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Lung inflammation and thrombogenic responses in a time course study of *Csb* mice exposed to ozone

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ABSTRACT: Ozone is a well-known oxidant air pollutant, inhalation of which can result in oxidative stress, and lead to pulmonary inflammation.

The aim of this study was to evaluate the time-course events after a single ozone exposure in transcription-coupled repair defective *Csb* and wild type mice.

Mice were exposed for 3 h to 2 ppm ozone and biological parameters related to oxidative stress and inflammation were examined in the lungs at 0, 4, 9, 24 and 48 h after exposure. In addition the procoagulant and thrombomodulin activities were explored by a combination of assays for tissue factor and thrombin generation.

This study revealed a significant biological response to ozone, for both *Csb* and wild type mice. The onset of inflammation in *Csb* mice, as indicated by an increase in interleukin-6, tumor necrosis factor-α and total cell influx, occurred earlier compared with those seen in wild type mice. On the other hand, *Csb* mice showed a delayed antioxidant reaction compared with wild type mice. Both genotypes developed a procoagulant reaction characterized by a stably increased tissue factor activity and a progressive increase in thrombin generation after 2 days.

These experiments have shown that ozone, a well-known toxic substance from the environment, induces not only inflammation, but also procoagulant reactions in the lungs of mice. These results have implications for understanding the systemic effects induced by oxidant air pollutants. Copyright © 2008 John Wiley & Sons, Ltd.

KEY WORDS: ozone; time-course; inflammation; thrombogenicity

Introduction

Ozone is a primary component of photochemical smog. Ozone-induced pulmonary injury has been well investigated for more than three decades and is characterized by oxidative stress mediated processes resulting in damage of lung epithelial cells, inflammation, impaired lung function and airway hyper responsiveness (Shore *et al*., 2001).

Cockayne syndrome group B (*Csb*) mice, being repair-deficient in transcription coupled repair are sensitive to oxidative stressors such as paraquat and gamma radiation (de Waard *et al*., 2003; 2004). A recent study investigated acute lung injury caused by the oxidant ozone in *Csb* mice (Kooter *et al*., 2007). Besides significant biological responses, additionally a higher level of tumor necrosis factor α (TNF-α) was observed in *Csb* mice compared with repair-proficient counterparts after ozone exposure. In addition, a trend was observed that *Csb* mice showed a lower number of differentially expressed genes which had a lower fold ratio towards ozone exposure compared with the repair-proficient mice.

The aforementioned study was designed to study gene expression at only the 4 h time point after ozone exposure. The present study tests the hypothesis that repair defective *Csb* mice are more sensitive compared with their wild-type mice in their time-course response to ozone. More specifically it was designed to characterize the time course of oxidative stressor ozone over 0 to 48 h post exposure to follow both inflammation and the relationship with thrombogenic responses. Thrombogenic responses due to ozone exposure are less well studied in an experimental setting. In human subjects it has been shown that urban air pollution, and in particular ozone, is associated with cardiovascular events such as blood coagulation and ischemic stroke in human subjects (Chuang *et al*., 2007; Henrotin *et al*., 2007). Another study, performed at relatively low ozone levels, showed,

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however, no activation of the clotting system, systemic inflammatory changes and/or endothelial damage (Hermans *et al*., 2005).

Tissue factor, in particular, was reported to increase in human volunteers after 1 h of a 2 h 0.4 ppm ozone exposure (Devlin *et al*., 1996). For an *in vivo* mouse study it was recently reported that ozone induces the expression of tissue factor (TF) mRNA in lung tissue (Kooter *et al*., 2007). In addition, it was shown that exposure of rodents to the oxidative air pollutant particulate matter (PM) can induce TF expression (Kooter *et al*., 2005). TF is synthesized in the alveolar macrophage population in the lung, and as a result of ozone inhalation both TF mRNA levels and TF activity are increased (McGee *et al*., 1990), thereby changing the haemostatic balance to procoagulant. The complex of TF and its ligand, coagulation factor VIIa (FVIIa), is not only the primary initiator of the coagulation cascade, but is also involved in major cellular processes such as inflammation, tumor growth and angiogenesis (Versteeg and Ruf, 2006). We recently explored the procoagulant and anticoagulant activities of TF and thrombomodulin (TM), respectively, by means of a modified thrombin generation assay (ten Cate *et al*., 2007). It was shown that both endotoxin and particulate matter induce TF and down regulate thrombomodulin activities *in vivo* thereby causing a shift towards a prothrombotic state.

The present study focused on time-course processes of oxidative stress mediated inflammation in lung tissue and lavaged cells. In addition, the influence of ozone exposure on the prothrombotic activity of lung tissue was studied in mice to improve our understanding of the contribution of oxidative air pollution in the development of systemic effects.

Materials and Methods

Animal Housing and Ozone Exposure

Repair-deficient *Csb* mice have a pure C57BL/6J genetic background, to which they have been backcrossed. Male *Csb* mice and their wild type C57BL/6J littermate mice were 12 weeks old and bred at the National Institute for Public Health and the Environment (RIVM).

