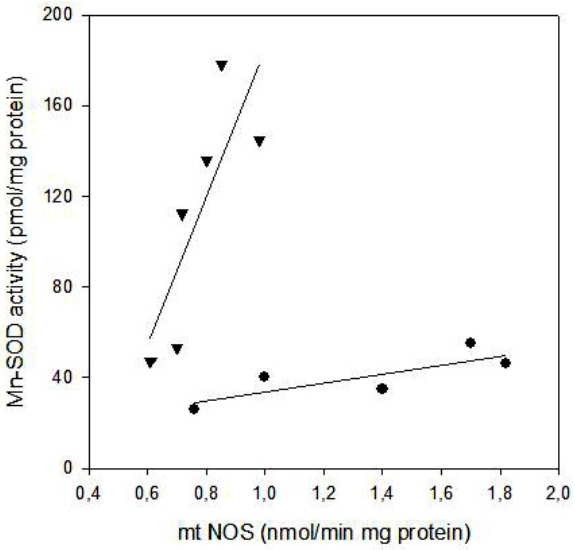

Figure 2. Linear regression analysis of mtNOS and MnSOD activities ($p > 0.01$). The corresponding slopes are: 326 pmol MnSOD/nmol NO min^{-1} (heart) and 20 pmol MnSOD/nmol NO min^{-1} (diaphragm).

Table 1. Effect of endotoxic shock on the tissue O_2 consumption

Organ/Treatment	O_2 uptake (ng-at O/min/g tissue)
Heart	
Control	983 ± 95
LPS, 6 hours	1435 ± 106 [1]
Diaphragm	
Control	543 ± 51
LPS, 6 hours	714 ± 31[1]

[1] $p < 0.01$ Alvarez, S., unpublished results

Table 2. Mitochondrial respiration supported by NAD-linked substrates in septic and endotoxic shock

Mitochondria/Condition	O_2 uptake (ng-at O/min/mg prot)	
	State 3	State 4
Rat muscle/septic shock[1]		
Control	149 ± 5	23 ± 2
Sepsis, 6 h	120 ± 8	24 ± 3
Sepsis, 12 h	97 ± 6	24 ± 8
Rat diaphragm/endotoxin[2]		
Control	200 ± 10	32 ± 2
Endotoxin, 12 h	183 ± 7	33 ± 3
Endotoxin, 24 h	120 ± 10	28 ± 2
Rat heart/endotoxin[3]		
Control	117 ± 5	33 ± 4
Endotoxin, 2 h	112 ± 7	38 ± 3
Endotoxin, 6 h	91 ± 3	39 ± 4
Human muscle/septic shock[4]		
Normal patients	171 ± 21	57 ± 10
Severe sepsis	81 ± 12	42 ± 8
Septic shock	65 ± 16	37 ± 9
Cardiogenic shock	119 ± 12	43 ± 7

Table reproduced with permission from (24); [1] 6 mM malate and 6 mM glutamate as substrates. [2] 2.5 mM malate and 10 mM pyruvate as substrates. [3] 1 mM malate and 10 mM glutamate as substrates. [4] 6 mM malate and 6 mM glutamate as substrates.

studies (23,35-38) that show that the mitochondrial respiratory rates supported by NAD-linked substrates are significantly decreased in rat and human skeletal muscle during sepsis and in rat heart and diaphragm after LPS administration. The respiratory impairment is due to an inhibition of electron transfer, as it can be inferred from the decreased state 3 respiratory rate, simultaneous with an unchanged state 4 respiration; meaning absence of uncoupling and the existence of normal ADP:O ratios. Results presented in Table 1 and 2 have to be interpreted as a tissue adaptation to the high energy demand (ATP production) driven by the sepsis syndrome. The high tissue O_2 consumption seems originated by an elevated mitochondrial subpopulation in state 3 in order to respond to the high ATP demand, despite the inhibition of mitochondrial respiration due to the increased mitochondrial NO production.

Findings of impaired O_2 utilization in septic patients and animals implicate a NO-mediated inhibition of the respiratory chain. It is noteworthy that, as recently reported, there is an association between skeletal muscle mitochondrial dysfunction, clinical severity, and poor outcome in human patients with septic shock (15). The severity of organ dysfunction and eventual poor outcome were associated with NO overproduction and increasing mitochondrial dysfunction (complex I inhibition and ATP depletion) (39).

3.4. Mitochondrial steady- state concentrations of O_2^-, H_2O_2, NO and $ONOO^-$ in sepsis.

The measurement of steady-state concentrations of O_2^-, H_2O_2, NO and $ONOO^-$ in biological systems is a difficult task due to the low intracellular concentrations (10^{-9} - 10^{-11} M), the instability, and the lack of selective and sensible assays. For situations of low intracellular concentrations of the involved chemical species, or when the enzymes do not follow a Michaelis-Menten dependence, the physicochemical approach of the steady state affords a useful tool, although the limitations of this method should be taken into account. The mitochondrial concentrations of O_2^-, H_2O_2, NO and $ONOO^-$ in heart and diaphragm of septic rats were calculated using this approach and are described elsewhere (16).

