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ORIGINAL ARTICLE

Particulate matter inhalation during hay storing activity induces systemic inflammation and platelet aggregation

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Abstract The aim of this study was to investigate possible pathomechanisms behind the cardiovascular morbidity caused by inhalation of particulate matter (PM_{10}). For that purpose, healthy volunteers were exposed to high PM_{10} concentrations during a 2 h hay storing activity. Blood was drawn in the evening before and after PM_{10} exposure and in the morning and evening of the day after exposure. The leukocyte count increased after PM_{10} exposure with an initial increase of segmented neutrophils followed by banded forms. *C*-reactive protein increased over time. Fibrinogen and plasma viscosity became increased in the evening of the day after PM_{10} exposure. Platelet aggregation was increased in the evening after PM_{10} exposure. At the same time von Willebrand factor and factor VIII were increased, reflecting endothelial activation. These results confirm that acute inhalative exposure to high PM_{10} concentrations during hay storage activity leads to a systemic inflammatory reaction, endothelial activation, and platelet aggregation.

Keywords *C*-reactive protein · Endothelium · Inflammation · Particulate matter · Platelets · Viscosity

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Introduction

Ambient air pollution is associated with increased cardiovascular mortality (Pope et al. 2004). Such air pollutants are gases or particulate matter (PM), the latter having gained special scientific and public interest in the last few years (Brook et al. 2004). The size of the PM determines the pathophysiological role. $PM_{10,}$ also called thoracic particles, have an aerodynamic diameter smaller than 10 µm. Upon inhalation they can reach the tracheobronchial tree; wheras larger particles (>10 µm) are held off by the nasal epithelium. The smaller the particles are, the deeper they penetrate into the lung periphery, eventually reaching the alveolar space.

Long-term epidemiological studies have consistently shown an association between air pollution and mortality for all causes as well as cardiovascular events (Dockery et al. 1993; Pope et al. 1995). Short-term exposure to high ambient PM_{10} pollution leads to higher hospitalisation rates for cardiovascular disease (Hoek et al. 2001), an increased risk for myocardial infarction (Zanobetti and Schwartz 2005), and ischemic stroke (Wellenius et al. 2005). An elevation in PM_{10} by 10 µg/m^3 was associated with a 4.5% increase in risk for acute ischemic coronary events (Pope et al. 2006), and a 0.2–0.3% increase in daily cardiopulmonary mortality (Dominici et al. 2005).

The pathophysiological mechanisms behind the cardiovascular effects of PM_{10} are not well understood. Direct effects may occur via agents, which readily cross the pulmonary epithelium into the circulation such as gases, and possibly ultrafine particles along with soluble constituents of $PM_{2.5}$ (Nemmar et al. 2002). Less acute, indirect effects may occur via a pulmonary oxidative stress with a subsequent systemic inflammatory reaction with increased leucocytes (Salvi et al. 1999), fibrinogen (Ghio et al. 2000) and

C-reactive protein (Ruckerl et al. 2006), which all may impair vascular functions and accelerate atherosclerosis. Inhalation of organic dust containing toxigenic moulds and mycotoxins during indoor exposure induces an immunological pulmonary reaction (Bünger et al. 2004; Gray et al. 2003).

We have previously shown that farmers in the Swiss Alps are exposed to very high PM_{10} concentrations during their work, especially hay storage (Cathomas et al. 2002). This gave us the unique opportunity to investigate the influence of a ~2 h exposure to such high concentrations of rural PM_{10} during hay storage in healthy volunteers on parameters of hemorheology, systemic inflammation, plasmatic coagulation and platelet function.

Methods

Study design

Fourteen healthy volunteers (six females, eight males, age 26–54 years, median 33.5 years) gave their informed consent to participate in the study, which had been approved by the local Ethics Committee. They were members of the medical staff, non-smokers, in good physical condition and accustomed to the intermediate altitudes of the area. They were exposed in groups of 2–3 to PM inhalation during hay storage with an electrically powered hay blower between 02.00 and 04.00 p.m. on two farms in the Swiss Alps of the Grison: Farm A in Scheid, at 1,300 m above sea level, and Farm B in Cresta, Avers valley, at 1,960 m. The physical activity consisted of loading the hay, which was brought into the barn periodically, from the floor into the blower. Between such periods of work they had large breaks, during which they were relaxing in the barn. The physical stress was more on the muscles than on the cardiovascular system and can be rated as moderate.

