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INFLUENCE OF DIESEL SOOT PARTICLES AND SULFITE ON FUNCTIONS OF POLYMORPHONUCLEAR LEUKOCYTES

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Aqueous suspensions of diesel soot particles in combination with sulfite influence certain functions of human polymorphonuclear neutrophils in vitro. Chemiluminescence, generated after activation by opsonized zymosan as well as oxygen uptake were decreased, whereas phagocytosis was increased. An enhancement of degranulation could not be observed. The single substances show little or no effects on the above properties. The results indicate that combinations of air pollutants such as diesel soot and sulfite may modulate vital functions of activated leukocytes *in vivo*.

KEY WORDS: Diesel particles, sulfite, polymorphonuclear leukocytes, oxygen activation, phagocytosis.

ABBREVIATIONS: BSA, bovine serum albumin; DP, diesel soot particle; EDTA, ethylenediaminetetraacetate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocytes; PMSF, phenylmethylsulfonyl fluoride; SH-group, sulfhydryl group, RPMI.

INTRODUCTION

In recent decades it became quite clear that several human lung dysfunctions are based on oxidatively induced cell damage.

Due to their oxygen-rich environment, lung cells exhibit an extremely well balanced system of antioxidative capacities. Thus, the control of redox balance is of utmost importance for the maintenance of normal functions. The lung has a large epithelial surface. The tracheobronchial tree and the alveolar space in addition to normal oxidative processes are especially exposed to several airborne reactive compounds such as air pollutants, cigarette smoke as well as promotors of inflammation of secondary origin, e.g. activated alveolar macrophages. It is not surprising, therefore, that the antioxidative system of the lung has been in the focus of interest and thus corresponding research activities.[1]

As shown by Heinrich *et al.*[2] the redox balance of the lung can be shifted by inhalation of diesel soot particles. In animal experiments these particles may provoke inflammatory responses accompanied by thickening of the alveolar septa connected with alveolar hypoplasia. In parallel, a decrease of lung clearance was observed. The same authors[3] also demonstrated that unfiltered diesel soot could provoke adenocarcinoma in the lung. That the damage brought about by diesel soot particles is based on oxidative reactions was already shown by Henderson *et al.*[4] and Li.[5] Using

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biochemical model reactions we recently demonstrated[6,7] that aqueous suspensions of diesel soot particles are able to oxidize biological target molecules. Thus, diesel soot particles react as organic catalysts and are able to activate oxygen at the expense of certain electron donors such as cysteine or sulfite. In this context it is important to note that aqueous solutions of SO_2 as one of the main components of urban air pollution seem to cooperate synergistically with the toxicity of diesel soot particles. Thus, there is good reason to speculate that these two major components of air pollution might be responsible for certain bronchial diseases and lung dysfunctions, especially in sensitive persons. Due to the particle size of diesel soot, phagocytic cells must be of special importance. In the lung, long-living alveolar macrophages together with short-living neutrophil granulocytes cooperate in the complex defense system which is activated upon infection or other stress situations.[8] During these processes surface receptors of the leukocytes activate a chain of events comprising the following functions:

1. phagocytosis of pathogens or other particles;
2. activation of the aereobic metabolism ("respiratory burst") and
3. degranulation inside the leukocyte and finally degradation of the ingested particle.

For the microbicid activity several enzymes are of great importance including membrane activities where at the expense of NADPH an electron transport system is functioning under formation of superoxide and hydrogen peroxide. The myeloperoxidase system activates halogens (mainly Cl^-) at the expense of H_2O_2, forming hypochloric acid and other strongly reactive metabolites such as singlet oxygen and chloramines.[9] During degranulation proteolytic enzymes such as serine proteases and metalloproteinases are excreted, catalysing the degradation of connective tissues in addition to the ingested pathogen.[10] In this context, myeloperoxidase is also of great importance since it inactivates α1-proteinase-inhibitor thus inducing tissue damage.[11] On the other hand, the primarily inactive form of excreted metalloproteinases (for example, collagenase) may be activated through the function of hypochloric acid.[12]

In the following, we report on *in vitro* experiments with isolated human and porcine neutrophiles showing that under the influence of diesel soot particles and sulfite the above indicated granulocyte functions are significantly altered.