During the acclimatization period of at least 7 days, as well as during exposure and recovery period, the animals were housed in 0.2 m^3 stainless steel and Lexan inhalation chambers in the RIVM inhalation facilities (Marra and Rombout, 1990). *Csb* and their wild type mice (7 per group) were exposed for 3 h to either 2 ppm ozone (target concentration) or clean air during the animals' dark cycle, in which the animals are active. Experimental conditions during the air and ozone exposure were as described previously (Kooter *et al*., 2007). Experiments were approved by the Animal Ethics Committee (IUCAC) of the Dutch National Vaccine Institute (NVI), Bilthoven, the Netherlands. The exposure level and duration for our protocol were based upon the popularity of this mouse model as a model of ozone/oxidant injury to the lower respiratory tract (Kleeberger *et al*., 1990; Shore *et al*., 2001).

Necropsy

Necropsy was performed 0, 4, 9, 24 and 48 h post-exposure. At necropsy, the animals were weighed and anaesthetized with a mixture of ketamine (100 mg ml^{-1}, Aesculaap, Boxtel, the Netherlands), xylazine (20 mg ml^{-1}, Bayer, Leverkusen, Germany) and saline in a ratio of 10:4:14. Injection of the anaesthetic was applied i.p. (2 ml kg^{-1} body weight), resulting in a dose of 70 mg kg^{-1} body weight for ketamine and 6 mg kg^{-1} body weight for xylazine. The animals were killed by exsanguination via the abdominal aorta. Saline perfusion of the lungs was performed via the right cardiac ventricle to remove blood. Lungs were lavaged (three in and out lavages using the same fluid) with a volume of saline corresponding to 40 ml kg^{-1} body weight at 37 °C to obtain bronchoalveolar lavage fluid (BALF). A second wash was performed with an amount of saline which corresponds to the recovered volume of the first lavage. The recovered BALF was placed on ice until further processing. The left and right lungs were dissected, weighed and frozen in liquid nitrogen.

BALF Analysis

BALF analysis was performed as reported previously, wherein the used lavage volume was adjusted to the mouse body weight (Kooter *et al*., 2007). In addition interleukin-6 (IL-6) was determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Biosource, Etten-Leur, the Netherlands). The levels of uric acid (UA-B) were determined using a reagent kit obtained from Roche (Almere, the Netherlands).

Lung Homogenate Analysis

Oxidative Stress

Frozen left lungs were homogenized in 120 mM KCl, 30 mM phosphate buffer (pH 7.2), supplemented with protein inhibitors (1 μg ml^{-1} leupeptin, 1 μg ml^{-1} aprotinin, 10 μg ml^{-1} soybean trypsin inhibitor, 1 μg ml^{-1} pepstatin and 0.5 mM phenylmethylsulphonyl fluoride (PMSF)) at 0–4 °C. The suspensions were centrifuged at 600 *g* for 10 min at 0–4 °C to remove nuclei and cell debris. The pellets were discarded and the supernatants were used as homogenates according to Rhoden *et al*. (2004).

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Malonic dialdehyde (MDA) was measured using high performance liquid chromatography (HPLC) with fluorescence detection (Varian Ass, Middelburg, Netherlands) (Nielsen *et al*., 1997). Glutathione peroxidase as a marker for oxidative stress (GPx) was measured on an autoanalyser (Hitachi 912, Roche, Almere, Netherlands) with a kit from Randox, Crumlin, United Kingdom. Superoxide dismutase (SOD) as a marker for oxidative stress was measured on an autoanalyser (Hitachi 912, Roche, Almere, Netherlands) with a kit from Randox, Crumlin, United Kingdom. Reactive carbonyl due to oxidized protein and as a result of oxidative stress was measured using an ELISA kit from Zentech, Papatoetoe, New Zealand. Total protein was measured on an autoanalyser (Hitachi 912, Roche, Almere, Netherlands) with a bicinchoninic acid (BCA) kit from Pierce, Etten Leur, Netherlands. Hemoxygenase 1 (HO-1) was measured according to Araujo *et al*. (2003), but with the adaptation that bound horseradish peroxidase (HRP) was detected using 3,3′,5,5′tetramethyl benzidine (Sigma-Aldrich) in substrate using 450 nm as the detection wavelength corrected for the background at 620 nm.

Thrombogenicity

Frozen right lung was freeze-dried for 3 days, pulverized, and subsequently the lung powder was divided for RNA isolation and protein analyses. Fractions for protein analyses were solved in 50 mM *n*-octyl ß-D-glucopyranoside (Sigma-Aldrich) in Hepes-NaCl buffer (HN, 25 mM Hepes, 175 mM NaCl, pH 7.7), vortexed, centrifuged twice (10 min, 13 000 rpm). Total protein content of the tissue homogenate was spectrophotometrically determined using the Biorad DC Protein Assay system according to the manufacturer's instructions (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands).