Quantification of PM exposure

To collect the organic dust, an Aerosol Sampler DHA-80 (Digitel, Hegnau, Switzerland) was used, which was positioned in similar distance from the hay blower and level above ground as the faces of the working volunteers were. The filters were weighed before and after exposure at the same conditions (room temperature 20°C). The results were related to the filtered air volume and given as $\mu g/m^3$. On the first day at farm B we additionally used a cascade impactor (Anderson Mark II, Particle Size Stack Sampler) to analyse the distribution of the aerodynamic diameters of the particles. The filters were desiccated for 48 h under defined conditions and weighed with an analytical balance (Mettler® Toledo, Greifensee, Switzerland).

Blood sampling

Blood was sampled on four different occasions: at 08.00 p.m. on the day before exposure (Day 0, baseline value), 08.00 p.m. on the day of exposure (Day 1), i.e. 6 h after the end of PM_{10} exposure, and in the morning of the day after exposure (Day 2) at 08.00 a.m. and at 08.00 p.m. Each time 5 ml tubes containing 0.106 mol/l buffered sodium citrate, a 4.5 ml tube with lithium–heparin, a 4.5 ml tube for serum and two 4 ml tubes containing EDTA-K (ethylene-diamine-tetraacetic acid) were drawn. Plasma and serum samples were deepfrozen at −80°C.

Hematologic parameters

Erythrocyte and erythrocyte indices, leucocyte and platelet count were measured in blood anticoagulated with EDTA with an electronic particle counter (Sysmex K-1000, Kobe, Japan). Blood smears were prepared, air dried and stained with May-Grünwald-Giemsa.

Blood and plasma viscosity

Whole blood was incubated at 37.0°C for 10 min and viscosity was measured with a Couette-type viscometer (Contraves LS-30, Mettler-Toledo, Greifensee, Switzerland). We used a high shear rate of 94.5 s^{-1} and a low shear rate of 0.1 s^{-1} for whole blood, plasma was measured at 11 s^{-1}.

Platelet function analysis

Platelet aggregatory function was tested with a platelet function analyzer (PFA-100®, Dade Behring, Düdingen, Switzerland). In this instrument blood is aspirated into a 150 µm membrane pore coated with collagen and either epinephrine bitartrate (EPI cartridge) or adenosine diphosphate (ADP cartridge) for platelet activation. Platelets adhere to the pore surface, become activated, then aggregate and finally form an occluding platelet plug in the pore, which stops blood flow (Closure time CT). All measurements were done in duplicate and the mean value was calculated.

Inflammatory parameters

The plasma concentrations of tumour necrosis factor alpha (TNF-alpha) and interleukin-6 (IL-6) were measured by ELISA (human IL-6, TNF-alpha, R&D Systems, Minneapolis, MN, USA) following the instructions of the manufacturer. *C*-reactive protein (CRP) was measured with a high-sensitivity test (hs-CRP) using latex enhanced nephelometry (BN II, Behring Diagnostics Inc. Marburg, Germany).

Coagulation parameters

Factor VIII (FVIII) activity was assayed by a standard PTT-based commercial test (Dade Behring, Marburg, Germany) using Prothrombin SL as the contact activating agent. A functional determination of fibrinogen (Multifibren U, Dade Behring, Marburg, Germany) and the ristocetin cofactor activity of von Willebrand factor (BC von Willebrand Reagent, Dade Behring, Marburg Germany) were performed. Plasma thrombin–antithrombin (TAT) complexes were determined using a quantitative enzymoimmunoassay (Enzygnost TAT micro; Dade Behring, Marburg, Germany). Human soluble P selectin (sP-selectin) was measured by a quantitative sandwich solid phase ELISA using a monoclonal antibody directed against soluble P selectin (R&D Systems, Minneapolis, MN, USA).

Statistics

A GraphPad prism Version 4.0 for Windows was used for statistical analysis. A one-way analysis of variance (ANOVA) for non-parametric data with Dunn's Multiple Comparison Test for the comparison with baseline values before PM_{10} exposure were used. A P-value < 0.05 was regarded as statistically significant.