MATERIALS AND METHODS

Reagents

Histopaque 1119, collagen typ VIII, luminol, PMFS, RPMI 1640 (with glutathione, without sodium bicarbonate), superoxide dismutase and zymosan A were obtained from Sigma; methylene blue, sodium sulfite, EDTA, trypan blue and Türks solution were purchased from Merck; dextran T 500 was from Pharmacia, Liquemin N 25000 (Heparin) was from Hoffmann La Roche and acridine orange was from Serva. All other chemicals were of the highest grade of purity available (Merck).

Diesel soot particles (DP) were carefully removed with a small brush from the exhaust pipe of a fire brigade motorcar (Magirus, Fire brigade, Lohr/Main). All experiments with DP were done with the same batch of soot.

Buffer Solutions and Staining Liquids

PBS-buffer: 1 l contains 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.2 g KH_2PO_4, 100 mg $MgCl_2$, 73.5 mg $CaCl_2$, pH 7.2; Sörensen buffer: a solution of 0.17 M Na_2HPO_4 was titrated with a solution of 0.13 M KH_2PO_4 at a pH of 7.70; acridine orange solution: 0.1% acridine orange in Sörensen buffer; methylene blue solution: 0.2% methylene blue in distilled water; Coomassie blue solution: 0.15% Coomassie brilliant blue R 250, 45.5% methanol, 9% acetic acid;

Preparation of Neutrophils

Neutrophils were harvested from fresh human blood obtained from healthy adult donors. 20 ml heparinized blood were mixed with 10 ml dextran solution (1.5%) before sedimentation of the erythrocytes. After 30 min the leukocyte enriched supernatant was further purified by 20 min centrifugation over histopaque at 1000 × g. Contaminating erythrocytes were removed by 45 sec hypotonic lysis in distilled water. The cell suspension was returned to isotonicity by addition of 2.7% NaCl-solution. After centrifugation (10 min) at 400 × g the cells were suspended in RPMI. Cell viabilities were assessed by trypan blue dye exclusion and cell numbers were quantified with a hemocytometer. Cell concentration was adjusted to 3×10^6 viable cells/ml. In the collagenase test the cell concentration was 40×10^6 cells/ml.

For the isolation of porcine granulocytes 15 ml heparinized blood (obtained from the slaughter house) were diluted 1:2 with PBS-buffer and centrifuged at 1000 × g. The PRC-(packed red cell)-layer was mixed with an equal volume of PBS-buffer and the erythrocytes were removed twice by hypotonic lysis. The remaining granulocytes were centrifuged at 125 × g and resuspended in RPMI.

Preparation of Opsonized Zymosan

Zymosan particles were suspended in PBS-buffer (10 mg/ml) and incubated with fresh autologous serum at 37°C. After 60 min the supernatant was centrifuged and the opsonized zymosan was resuspended in PBS-buffer at a final concentration of 10 mg/ml.

Preparation of Opsonized Yeast

Commercial yeast (*Saccharomyces cerevisiae*) was suspended in physiological NaCl-solution and boiled for 30 min. After cooling the suspension was filtered twice through sterile gauze. The cell concentration was adjusted to 3×10^7/ml. 2.5 ml of this solution were incubated with 1 ml serum for 60 min at 37°C. After addition of 5 ml PBS-buffer the supernatant was centrifuged at 1000 × g and the cell concentration of the opsonized yeast was readjusted to 3×10^7 cells/ml.