Calibrated Automated Thrombogram

The calibrated automated thrombogram (CAT, Thrombinoscope, the Netherlands) was used to determine the contribution of mouse lung homogenates to thrombin generation in platelet-poor plasma. The protocol from Hemker *et al*. (2002) was adapted. 15 µl of tissue homogenate (5 mg ml^{-1} total protein) was added to 80 µl of platelet-poor pooled human plasma (University Hospital Maastricht), which consisted of plasma from 80 healthy volunteers. No additional TF or phospholipids were added and triggering of thrombin generation therefore depended on molecules present in the lung homogenate. The following parameters were derived from a typical thrombin generation curve: lag time, peak height and endogenous thrombin potential (ETP, the area under the curve). In a previous study (Frederix *et al*. unpublished observations) it has been shown that the lag time was shortened by high levels of tissue factor activity in tissue and both the ETP and peak height were inversely correlated with the presence of thrombomodulin in tissue. Overall, the thrombogenicity, defined as a thrombin generation curve with a short lag time and high ETP and peak height, reflects the sum of procoagulant tissue factor activity and anti-coagulant thrombomodulin in tissues. Intra- and inter-assay coefficients of variance were < 8% for all parameters measured.

Tissue Factor Activity Measurement

Tissue factor activities in lung homogenates were determined using a home-made activity assay as described previously (Cosemans *et al*., 2005). In brief, dissolved tissue homogenates with a concentration of 1 mg ml^{-1} total protein were diluted 20 times in Hepes–NaCl (HN) buffer (25 mM HEPES, 175 mM NaCl, pH 7.7). A reference curve was prepared with Innovin (Dade Behring Holding GmbH, Liederbach, Germany), starting with 5 pM and diluted serially 7 times, also in HN buffer. Samples were incubated for 45 min at 37 °C in the presence of recombinant factor VII (FVII) (Novo Nordisk, Bagsværd, Denmark), 0.2 mM 20/80 PS/PC vesicles, 1 U ml^{-1} bovine factor X (Sigma-Aldrich) and 100 mM Ca^{2+}. The formation of factor Xa was then measured kinetically using the chromogenic substrate S 2765 (Chromogenix, final concentration of 0.7 mg ml^{-1} diluted in 50 mM Tris-HCl, 175 nM NaCl, 30 mM Na_2EDTA, pH 7.4) by measuring the optical density (OD) at 405 nm each 15 s, for 15 min at 37 °C.

mRNA Quantification

Total RNA was isolated using a Trizol method according to the single-step method previously described (Chomczynski and Rymaszewski, 2006). Minor modifications of this protocol were the use of a commercial guanidinium thiocyanate solution (Tri Reagent, Sigma-Aldrich). Five to ten mg freeze-dried tissue was solved in 1 ml Tri Reagent. Samples were stored at −80 °C for precipitation steps, RNA was washed with 80% (v/v) ethanol and concentrations were spectrophotometrically measured in diethylpyrocarbonate (DEPC) treated water. cDNA was synthesized using the Avian Enhanced First Strand Synthesis kit (Sigma-Aldrich) according to the manufacturer's instructions. mRNA levels for TF and TM were measured on a light cycler system 1.2 (Roche, Woerden, The Netherlands) using the SYBR premix Ex Taq kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions using the following primers: TM forward 5′-GTCACGGTCTCGACAG, TM reverse 5′-GCAGCGTTTGAAAGTCC, TF forward 5′-GAAGAACACCCCGTCG, TF reverse 5′-GTTCGTCCTAACGTGACA. Quantification was done relative to household gene glyceraldehyde 3 phosphate dehydrogenase (GAPDH) using forward 5′-TCCCAGAGCTGAACGG and reverse 5′-GAAGTCGCAGGAGACA primers. Levels of TF and TM are expressed as ratios of TF/GAPDH or TM/GAPDH and each data point represents an average of three measurements.

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Statistical Analysis

All calculations were carried out using S-Plus (Mathsoft, release/version number: S-Plus 2000 prof. release 3). All data of biological effect parameters were log-transformed and, subsequently, a two-way analysis of variance (ANOVA) was performed. Two-way ANOVA analyses were used to assess differences due to ozone exposure, genotype and their interaction on the complete data set. Values of $P < 0.05$ were considered statistically significant.

Results

Body and Organ Weights

Body weight was lowered due to exposure to ozone especially at the 24 h and 48 h time points (reduction of 5%, 9%, and 12%, 6% for respectively the wild type and *Csb* mice). Both the body and organ (heart and lung) weights were reduced by the factor genotype, showing lower weights for all repair-deficient *Csb* mice compared with all wild type mice, as expected and reported before (Kooter *et al*., 2007). At the 0 h time point, for the mice exposed to clean air, the weights of body, heart and lung were respectively 13%, 15% and 13% lower in the *Csb* mice compared with the wild type mice, indicating strain differences between the two mice strains.