Results

PM_{10} measurements

The PM_{10} concentrations measured near the hay blower during the work of the participants are shown in Table 1. High values were found with a substantial day-to-day variation depending on the weather conditions with low values on days with high humidity and a very high value on the last day, which was after several very warm and dry days. Exposure times varied from 85 to 120 min. The resulting total PM_{10} exposure (PM_{10} concentration × time) is also shown in Table 1. No significant correlations were found between the degree of PM_{10} exposure and any of the parameters below. This may be due to the relatively low number of participants and sometimes large variations, which holds true as well for other parameters mentioned below.

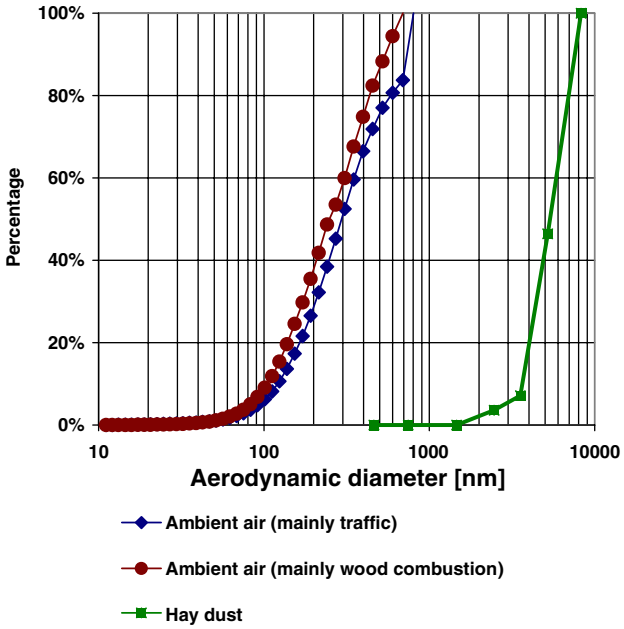

Fig. 1 Cumulative distribution of the particle aerodynamic diameter of PM_{10} collected with a cascade impactor during hay storage. For comparison, the particle diameter distributions of ambient air polluted by traffic and wood combustion, measured with the same instrument on other occasions, are shown

The particle diameter distribution (PM_{10}-mass) measured in this study near a hay blower is shown in Fig. 1. The PM aerodynamic diameter was between 2 and 10 µm, which is about 10 times higher than the diameters measured in air polluted by traffic or wood combustion, as shown in the same figure.

Hematological data

Erythrocyte count, erythrocyte indices and platelet count were not affected by PM_{10} exposure (data not shown). The leuco-

Table 1 Date of investigation, PM_{10} concentrations, exposure times, total PM_{10} exposure and number of exposed volunteers working in two farms with a hay blower on six different days

Date	PM_{10} (µg/m^3)	Exposure time (min)	Cumulative PM_{10} exposure (mg min/m^3)	Volunteers (n)
Farm A				
21/07/2005	10,103	110	1,111	2
21/07/2005	3,960	100	396	3
21/07/2005	7,129	85	606	3
Farm B				
21/07/2005	2,684	107	287	2
21/07/2005	3,695	108	399	2
09/08/2005	14,525	120	1,743	2

Table 2 Differential leucocyte count in absolute numbers before (Day 0) and after PM_{10} exposure (mean ± SD)

Leucocytes ($\times 10^3/\mu l$)	Day 0	Day 1	Day 2	
	08.00 p.m.	08.00 p.m.	08.00 a.m.	08.00 p.m.
Polymorphonuclear leucocytes	3.27 ± 1.01	5.34 ± 1.23***	4.76 ± 1.82	3.77 ± 1.23
Banded neutrophils	0.53 ± 0.34	1.09 ± 0.66	1.24 ± 0.64**	0.88 ± 0.51
Segmented neutrophils	2.76 ± 0.97	4.17 ± 1.49**	3.52 ± 1.50	2.90 ± 0.99
Eosinophils	0.09 ± 0.08	0.19 ± 0.18	0.19 ± 0.20	0.14 ± 0.12
Basophils	0.12 ± 0.28	0.03 ± 0.08	0.06 ± 0.06	0.01 ± 0.02
Monocytes	0.12 ± 0.14	0.32 ± 0.28	0.24 ± 0.15	0.24 ± 0.24
Lymphocytes	2.83 ± 0.95	3.01 ± 1.09	2.37 ± 1.02	2.79 ± 0.73

** $P < 0.01$, *** $P < 0.001$ compared with baseline on Day 0 (ANOVA)

cyte count was significantly increased in the evening after PM_{10} exposure due to an increase of polymorphonuclear cells, which is shown in Table 2. An initial rise of segmented neutrophils in the evening after the PM_{10} exposure was followed by an increase of banded neutrophils in the next morning (Fig. 2). Eosinophils remained unaffected by PM_{10} exposure.