Phagocytosis-test

As described by Stahl[8] neutrophils were incubated with opsonized yeast at 37°C. After 20 min the ingestion process was stopped with EDTA (10 mM) and the samples were stored on ice. For determination of the phagocytosis rate of fluorescence-microscopic

method was used. The samples were stained with acridinorange and 5 μl of this solution was mixed with 5 μl of methylene blue for quenching the fluorescence of not ingested yeast cells. The number of incorporated yeast cells was determined with approximately 100 granulocytes of each sample and the phagocytosis index (PI) was calculated by the following formula:

$$\frac{\text{sum of incorporated yeast cells of 100 neutrophils}}{100} = \text{PI}$$

The index of the control was set as 100% and all other values were given as percent of the control. The influence on phagocytosis of DP, sulfite and combinations was tested after preincubation with white blood cells. 300 μl cell suspension were treated with

a. 600 μl DP-suspension (400 μg, in RPMI),
b. 600 μl sulfite (5×10^{-4} M, in RPMI),
c. 600 μl DP/sulfite and
d. 600 μl medium as control

for 30 min at 37°C.

Chemiluminescence-test

Human neutrophils were preincubated with DP, sulfite and DP/sulfite under the *same* conditions as described for the phagocytosis test. After preincubation 200 μl of the samples were mixed with 20 μl luminol (1 mM) and the reaction was started by addition of 20 μl opsonized zymosan. This reaction mixtures contain about 6,600 cells, 88.9 μg DP, 0.11 mM sulfite, 0.08 mM luminol and 0.2 mg zymosan. Chemiluminescence was measured with an Amersham Light Speed Luminometer, using titer plates as sample carrier. The reaction was observed for about 45 min. The values of the references (samples without zymosan) were subtracted from the real values. The highest value of the control was set as 100%, all other values were calculated as percent of the control. The test was carried out with blood of four different donors with four parallels of each sample. In preliminary experiments it was shown that the luminol dependent chemiluminescence appears only in samples, containing activated leukocytes. The light emission was identical in samples containing untreated cells alone or in combination with DP, sulfite or DP/sulfite. The cell-derived chemiluminescence was blocked by 100% after addition of superoxide dismutase.

Oxygen Uptake

Oxygen consumption of activated neutrophils was measured with an oxygen electrode. Human granulocytes were preincubated as described for the phagocytosis test. 50 μl opsonized zymosan were added to 500 μl of the preincubation solutions and oxygen uptake was observed for about 25 min at 37°C.

Collagenase-test

900 μl of human granulocyte suspension (40 Mio cells/ml) were preincubated with the

test solutions for 30 min at 37°C. In accordance with Weiss[12] *et al.* 200 μl of opsonized zymosan were added and the supernatants were centrifuged at 1250 × g for 25 min. 500 μl of the supernatant were removed and treated with an equal volume of the serine proteinase inhibitor phenylmethylsulfonyl fluoride (2 mM). 800 μl of this mixture were examined for collagenolytic activity by analysis of the products formed after incubation (24 hours) of the supernatants with soluble type I collagen (0.15 mg) at pH 7.4 and 25°C. The samples were reduced, heat-denaturated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 5% stacking and a 10% resolving gel. Products were stained with Coomassie blue.

Detection of Sulfhydryl Groups

The amount of free sulfhydryl groups in BSA and glutathione was determined according to Ellman.[13] The reaction mixtures contained 100 mM phosphate buffer, pH 7.8; 400 μg DP; 0.5 mM sulfite and 50 μM glutathione or 4 mg BSA respectively. After 60 min incubation at 37°C, 100 μl 5,5-dithiobis-(2-nitrobenzoic acid) (10 mM) were added. The samples were centrifuged in a microcentrifuge for 5 min. Formation of 2-nitro-5-mercapto-benzoic acid was quantified spectrophotometrically at 312 nm. The values of controls (untreated BSA or glutathione at incubation time 0.0) were set as 100%. The other values were calculated as percent of the control.