BALF Analysis

Results for BALF cell differentiation are shown in Fig. 1. The total cell concentration in BALF was significantly decreased after 3 h exposure to ozone, i.e. at the 0 h time point, for both the *Csb* and wild type mice compared with the clean air exposed mice. Subsequently, a marked rise in BALF cell counts occurred which reached a summit for the *Csb* mice at the 9 h time point (180% of control, $P < 0.001$) and for the wild type mice at the 24 h time point (175% of control, $P < 0.01$). At the 48 h time points values were back to normal. PMNs increases were largely (> 95%) responsible for these changes in total cell concentration in BALF. At 4 h post exposure an increase of PMNs was observed for ozone exposed mice. This increase was significant for both genotypes at the 9 h and 24 h time points (Fig. 1B).

A two-way ANOVA performed on the complete data set revealed no significant effect due to genotype except for parameter lymphocytes, and a significant effect due to exposure to ozone for the following biochemical parameters in BALF: total protein, LDH, IL-6, TNF-α and UA. The results for total protein are shown in Fig. 2A. This indicator of epithelial damage in the lung increased significantly due to ozone exposure in both *Csb* and wild type mice at 0 and 4 h time points, reaching 130% of

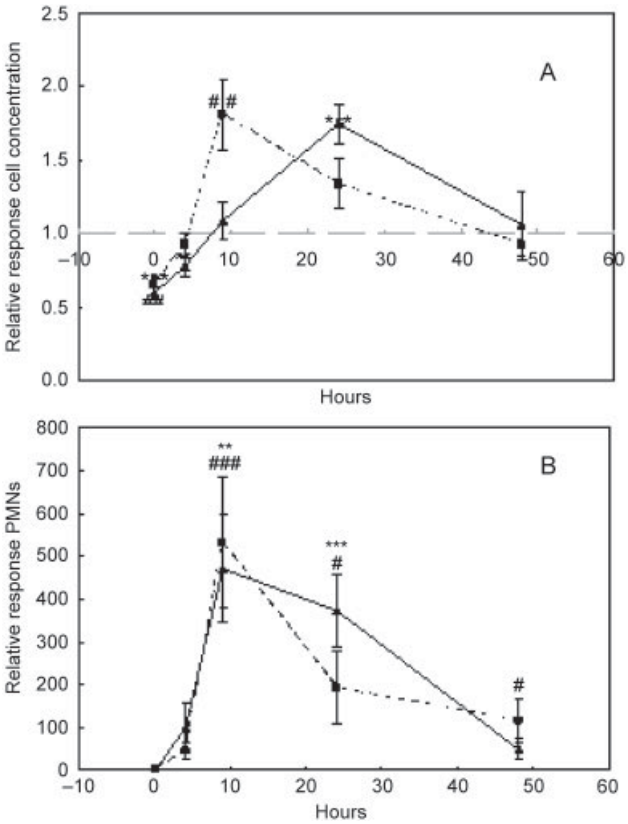

Figure 1. Relative effect on total cell (A) and polymorphonuclear neutrophils (PMNs) (B) concentrations in bronchoalveolar lavage fluid (BALF) at various times after termination of a 3 h exposure of wild type (solid line) and *Csb* (dashed line) mice ($n = 7$) to 2 ppm ozone. Relative effect is defined as the mean value of the ozone exposed group divided by the mean value of the sham exposed group at the same time point. Error bars indicate the standard error of the mean, corrected for the error introduced by the normalization. *, **, *** and #, ##, ### Significantly different from control at $P < 0.05$, < 0.01, < 0.001 for the wild type and *Csb* mice, respectively

control values. No significant differences between the genotypes were found. At the 24 and 48 h time points the highest protein levels were observed, reaching peaks between 200% and 300% of control values. LDH levels were mainly elevated immediately after exposure in both mice strains and returned rapidly to control values (Fig. 2B). Interestingly, increased IL-6 release seemed to occur already before the termination of the exposure in *Csb*, whereas in wild type mice this response developed much slower and reached a comparable maximum at a later time point (9 h). A similar response pattern was seen for TNF-α (Fig. 2D). The antioxidant UA showed a more variable response pattern and was significantly increased in wild type mice at 9 h time point, reaching up to 220% of control values. Whereas the response in *Csb* mice at the 24 h time point increased to 180% of control levels (Fig. 2E).