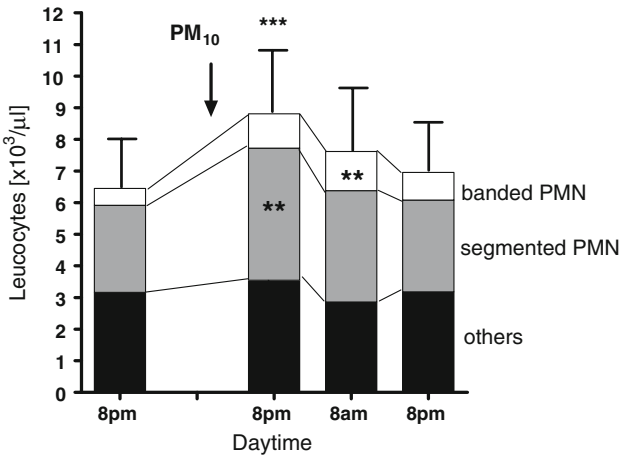

Fig. 2 Absolute and differential leucocyte count at 8 p.m. on day 0 (*baseline value*), at 8 p.m. on day 1 after PM10 exposure (*arrow*), and at 8 a.m. and 8 p.m. on day 2. Mean ± SD. * Denotes $P < 0.01$, *** $P < 0.001$ compared with baseline values (ANOVA)

Hemorheological data

Hemorheologic data are shown in Table 3. Whole blood viscosity, which is primarily determined by erythrocyte count as well as erythrocyte deformability and aggregation, was not affected by PM_{10} exposure. Plasma viscosity, which is determined by the concentration of large molecules such as immunoglobulins and fibrinogen, became elevated on day 2 at 8 p.m., and coincided with an increased fibrinogen level at that time (Table 3).

Inflammatory markers

Acute PM_{10} exposure induced a systemic inflammatory response, which is shown by the increase of hs-CRP over time (Fig. 3). The mean value of the whole group ($n = 14$) was significantly above the baseline value on the post expositional day at 08.00 p.m., i.e. 30 h after PM_{10} exposure. The large SD on day 2 reflects marked interindividual variations ranging from 0.58 to 38.00 mg/l at 08.00 p.m. These interindividual differences were not related to different PM_{10} concentrations (Table 1). Systemic serum concentrations of TNF-alpha and IL-6, were not affected by PM_{10} inhalation (data not shown).

Table 3 Hemorheologic parameters in 14 individuals exposed to high concentrations of PM_{10} on day 1

	Day 0	Day 1	Day 2	
	08.00 pm	08.00 pm	08.00 am	08.00 pm
Whole blood viscosity (mPa s)				
At 94.5 s^{-1}	4.43 ± 0.53	4.45 ± 0.47	4.51 ± 0.55	4.38 ± 0.49
At 0.1 s^{-1}	36.53 ± 8.22	35.51 ± 8.10	38.01 ± 9.89	35.32 ± 8.17
Plasma viscosity (mPa s)	1.25 ± 0.11	1.24 ± 0.09	1.27 ± 0.08	1.33 ± 0.08*
Fibrinogen (g/l)	2.62 ± 0.57	2.66 ± 0.72	2.87 ± 0.80	3.14 ± 0.90**

Mean ± SD, * $P < 0.05$, ** $P < 0.01$ compared with baseline on day 0 (ANOVA)