RESULTS

Phagocytosis-test

The phagocytosis rate of human neutrophils is clearly increased after incubation with a combination of diesel soot and sulfite whereas the single substances have no effect on the phagocytic behavior as shown in Table I. Due to the lack of fresh human blood the phagocytosis test was done with porcine blood in the same manner. Neutrophils were isolated from blood of three different pigs (blood samples I, II and III). Two or three parallels were made for each sample and the corresponding means were calculated as percent of the controls as described under Material and Methods. The percent values of the three samples (means) corresponding to the four different preincubation conditions are shown in Table II. Sulfite alone seems to decrease the phagocytosis whereas diesel soot has a slightly stimulatory effect. The phagocytosis rate of the combined reaction mixture is significantly enhanced by about 150%.

TABLE I
Rates of phagocytosis by human neutrophils

	ingested yeast cells per granulocyte									
	0	1	2	3	4	5	6	7	Index	in %
control	52	30	15	1	1				0.6	100
+ DP	60	24	7	4	4	1			0.7	106.0
+ sulfite	63	22	11	4					0.6	83.6
+ DP/sulfite	47	20	12	5	11	3	1	1	1.3	195.5

TABLE II
Rates of phagocytosis by porcine—PMN

		ingested yeast cells per granulocyte										
		0	1	2	3	4	5	6	7	Index	in %	x
control	I	66	18	13	2	1				0.5		
		68	25	4	3					0.5		
										0.5	100	100
	II	56	22	13	7	1	1			0.8		
		68	22	9	1	1				0.4		
		53	29	15	1	1				0.7		
										0.6	100	
	III	73	22	3		2				0.5		
		76	15	4	3	2				0.4		
		71	17	8	2	2				0.5		
										0.5	100	
+ DP	I	53	31	12	4					0.7		
		46	22	26	4	2				0.9		
										0.8	161.0	153.6 ±18.8
	II	44	29	16	6	3	2			1.0		
		54	26	13	7					0.7		
		60	18	14	6	2				0.7		
										0.8	132.3	
	III	61	23	7	4	4	1			0.7		
		60	26	11	2	1				0.6		
		54	23	15	7	1				0.8		
										0.7	167.6	
+ sulfite	I	72	22	2	3	1				0.4		
		72	24	3	1					0.3		
										0.4	72.0	73.1 ±3.8
	II	74	16	7	2		1			0.4		
		64	21	12	3					0.5		
		63	28	6	3					0.5		
										0.5	77.4	
	III	77	20	3						0.3		
		80	13	4	2		1			0.3		
		80	14	4	2					0.3		
										0.3	70.0	
+ DP/sulfite	I	39	18	11	15	6	6	5		1.7		
		46	21	12	4	12	3	1	1	1.3		
										1.5	302.0	269.2 ±38.1
	II	41	13	18	14	10	3	1		1.5		
		45	16	17	9	7	4	2		1.4		
		40	20	21	7	9	2	1		1.6		
										1.5	227.4	
	III	54	17	10	7	4	5	3		1.2		
		56	11	16	8	4	2	3		1.1		
		50	16	17	8	5	3	1		1.2		
										1.2	278.1	