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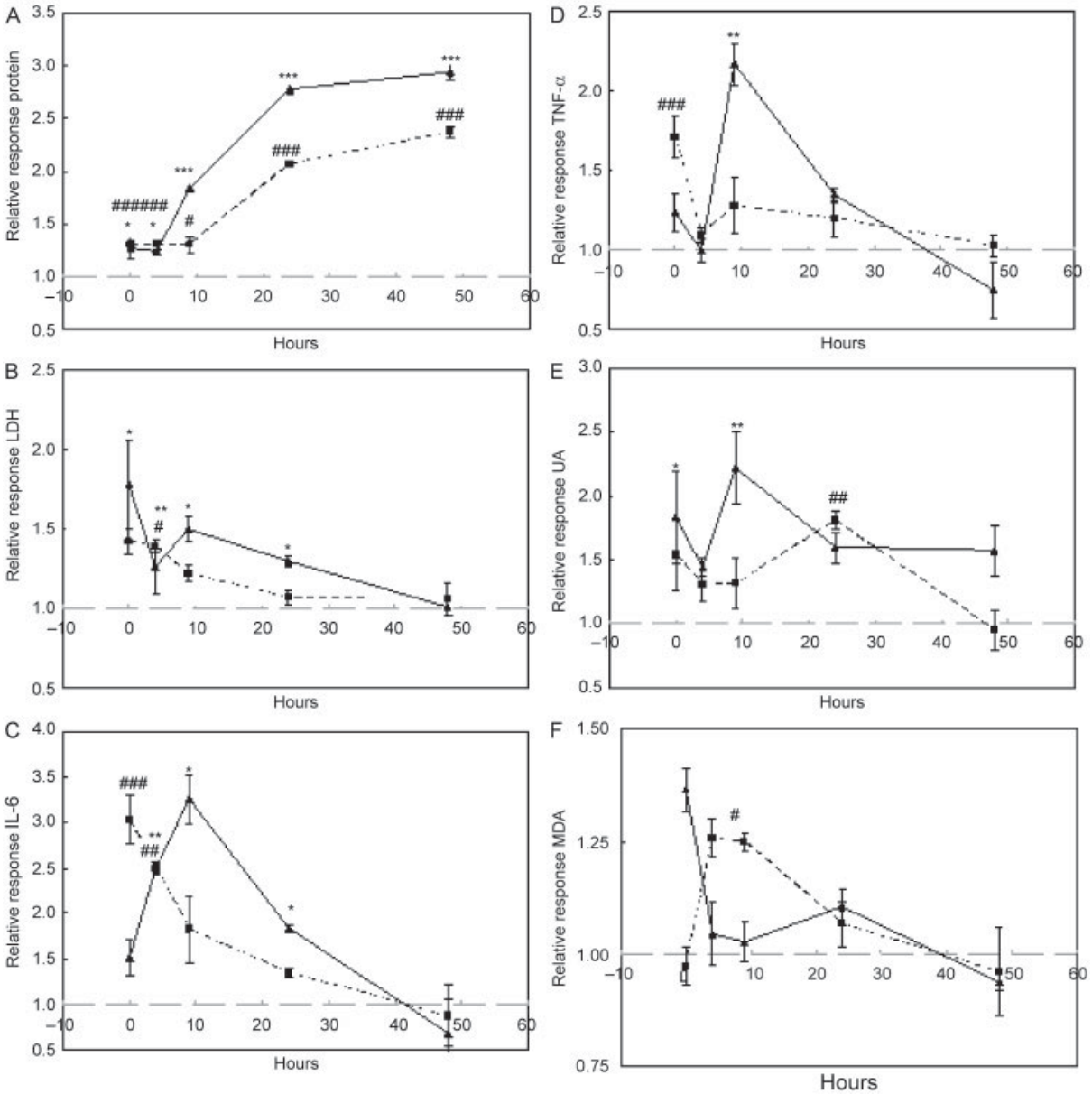

Figure 2. Relative effect on protein (A), LDH (B), IL-6 (C), TNF-α (D) and UA (E) in bronchoalveolar lavage fluid (BALF), and lung homogenate MDA (F) concentrations at various times after termination of a 3 h exposure of wild type (solid line) and *Csb* (dashed line) mice (n = 7) to 2 ppm ozone. Error bars indicate the standard error of the mean, corrected for the error introduced by the normalization. *, **, *** and #, ##, ### Significantly different from control at $P < 0.05$, < 0.01, < 0.001 for the wild type and *Csb* mice, respectively

Lung Homogenate Analysis

Oxidative Stress

A two-way ANOVA performed on the complete data set revealed no significant genotype effect, except for the parameter SOD, and no significant ozone effect for the oxidative stress parameters GPx, SOD, carbonyl or HO-1 measured in the lung homogenate tissue. However, lipid peroxidation as measured by MDA release in lung tissue was increased significantly to 125% of control in *Csb* mice at the 9 h time point (Fig. 2F); increases at other time points were not significant.

Thrombogenicity

The TF activity was significantly decreased immediately after the termination of the ozone exposure (0 h time point) to 78% of control for the *Csb* mice and to 38% of control for the wild type mice (Fig. 3A). Both at the