It is worth noting that O_2^- levels are not affected by endotoxic shock (1×10^{-10} M for heart and 5×10^{-10} M for diaphragm), whereas H_2O_2, NO and $ONOO^-$ levels are increased by 72%, 27% and 50% in heart and by 32%, 100% and and 130% in diaphragm (16). Concerning NO concentration, the calculated values (15-30 nM) are on the same order as those reported for diaphragm and heart (30-400 nM) (28,40) and as the 29 nM NO detected electrochemically with a NO electrode in a single heart mitochondrion after mtNOS activation with Ca^{2+} (41). The increase in the calculated NO concentration (for both organs) reflects the measured increases in mtNOS activity. The existence of a stable low mitochondrial $ONOO^-$ concentration (8- 15 nM) is indicated by the detection of nitrotyrosine in normal mitochondria (25).

4. INFLAMMATION BY AMBIENT AIR PARTICLES

4.1. Health effects of ambient air particles.

There is strong evidence that ambient air pollution particles currently present a serious risk to human health. Several epidemiological studies have shown associations between airbone particulate matter (PM) and adverse health effects. These studies have shown a significant association between exposure to PM and increased mortality and morbidity. Other important effects include aggravation of respiratory and cardiovascular disease, lung disease, decreased lung function, asthma attacks, and cardiovascular problems such as heart attacks and cardiac arrhythmia. These observations are indicated by increased hospital admissions, emergency room visits, absences from school or work, and restricted activity days. In a recent report, the World Health Organization estimates that inhalation of PM in ambient air is responsible for 500,000 excess deaths each year worldwide (42). Individuals particularly sensitive to particle exposure include older adults, people with heart and lung disease, and children (43).

PM consists of a mixture of particle components, including traffic and combustion-derived carbon-centered particles, secondary particles (nitrates and sulphates), wind-blown dust of geological origin potentially containing endotoxin, and biological particles (spores, pollen) with their associated allergens. PM are classified as coarse (with an aerodynamic diameter between 2.5–10 microns, PM_{10}), fine (aerodynamic diameter between 0.1-2.5 microns, $PM_{2.5}$) and ultrafine (aerodynamic diameter less than 0.1 micron). PM_{10} deposit predominantly in the lower airways (nose and throat) and are cleared by exhalation or mucociliary and swallowing. Fine and ultrafine particles can penetrate into the lower airways and alveoli and are more closely related with adverse health effects of PM than coarser particles (44).

Although the epidemiologic evidence is convincing, the biologic mechanisms by which PM evokes systemic effects remain to be defined. A number of hypotheses have been postulated to explain the adverse effects of PM. Some of these hypotheses have pointed out the role of particulate mass concentration, particle size and surface area, adsorbed transition metals, acids, sulfates and nitrates, elemental carbon, and co-pollutants (gases) (45). Other studies have focused on the participation of organic compounds. These include chemicals such as polycyclic aromatic hydrocarbons (PAHs), nitroderivatives of PAHs, oxygenated derivatives of PAHs (ketones, quinones and diones), heterocyclic compounds, aldehydes and aliphatic hydrocarbons (46).

4.2. Inflammatory response produced by particulate matter

It has been observed that the respiratory tract shows an inflammatory response after inhalation or intratracheal instillation of different pollutants such as residual oil fly ashes (ROFA), cooper particles (CMP), coal fly ashes (CFA) with an increase in the number of neutrophils, protein concentration and the production of reactive oxygen species (47,48). Cultured human alveolar macrophages produce TNF-alpha and proinflammatory cytokines such as granulocyte-macrophage colony-stimulating factor, IL-6, and IL-1 after phagocytosing PM (49).

A critical component of the inflammatory response to particles in the lungs is the release of cytokines from activated macrophages and lung epithelial cells, resulting in neutrophil recruitment. This response may be caused by the deposition of PM into the alveolar space in the lungs, inducing the release of cytokines from alveolar macrophages. The release of proinflammatory mediators from PM-exposed macrophages appears to be important in stimulating cytokine release from lung epithelial cells, thus amplifying the inflammatory response (50).

Attention has been focused on transition metals and ultrafine particles as components of PM that may be responsible of the inflammatory response. Each of these individual components of PM has been shown to produce an inflammatory response after exposure in animal models (51,52).