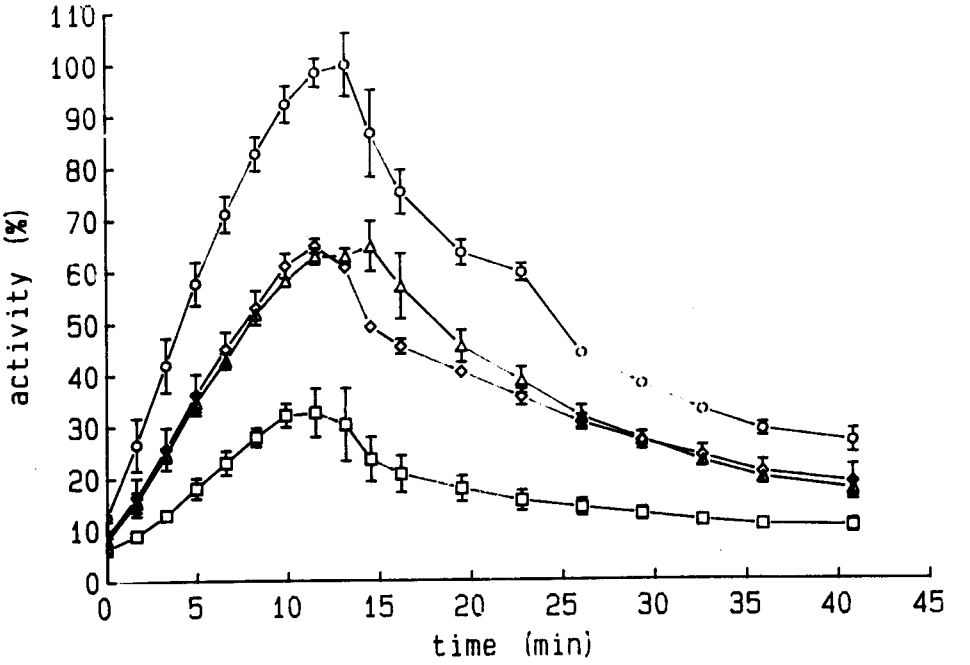

FIGURE 1 Chemiluminescence, generated by human neutrophils treated with diesel soot and sulfite. Experimental conditions were as described in Materials and Methods. Reaction mixtures contained about 6,600 neutrophils, 88.90 μg DP, 0.11 mM sulfite, 0.08 mM luminol and 0.2 mg zymosan. Standard deviations for $n = 4$. Controls, (○); plus DP, (△); plus sulfite, (◇); plus DP and sulfite, (□).

Chemiluminescence-test

In contrast to phagocytosis the chemiluminescence of stimulated neutrophils is inhibited by diesel soot, sulfite and their combinations where the combination shows the largest effect. At the maxima of the controls (100%) the values of the combined samples are 47.1 ± 4.1% (sample I), 34.8 ± 2.8% (sample II), 22.4 ± 6.2% (sample III) and 30.3 ± 7.1% (sample IV). Figure 1 shows the results with sample IV.

Oxygen Uptake

In agreement with the inhibition of chemiluminescence oxygen uptake of activated granulocytes is reduced after treatment with diesel soot, sulfite or the combination. The rates of oxygen uptake are shown in Table III.

TABLE III
Oxygen uptake by human neutrophils

Treatment	O_2 uptake (nmol/min)
control	4.10
cells + sulfite (5×10^{-4} M)	3.81
cells + DP (400 μg)	3.60
cells + DP/sulfite	2.16

control contains about 16,600 neutrophils in RPMI and 50 μl opsonized zymosan; n = 2

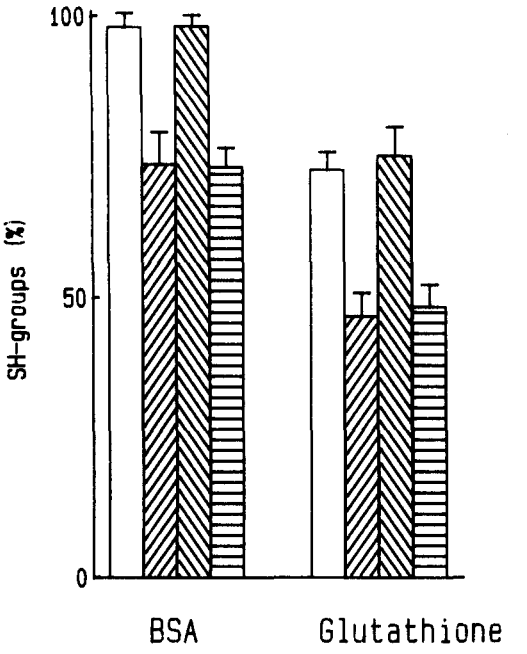

FIGURE 2 Loss of free SH-groups in BSA and glutathione. Experimental conditions were as described in Materials and Methods. Reaction mixtures contained 100 mM phosphate buffer, pH 7.8; 400 μg DP; 0.5 mM sulfite and 4 mg BSA or 50 μM glutathione, respectively. Standard deviations for $n = 4$. Controls, open bars; plus DP, ▨; plus sulfite, ▧; plus DP and sulfite, ■.