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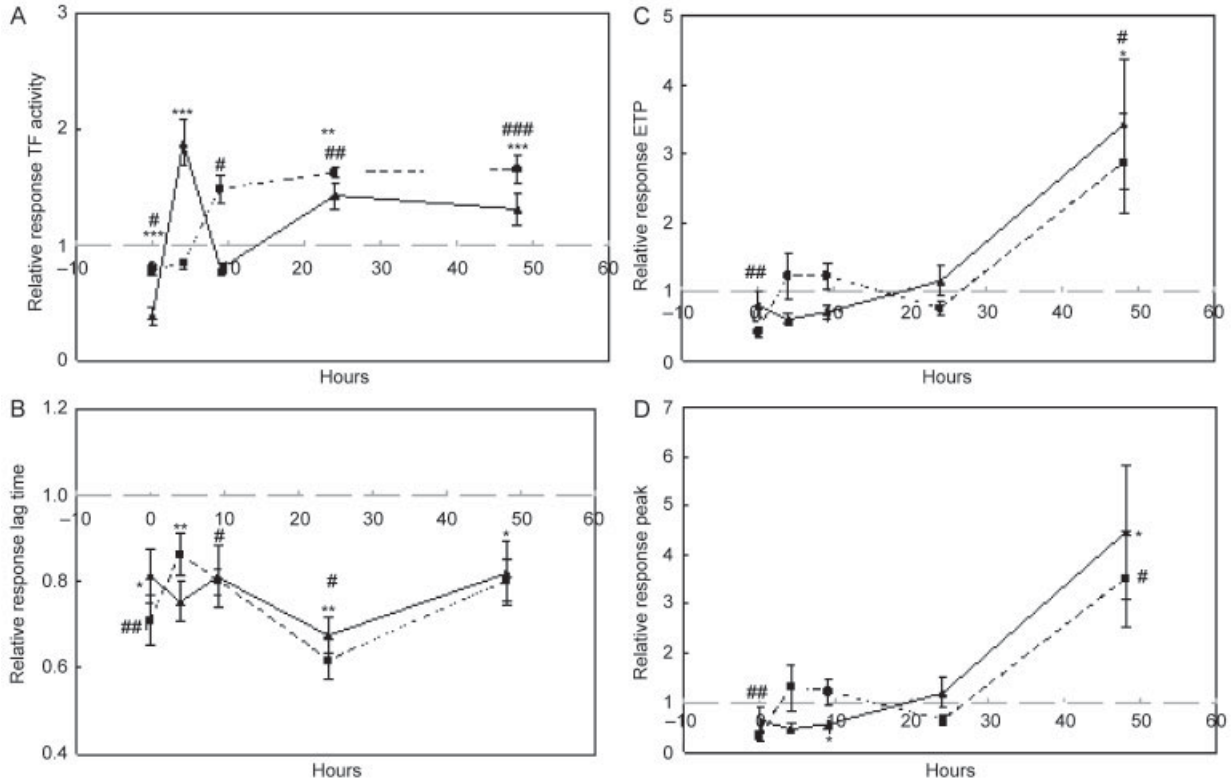

Figure 3. Relative effect of TF activity (A), thrombogenicity lag time (B), ETP (C), and peak (D) in lung homogenate tissue at various times after termination of a 3 h exposure of wild type (solid line) and *Csb* (dashed line) mice (n = 7) to 2 ppm ozone. Error bars indicate the standard error of the mean, corrected for the error introduced by the normalization. *, **, *** and #, ##, ### Significantly different from control at $P < 0.05$, < 0.01, < 0.001 for the wild type and *Csb* mice, respectively

24 and 48 h time points after ozone exposure, there was a significant increase in TF activity observed both for the *Csb* mice (163% of control) and wild type mice (136% of control).

The increase in TF activity was reflected by decreased lag times (Fig. 3B): the thrombin generation assay showed a reduction of about 75% at all time points due to ozone exposures for both types of mice. Both the ETP and peak height were altered due to ozone exposure (Fig. 3C, D). Due to the ozone exposure the peak height was significantly reduced to 30% of control for the *Csb* mice and to 65% and 30% in wild type mice at respectively the 0 h time point, and 48 h post exposure. Furthermore a significant increase in peak height was observed for both *Csb* mice, 350% of control and wild type mice, 450% of control. The ETP values correlated well with peak height, indicating a thrombotic state.

Using real time quantitative mRNA analysis of TF and TM the findings from previous gene expression profiles were verified. TF mRNA expression was increased in response to ozone exposure, whereas TM mRNA was decreased at 9 h post exposure (Fig. 4). At all other time points no differences were observed (data not shown).

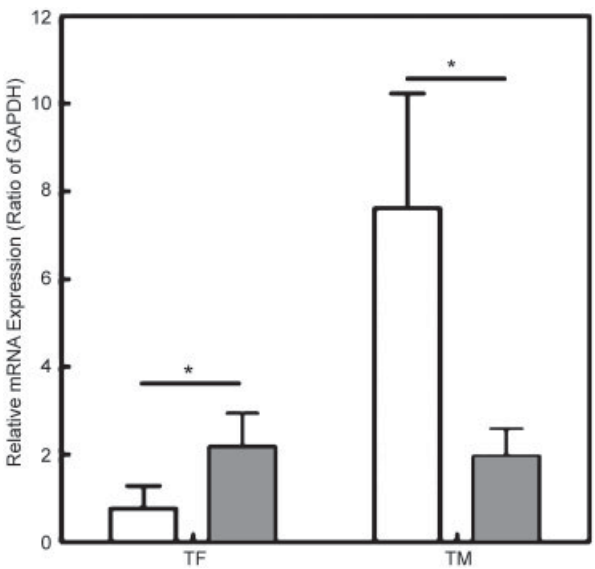

Figure 4. Relative mRNA gene expression (ratio of GAPDH) of TF and TM in lung tissue of wild type mice (n = 4) for clean air (white bars) and 2 ppm ozone exposed (grey bars) groups as determined by quantitative real-time polymerase chain reactions at 9 h after exposure. Error bars indicate the standard error of the mean. * Significantly different at $P < 0.05$