Trace amounts of bacterial endotoxin present in PM has also been postulated to induce inflammatory effects. It has been reported that low levels of endotoxin may activate lung macrophages which in turn produce macrophage inflammatory protein-2 (MIP-2) and TNF-alpha (53).

The inflammatory genes induced after exposure to PM (TNF-alpha and IL-8, for example) are regulated by redox sensitive transcription factors such as NF-kappaB, AP-1 and C/EBP. NF-kappaB is one of key transcription factors that are involved in the inflammatory responses to the PM in the lungs (see Figure 1). The result of NF-kappaB activation is the generation of proinflammatory cytokines such as TNF-alpha, IL-6, IL-8 by airway and alveolar epithelial cells (54). Quay et al reported that ROFA activated NF-kappaB via a process thought to be related to the presence of vanadium in the particles; this effect could be inhibited by the addition of the antioxidants deferoxamine and N-acetyl cysteine (55).

Taken all this data into consideration, the probable sequence of events for pollutant-induced lung inflammation involves the following: (a) injury to epithelial cells by reactive oxygen species, possibly enhanced in the presence of metals via Haber-Weiss and Fenton chemistry, accompanied by activation of nuclear regulatory factors, leading to elaboration of proinflammatory cytokines, including IL-8 and IL-6, and increased expression of NOS, with increased NO in exhaled air; (b) activation of vascular endothelium and circulating leukocytes. Emigration of inflammatory cells from blood to tissue sites involves up-regulation of adhesion molecules and other markers on vascular endothelium and on circulating leukocytes; and (c) the process of leukocyte-endothelial binding include increased expression of adhesion molecules followed by shedding of adhesion molecules as cells "tether and roll", leukocyte activation, stable adhesion, and transmigration through the epithelium.

Table 3. Oxidative stress parameters in mice lung homogenates exposed to airborne pollution particles

Oxidative stress parameter	Control	ROFA
TBARS (pmol/mg prot)	3.7 ± 0.2	12.4 ± 1.6[1]
Superoxide dismutase (U SOD/mg prot)	1.4 ± 0.1	0.7 ± 0.1 [1]
Catalase (pmol/mg prot)	0.38 ± 0.03	0.19 ± 0.03 [1]
Thiol content (nmol/mg prot)	3.8 ± 0.6	3.1 ± 0.2
Ascorbic acid (micro-mol/mg prot)	0.98 ± 0.05	0.43 ± 0.09 [1]

[1] $p < 0.01$ Evelson & Tasat, unpublished results

4.3. Oxidative stress by ambient air particles.

Oxidative stress and oxidative damage are central hypothetical mechanisms for the adverse effects of PM. Our laboratory studied the occurrence of oxidative stress and damage in mice lung. The exposure consisted of a nasal instillation (0.17 mg / kg body weight) of a ROFA suspension 3 times a day, 3 times a week, during one week. Thiobarbituric acid reactive substances (TBARS) levels, the activity of antioxidant enzymes (superoxide dismutase and catalase), GSH and ascorbic acid levels were measured. The results are shown in Table 3. The results suggest the occurrence of both oxidative stress and oxidative damage with an increase in the level of oxidation products (indicated by the increase in TBARS) and a decrease in the levels of antioxidants (shown by the decrease in activities of superoxide dismutase and catalase and the levels of ascorbic acid).

The mechanisms by which PM causes oxidative stress is still unknown but the phenomenon may be attributed to different causes, that include: (a) generation of oxidants at the particle surface, (b) release of metals or organic components from the particle, and (c) triggering of an inflammatory response.

The direct generation of reactive oxygen species at the surface of the particles is supported by the concept that the particle surface offers a unique physicochemical interface to catalyze reactions resulting in oxidant production. The interaction of PM with membrane components was recognized by the presence of free radicals and oxidants on the particle surface (56).

The release of compounds such as transition metals or organic compounds from thr particles has been documented. The involvement of transition metals, such as Fe, Va, Cr, Mn, Co, Ni and Cu, which are able to catalyze Fenton-type reactions and generate hydroxyl radicals, has been proposed. Indeed, the production of oxidative damage, as measured by TBARS levels, shows significant correlations with the overall metal content and with the content of individual metals such as Fe and Va. This effect was inhibited by the presence of antioxidants such as dimethylthiourea and deferoxamine (57-59).

The organic fraction of PM contains quinones that can act as catalysts to produce oxidant species directly through redox cycling and may play a role in the oxidative stress produced by PM. PAHs can also induce oxidative stress indirectly through biotransformation by cytochrome P450 to generate redox active quinones (60). Since several of the components of PM can generate oxidative stress, there is a potential for additive or synergistic interaction between the components. Recently, Wilson et al showed that ultrafine particles and metals can interact in a cell-free environment to generate oxidant species, as measured with the probe dichlorofluorescein, and in the lung to generate inflammation as shown by increased neutrophil influx (61).