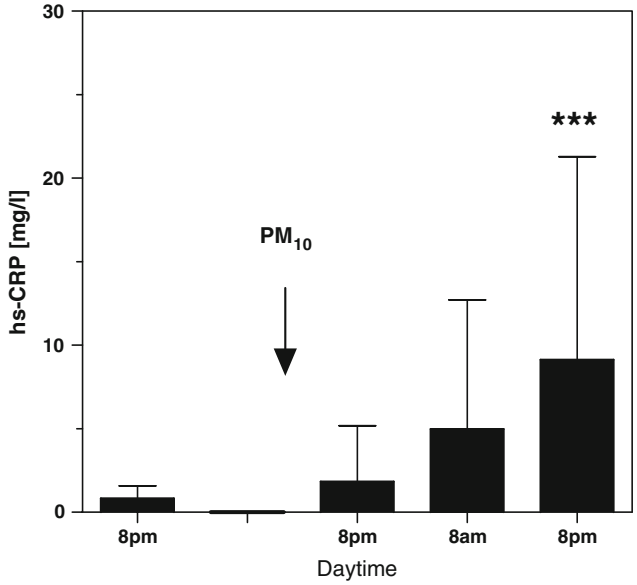

Fig. 3 Serum levels of high sensitivity *C*-reactive protein (hs-CRP) before and different time points after PM_{10} exposure. Mean ± SD, $n = 14$. *** Denotes $P < 0.001$ compared with baseline value (ANOVA)

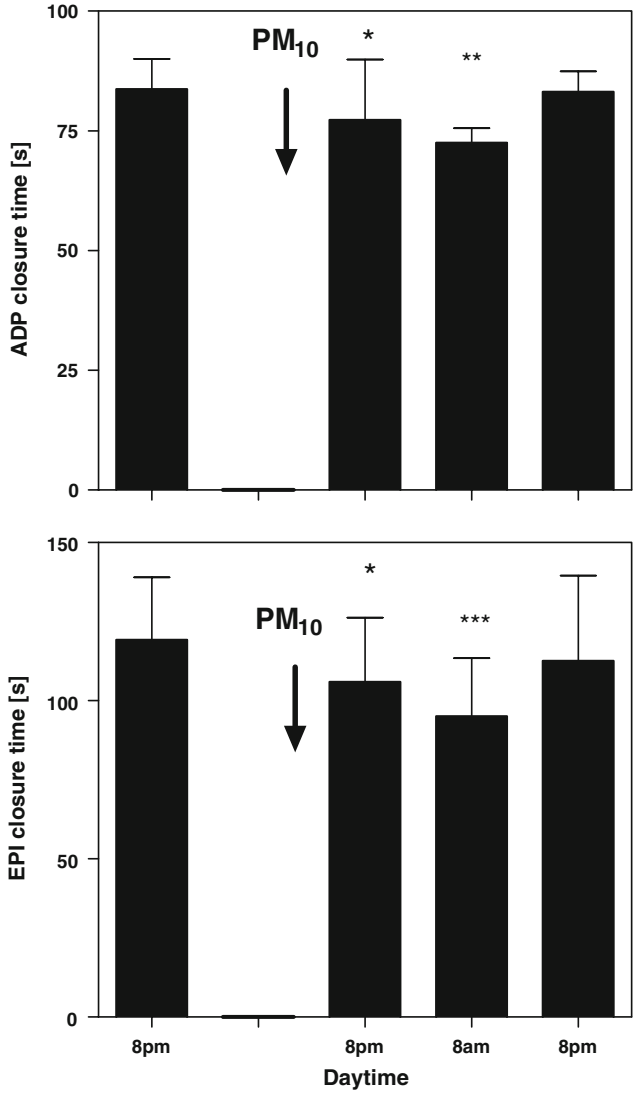

Fig. 4 Pore closing time by occluding platelet aggregates using either ADP cartridges (*above*) or EPI cartridges (*below*) on the platelet function analyser PFA-100® (see "Methods"). Values (mean ± SD, $n = 14$) are before and various time intervals after PM_{10} exposure. Shorter closure times indicate increased platelet aggregation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with baseline values (ANOVA)

Platelet aggregation

The platelet aggregatory function as measured with the PFA-100® instrument is shown in Fig. 4. The shorter closure times (CT) with either ADP or epinephrine as a platelet activator indicate increased platelet aggregation after PM_{10} exposure. sP-selectin, which is released during platelet aggregation, tended to be higher immediately after PM_{10} exposure (23.6 ± 8.0, 28.1 ± 18.0, 25.2 ± 10.1, and 24.2 ± 11.0 ng/ml, respectively).

Coagulation proteins

The serum levels of vWF and FVIII, which are stored in endothelial cells and released into the circulation upon endothelial cell activation, were increased after PM_{10} exposure (Fig. 5). The plasmatic coagulation cascade was not initiated by PM_{10} exposure, as measured by the unchanged levels of thrombin–antithrombin complexes (TAT: 4.5 ± 8.4, 2.7 ± 1.5, 6.2 ± 12.7, and 2.1 ± 0.6 ng/ml at the four different time points of blood sampling, respectively).