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Discussion

The present study tested the hypothesis that repair defective *Csb* mice are more sensitive compared with their wild-type mice in their response to ozone, by following both inflammation and the relationship with thrombogenic responses. This time course study indicated that inhalation of the environmental oxidative stressor ozone results in an earlier pro-inflammatory response and a delayed antioxidant reaction in Csb mice compared with wild type mice. This inflammatory response is followed by a procoagulant reaction in the lung, similar for both the *Csb* mice as well the wild type mice, due to a disbalance in TF and TM.

The balance between the oxidant and antioxidant potential has been the subject of many inhalation studies (Jang *et al*., 2005; Li *et al*., 2002). Antioxidants present within lung epithelial lining fluids constitute an initial line of defense against inhaled environmental oxidants such as ozone, tobacco smoke and particulate matter. In addition induction of several enzymes as well as increased expression of genes that code for these enzymes have been reported such as GPx, SOD and HO-1 (Kooter *et al*., 2005; Rietjens *et al*., 1985). In the present study, uric acid was significantly increased in BALF following ozone exposure both for the *Csb* and wild type mice. This is in accordance with findings that uric acid is the prime antioxidant responding to an ozone challenge, with larger increases compared with for example glutathione or ascorbic acid (Jang *et al*., 2005). In the lung homogenate no antioxidant response was observed by measuring GPx and SOD activity levels. Also MDA levels were hardly increased by the exposure and only found to be significantly increased for the *Csb* mice at the 9 h time point. Similar findings have been published by Jang *et al*. who exposed BALB/c mice for 3 h to 0.12, 0.5, 1 and 2 ppm ozone (Jang *et al*., 2005). They observed a clear–dose response relationship for the antioxidants uric acid, γ- and α-tocopherol, and increases for MDA levels were only detected at the highest concentration. In addition no significant increase in accumulation of oxidized proteins was found for either genotype in our study. The absence of an effect of the ozone exposure on the oxidative stress parameters GPx, SOD, carbonyl and HO-1 is very likely caused by the rather high concentration of ozone. As described by Li *et al*. (2002), oxidative stress can be viewed as a biological emergency that elicits a range of cellular responses. The first and most sensitive responses are the induction of antioxidant and phase-2 metabolizing enzymes, which are often regulated by the antioxidant response element (ARE) in the promoter of the corresponding genes of these enzymes (Itoh *et al*., 1997; Takizawa *et al*., 1999). It has been suggested that the failure of these antioxidant and detoxifying mechanisms to control the level of oxidative stress will lead to more damaging responses in the next step. This next step may be the initiation of inflammation and finally the activation of programmed cell death (apoptosis) leading to pulmonary and cardiovascular disease (Fig. 5). The concentration used in this study might have resulted in such a state of oxidative stress that indeed failure of the antioxidant and detoxifying mechanisms in the first tier lead to more damaging responses of inflammation in the second tier, as described by Li *et al*. (2002). The results of this study therefore might reflect effects of mainly this second tier.

In analogy with wild type data of Graham *et al*. (2001), our current data also show that ozone exposure results in a time dependent increase in inflammatory markers such as IL-6, TNF-α and PMNs influx in the BALF upon ozone exposure. These markers represent evidence that the pulmonary reaction of the *Csb* mice preceded the reaction in wild type mice. For the *Csb* mice the most prominent IL-6 increase was at the 0 and 4 h time point of analysis,

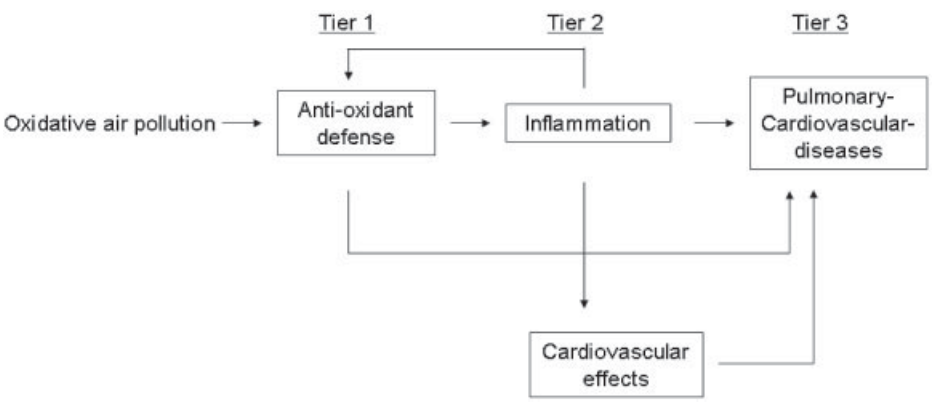

Figure 5. Oxidative stress model in response to oxidative air pollution. Different levels of oxidative stress can be distinguished. At a lower level of oxidative stress (tier 1), antioxidant enzymes are induced. At an intermediate level (tier 2), an inflammation response is induced and at the highest level (tier 3), cytotoxicity results in pulmonary and cardiovascular diseases