Activation of inflammatory cells capable of generating reactive oxygen and nitrogen species is other proposed mechanism: chronic inflammatory lung diseases are characterized by activation of epithelial cells and resident macrophages and the recruitment and activation of neutrophils, eosinophils, monocytes, and lymphocytes. It is understood that the oxidative stress caused by the activation of the inflammatory system, comprised by alveolar macrophages and neutrophils, plays an important role in the deleterious effects of PM in multicellular organisms. Reactive oxygen species are generated during phagocytosis of the particles, leading to enhancement of oxidative stress and triggering of the inflammatory response (45).

4.4. NO metabolism

Nitric oxide regulates pulmonary vascular and airway tone and plays an important role in lung host defense against bacteria. However, NO may be cytotoxic by inhibiting critical enzymes such as mitochondrial cytochrome oxidase and ubiquinol-cytochrome c reductase (complex III). Other enzymes are also tagets for increased NO steady state levels, mitochondrial aconitase and ribonucleotide reductase. Moreover, NO may exhibit cytotoxic actions by S-nitrosylation of thiol groups or by binding to iron-sulfur centers. In addition, NO reacts with O_2^- to form peroxynitrite, which can nitrate and oxidize key enzymes and amino acids in lung proteins such as surfactant protein A, and inhibit their functions (62).

The environmental particles that after inhalation are internalized by phagocytosis trigger the release of reactive oxygen species and inflammatory mediators in both the alveolar macrophages and the airway epithelial cells. In addition, NO was suggested to play a role in the cellular toxicity of the particles. However, results are scarce and the experimental data dealing with this subject is controversial.

Preliminary results from our laboratory using the exposure protocol described in point 4.3, showed no changes in the production of NO in lung homogenates (data not shown). Various studies (reviewed in ref. 63) have shown that inhalation or intratracheal instillation of respirable mineral dusts or asbestos fibers increased the levels of NO and nitrotyrosine. However, murine macrophages exposed to aqueous suspensions of PM show a decrease in NO production, directly correlated with a decrease in the expression of iNOS as determined by Western blot analysis (64).

On the other side, exposure to diesel exhaust particles (DEP) in mice was shown to double the level of NO in the exhaled air. DEP exposure also increased the

level of eNOS in the airway epithelium and iNOS in the macrophages. Pretreatment with N-G-monomethyl L-arginine, a nonspecific inhibitor of NO synthases, significantly reduced the airway hyper-response induced by DEP (65). Takano *et al* investigated the effects of three different agents on eosinophilic airway inflammation induced by the intratracheal instillation of DEP in mice: L-arginine, L-NAME, and aminoguanidine. Airway inflammation induced by DEP was aggravated by the administration of L-arginine or L-NAME, whereas it was reduced by aminoguanidine. NO produced from iNOS may participate in the pathogenesis of DEP-induced airway inflammation, while NO derived from eNOS may afford protection against the airway inflammation induced by DEP (66).

5. CONCLUSIONS AND PERSPECTIVES

The molecular and cellular mechanisms by which sepsis leads to organ dysfunction remain to be established. Although microvascular flow abnormalities occur, findings of decreased mitochondrial O_2 consumption despite elevated tissue O_2 tensions (cytopathic hypoxia, 67)) an with minimal cell death despite marked functional and biochemical derangements, suggest that the problem lies more in cellular O_2 utilization rather than a in O_2 delivery. As mitochondria utilize 95% of total body O_2 consumption to generate ATP, organ dysfunction seems a consequence of an impaired bioenergetic process. Thus, mitochondria play a central role in the intracellular events associated with inflammation and septic shock, as active sources and sensitive targets of NO.

Associated to the concept of endotoxin-driven mitochondrial dysfunction leading to impaired organ function, new therapeutic opportunities should be considered. For example, antioxidant molecules that are generally targeted to mitochondria and manipulation of proteins acting on mitochondrial membrane pores are demonstrated to preserve mitochondrial integrity during conditions associated with oxidative stress, such as sepsis. Additional investigations detailing the mechanisms and timing of mitochondrial injury during sepsis are necessary before developing effective protection strategies for mitochondrial function with application in the clinical setting.

The mechanisms of PM health effects are still poorly understood. However, studies in isolated cells and in animal models (by inhalation or instillation) suggest a variety of possible mechanisms, including direct effects of particle components and indirect effects due to pro-inflammatory mediators. It is known that transition metals, organic compounds and bacterial endotoxins are some PM constituents that mediate toxic effects. The current hypothesis is that the lung inflammation induced by inhalation of ambient particles implies a systemic inflammatory response, with endothelial activation and oxidative and nitrosative stress. These changes contribute to the increase in morbidity and mortality associated to polluted areas.

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