Collagenase-test

There is no visible influence of collagen decomposition after treatment of PMN with diesel soot particles and sulfite (data not shown).

Alteration of SH-Groups

As shown in Figure 2 the amounts of free SH-groups were significantly decreased under the influence of DP. Sulfite alone or in combination with DP was without effect. 30% of the SH-groups in BSA and 50% of the SH-groups in glutathione were oxidized in the presence of DP, whereas the controls showed oxidizing rates of 2% and 25%, respectively.

DISCUSSION

The rate of phagocytosis of polymorphonuclear leukocytes (PMN) is increased by a combination of diesel soot particles and sulfite. This increase may be explained by a stimulation of the function of the contractile apparatus. The mechanism responsible for this stimulation has yet to be elaborated. Degranulation of the PMN should be accompanied by an increased release of collagenase. According to our PAGE-analysis there is no indication for such an increase. This again might be due to several reasons;

it seems very likely that the sensitivity of the PAGE-color test is not high enough. The detection limit of this procedure is about 0.1 μg protein. In contrast to phagocytosis, the respiratory burst of the treated leukocytes seems to be inhibited under the influence of the combined air pollutants. This effect again may be due to several reasons. One of them might be the reaction of diesel soot particles (in presence of sulfite) with cysteine in the catalytic center of protein kinase C which in turn is involved in the activation of the respiratory burst. This point is all but clear, however, since Christiansen[14] emphasized that a simple correlation between the two parameters, namely the activation of protein kinase C and stimulation of the NADPH-oxidase does not exist, and that other mechanisms for oxidase activation must be operating. Witz *et al.*[15] correlated an altered level of free sulfhydryl groups on the surface of PMNs with decreased superoxide generation. Our own results are not in disagreement with such an assumption, since we also could show that aqueous suspensions of diesel soot particles decrease the content of SH-groups in bovine serum albumin and glutathione. Modification of surface SH-groups can not be the only reason of the reduced chemiluminescence in the combined system (DP + sulfite), since the amount of oxidized SH-groups was not enhanced in the samples containing DP/sulfite compared to DP alone. The diminished superoxide formation shown as inhibition of chemiluminescence may have secondary effects on the function of the activated leukocytes. In this context reduced formation of hypochloric acid and thus reduced microbicidal activities of the granulocytes has to be discussed. In addition, regulation of myeloperoxidase activity by superoxide[16] and influence of superoxide generation being controlled by myeloperoxidase[17] indicates a very complex system of regulation of the microbicidal activities in white blood cells. According to Borregaard[18] the cooperation of degranulation and induction of respiratory burst is one of the prerequisites for the correct function of white blood cells. This fact seems to be indicated by the observation that about 50% of the flavoprotein and approximately 90% of cytochrome b_{558} are located in specific granula which upon degranulation are incorporated into the membrane, thus producing a functional NADPH-oxidase. Cytochrome b_{558} seems to function as one component of the terminal redox components in a short electron transport chain of the NADPH-oxidase complex similar to the P_{450}-redox system. According to Badwey and Karnovsky[19] the respiratory burst may be independent of degranulation but dependent on the contact between leukocyte membranes and stimulating agents. This assumption is supported by the fact that neutrophils without detectable cytochrome b are able to produce superoxide with almost normal rates.[9] Our results support the latter assumption, since we observe that after incubation of diesel soot/sulfite with neutrophils the respiratory burst seems to be strongly diminished, phagocytosis on the other hand seems to be stimulated, possibly indicating that phagocytosis is an NADPH-oxidase-independent process. It is also indicated by several authors that during the regulation of NADPH-oxidase and also of collagenase activity, myeloperoxidase seems to possess a key function. Therefore, the effects of diesel soot and/or sulfite on myeloperoxidase have to be elaborated. Nevertheless, our results indicate that vital functions of activated leukocytes are influenced by combinations of air pollutants such as diesel soot particles and sulfite.

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