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whereas for the wild type mice it was at the 4 and 9 h time point. In accordance with our previous study, the relative increase of TNF-α in the BALF was higher for the *Csb* mice than observed for its wild type littermates shortly after exposure (Kooter *et al*., 2007). In contrast, at the 9 h time point a relative large increase in TNF-α in the BALF of the wild type mice was observed. The PMNs influx peaked at the 9 h and 24 h time point for *Csb* and wild type, preceding the peaks in protein concentration at 48 and 24 h post exposure, respectively. Similar findings were obtained for rats exposed for 2 h to 1 ppm ozone (Bhalla and Gupta, 2000). The initial small increase of protein at time points 0 h and 4 h is likely a consequence of the direct effect of ozone causing an increase in mucosal permeability, whereas oxidants and other toxic mediators released by activated PMNs contribute to the augmented response at the 24 h and 48 h time points, in accordance with previous data (Bhalla and Gupta, 2000).

The *Csb* gene functions in transcription-coupled repair of nucleotide excision repair (NER) and possibly also in base excision repair (BER), and in doing so, it is involved in removing transcription blocking lesions such as oxidative DNA damage induced by oxidative stress. From previous studies it is known that there is in general less transcriptional activity in repair-deficient *Csb* mice, which is compatible with the idea that, owing to enhanced DNA damage, especially in actively transcribed genes, transcription is hampered somewhat in *Csb* mice since lesions persist (Kooter *et al*., 2007). This is supported by at least two events as observed in this study for the *Csb* mice. First of all, the earlier pro-inflammatory reaction as indicated by the release of IL-6 and TNF-α can be regarded as a sign for increased susceptibility. These factors are stored in granules and no transcription is required for initial activation of these factors. Secondly, a delayed or suppressed protective antioxidant UA release was noted for which transcription of the gene coding for xanthine dehydrogenase is required. This gene which has a role in the formation of the powerful antioxidant UA has been shown to be expressed at lower rates in *Csb* than in its wild type mice (Kooter *et al*., 2007). On the other hand, the difference in pro-inflammatory mediators was not reflected in the influx of PMNs in the BALF. Apparently, the assumption that transcription-coupled repair defective mice would accelerate adverse effects due to oxidative stress is validated by this study by some but not all markers measured.

The pro-inflammatory responses in the lung were followed by a procoagulant reaction, as indicated by concurrent increases in TF activity and delayed increases in the amount of thrombin generation, as indicated by peak and ETP values. TF activity measured immediately after exposure to ozone decreased, subsequently increased 10 h after exposure and remained at a fairly stable level towards 48 h. This reaction is probably reflected by the course of the lag time in the thrombin generation, which remains constant but decreased upon ozone exposure, suggesting increased TF levels. mRNA analyses confirm increased TF gene expression levels after ozone exposure. Thrombin generation peak values and ETP gradually increased and reached the highest values at 48 h: more than a 300% increase in thrombin generation. Since longer follow up is lacking we cannot determine whether these time points reflect maximum levels. The discrepant behaviour may indicate the loss in TM activity, rather than an increase in TF activity. In previous experiments it was determined that the lungs have a profound inhibitory activity towards thrombin generation, due to TM activity. Upon exposure to endotoxin or PM, the anticoagulant activity is lost and it is likely that the same loss in TM, as confirmed by gene expression, also causes the protracted upregulation of thrombin generation level after ozone exposure (ten Cate *et al*., 2007). The loss of TM's thrombin scavenging function upon inflammation may contribute to a greater availability of thrombin for other receptors including protease activated receptor 1 (PAR-1) that may mediate additional pro-inflammatory signaling pathways in lung tissue. Although these findings demonstrate a systemic effect due to ozone exposure, the implications for development of cardiovascular effect remains to be investigated.

In conclusion, this study demonstrated a significant biological time-dependent response to the oxidative stressor ozone, for both the repair-deficient *Csb* mice and wild type mice. Although major differences in the time-course response to ozone between the two mice strains are not present, under the current experimental conditions there was a clear sign that the increase in pro-inflammatory mediators in BALF of *Csb* mice preceded in time compared with the wild type mice. Although this was also true for total cell numbers in BALF, the PMNs numbers were about the same in both types of mice and the inflammation in *Csb* mice returned to base line values more quickly. In addition a delayed antioxidant reaction was observed for the *Csb* mice compared with the wild type mice. Both genotypes developed a procoagulant reaction characterized by a stably increased TF activity and a progressive increase in thrombin generation. These experiments show that ozone, a well known toxic substance from the environment, induces pro-inflammatory and procoagulant reactions in the lungs of mice. The inflammation might be responsible for the activation of the coagulation system, which deserves further mechanistic studies.

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