Discussion

Increasing evidence suggests that PM air pollution may adversely affect the cardiovascular system (Brook et al. 2004; Peters 2005). An association between acute PM_{10} exposure and acute myocardial infarction (Lanki et al. 2006) and hospital admission (Barnett et al. 2006) has been described. The sources of those particles were related to traffic and combustion. We had shown before (Cathomas et al. 2002) and confirm in this study that famers in our Swiss mountain area are exposed to very high concentrations of PM_{10} of another source during normal activities such as hay storage in their barns. We found that these coarse PM_{10} induced biological responses with a possible impact on health.

This PM_{10} exposure induced systemic responses such as leucocytosis. The initial increase of neutrophils with a preserved ratio of banded-to-segmented cells indicates primarily a demargination of leucocytes from the endothelium. The later relative predominance of banded neutrophils, i.e. less

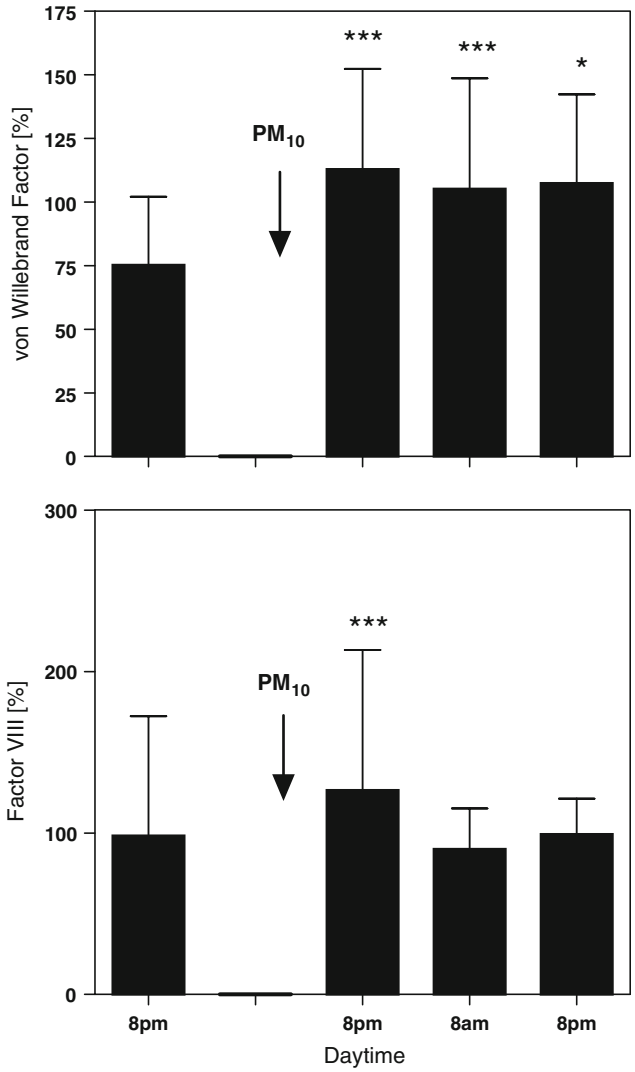

Fig. 5 Influence of PM_{10} exposure on von Willebrand factor (*above*) and coagulation factor VIII (*below*). $n = 14$. * $P < 0.05$, *** $P < 0.001$ compared with baseline values (ANOVA)

mature cells, indicates a stimulation of the release from the bone marrow. In this context, the observation of increased levels of granulocyte/macrophage-colony stimulating factor (GM-CSF) after PM_{10} exposure by others is most interesting (Ishii et al. 2005).

C-reactive protein was increased three-fold at the last blood withdrawal, i.e. 30 h after PM_{10} exposure. CRP elevation has been described before (Ruckerl et al. 2006) with a similar time lag of 2 days, which reflects the time needed for gene transcription and de novo synthesis of CRP after triggering. The CRP increase indicates an acute phase reaction, which explains the increase of another acute phase reactant, namely fibrinogen, at the same time >24 h after PM_{10} exposure and confirms earlier observations (Ghio et al. 2000). Elevated CRP levels are associated with coronary heart disease (Danesh et al. 2000). Fibrinogen increases plasma viscosity, as seen in our study, which impairs blood flow and might be a contributing factor to the higher mortality during air pollution episodes (Peters et al. 2000).

It seems surprising that the systemic levels of inflammatory cytokines TNF-alpha and IL-6 were not affected. In vitro studies on cultured alveolar macrophages and lung epithelial cells had shown a stimulation of TNF-alpha (Jimenez et al. 2002) and IL-6 (van Eeden et al. 2001).

Platelet aggregation was increased after PM_{10} inhalation, which was seen by the faster pore closure in the PFA-100® instrument (Fig. 4) in the evening after exposure and even more marked in the next morning. Note that platelet aggregatory function is subjected to circadian changes with most activity in the morning, what we (Lehmann et al. 2006) and others (Dalby et al. 2000) had observed before. Platelet aggregation is the key event in acute occlusion of an artery. The increased aggregatory function of platelets after PM_{10} exposure may be a factor contributing to the higher incidence of cardiovascular events and mortality under these circumstances.

The PFA-100® instrument is sensitive to changes in circulating vWF, which is the bridging molecule between platelet glycoprotein Ib/IX and collagen, inducing platelet adhesion to the vessel wall. The increase in vWF observed in this study (Fig. 5) may explain the accelerated platelet aggregation in the PFA-100® instrument, and is indicative for an endothelial activation, which is in line with earlier observations (Ahmadizad et al. 2006; Törnqvist et al. 2007). Such endothelial cell activation with increase of circulating vWF is an independent risk factor for acute coronary events (Morange et al. 2004; Lee et al. 2005). The fact that sP-selectin, a marker of in vivo platelet aggregation and consumption, was hardly changed, suggests that platelet aggregation and consumption had not occurred in vivo in our healthy volunteers, despite the increase platelet aggregability, probably because they had intact vessel walls without exposure of subendothelial collagen required for platelet adhesion. The unchanged levels of TAT indicate that plasmatic coagulation was not affected, which is in general agreement with recent investigations in humans (Ruckerl et al. 2006).

The mechanisms by which PM_{10} induce these systemic reactions, which are independent of particle-induced effects on the lungs, are poorly understood. Our analysis of the particle size distribution (Fig. 1) revealed that these rural air pollutants are much larger than the particles generated by (diesel) motor vehicles and wood combustion, which measure about 10 times less. The composition of our rural PM_{10} has not been analysed so far, but may be quite different from other PM_{10}, e.g. generated by motor vehicles, and contain more organic particles. Fine and ultrafine particles may pass rapidly into the systemic circulation (Nemmar

et al. 2002), although probably only as a small fraction (Mills et al. 2006). Our results would now suggest that even course particles of a few µm may elict systemic effects. Possible mechanisms could be a direct passage of such course PM, which, however, has not been documented so far, phagocytosis by alveolar macrophages, which then transport these particles via lymph nodes into the systemic circulation (Peters et al. 2006), or an induction of some second messenger by the PM_{10} in alveolar epithelial cells, which would induce an indirect systemic response.

The question arises as to whether the physical activity of hay storing itself affected the systemic reactions seen in our study. It has recently been shown that moderate intensity exercise, i.e. 30 min. at 50% maximal oxygen uptake, does not influence circulating neutrophils, lymphocytes, monocytes, serum IL-6 and *C*-reactive protein (Markovitch et al. 2008). With regard to platelet function, moderate exercise did not affect platelet aggregation (Ahmadizad et al. 2006; Winther et al. 1992), or even decreased it, which has been shown by several groups (Petidis et al. 2008; Tozzi-Ciancarelli et al. 2002; Wang et al. 1994). Taken together, it seems rather unlikely that the moderate physical activity during hay storing contributed significantly to the measured effects, it may even have decreased the effect on platelet aggregation as suggested by the work of Petidis et al. (2008).

We conclude that acute exposure to very high concentrations of rural PM_{10} during hay storage led to systemic inflammation, increased platelet aggregation and endothelial cell activation. All these three mechanisms could contribute to cardiovascular diseases and complications. Our observations would suggest that farmers may be at risk for cardiovascular events, which has however, not been confirmed by epidemiological studies so far. A better protection by wearing a mask during work with a high PM_{10} exposure such as hay storage may thus be warranted. The traditional hay storage in the Swiss mountains resulting in very high short-term rural PM_{10}-concentrations may serve as a model to further study acute PM_{10} effects.

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