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Superoxide anion release induced by platelet-activating factor is increased in human alveolar macrophages from smokers

T. Schaberg*, H. Haller**, M. Rau, D. Kaiser, M. Fassbender, H. Lode

Superoxide anion release induced by platelet-activating factor is increased in human alveolar macrophages from smokers. T. Schaberg, H. Haller, M. Rau, D. Kaiser, M. Fassbender, H. Lode.

ABSTRACT: This study was designed to investigate the effects of the platelet-activating factor (PAF) on the superoxide anion production (O_2^-) of human alveolar macrophages (AM) from nonsmoking (n=18) and smoking (n=30) subjects. Freshly isolated cells were stimulated with (PAF) or with a phorbol ester (phorbol 12-myristate 13 acetate (PMA)).

Stimulation with PAF led to a dose-dependent increase of O_2^- production by AM in both groups. The median effective dose (EC_{50}) for PAF action on O_2^- production of smoker AM was 0.5×10^{-8} M, compared to nonsmoker AM with an EC_{50} of 1.0×10^{-7} M. This effect of PAF was blockable by the PAF-antagonist WEB 2086 in a dose-dependent manner. Comparison of the relative increase of O_2^- production after PAF-stimulation showed that smoker cells were significantly more sensitive to PAF than nonsmoker cells ($p<0.01$). In contrast to the findings with PAF, the relative increase of O_2^- production after PMA-stimulation showed no differences between smoker and nonsmoker AM.

Our data suggest that AM from smoking subjects are more sensitive to PAF than AM from nonsmokers.

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Smoking habits may alter alveolar macrophage function [1], but whether alveolar macrophages (AM) from smokers have a higher or lower metabolic activity than AM from nonsmoking subjects is still under discussion. We have recently observed that the platelet-activating factor (PAF), 1-0-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine [2], is a potent stimulator of human alveolar macrophages [3, 4]. PAF is an important mediator in inflammatory and allergic diseases of the lung [5, 6]. It is secreted by platelets [7], macrophages [8], and endothelial cells [9] and has been found in the bronchoalveolar lavage fluid of patients with a hyperreactive bronchial responsiveness [10]. Physiological responses to PAF are bronchoconstriction and a long-lasting bronchial hyperresponsiveness in animals and humans [11–13], which are assumed to be involved in the changes of lung structure and function in smokers. We therefore designed a study to investigate the effects of PAF on human alveolar macrophages in smokers and nonsmokers. Activation of AM was measured by the physiological response of superoxide anion production by these cells.

We could show that the AM of smokers were significantly more sensitive to PAF than those from nonsmokers.

Materials and methods

Human subjects

Alveolar macrophages (AM) were obtained by bronchoalveolar lavage (BAL) from patients (28 male, 20 female, mean age 45±14 yrs) who underwent bronchoscopy in the course of diagnostic procedures performed for a solitary peripheral lung nodule (≤2 cm), history of minor haemoptysis, suspected localized airway stenosis, mediastinal mass or other minor radiological findings on their chest X-ray. Signed informed consent according to the Helsinki Declaration was obtained from each patient. This study was approved by the local Ethics Committee. None of the patients received corticosteroids orally or by inhalation, and any inhalation of other substances was stopped 12 h before the investigation. Smoking behaviour was determined by special interviews of all patients.

Subjects were classified as nonsmokers in this study if they had never smoked, or if they had stopped smoking at least 5 yrs before the investigation (total pack-years <8 yrs) (ex-smokers). All patients had normal lung function as determined by body-plethysmographic spirometry.

Bronchoalveolar lavage (BAL)

Patients received an intramuscular premedication consisting of atropine 0.5 mg, hydrocodone 7.5–15 mg and midazolone 1.5–3 mg 20 min before bronchoscopy. BAL was performed under local anaesthesia (xylocaine 1%) *via* a fibreoptic bronchoscope (FBF 5, Olympus, Japan), using 200 ml of sterile 0.9% saline at 30°C in 20 ml aliquots according to the recommendations of the American Thoracic Society (ATS) [14]. All patients undergoing BAL had macroscopically normal bronchi. BAL was always carried out in the contralateral lung of patients with lung nodules or other pathological X-ray findings, and before other bioptic procedures in order to avoid blood contamination.

Preparation of alveolar macrophages (AM)

Lavage fluid specimens were filtered through sterile gauze and centrifuged at 500 × g for 10 min at 4°C. The cells were washed three times in cold buffer (Hank's balanced salt solution (HBSS)), counted by a haemocytometer, and resuspended in RPMI 1640 to a concentration of 6×10^5 cells·ml^{-1}. Differential cell counts were performed using Pappenheim-stained cytocentrifuge preparations. Viability was determined by 0.1% trypan blue (Sigma Chemicals) dye exclusion. Separation of AM was achieved by adherence to a plastic surface. Two hundred µl of the cell suspension (1.2×10^5 cells) were placed in 96-well cell culture plates (Nunc, Roskilde, Denmark) and incubated at 37°C with 95% H_2O and 5% CO_2 for 2 h. After adherence the plates were washed three times with HBSS at 37°C. The macrophage population was visualized by nonspecific esterase staining of control wells, to ensure a uniform macrophage monolayer. Viability was determined at this time point again by 0.1% trypan blue.

At the end of the superoxide anion assay, cells were again washed with 37°C HBSS, treated with 200 µl of 1.0% trypsin in HBSS for 60 min at 4°C, and removed by carefully repeated suction-ejection pipetting. An aliquot of this cell suspension was counted using a haemocytometer. In our experience, the method described leads to homogeneous macrophage monolayers, which could be confirmed by a mean protein concentration of 6±0.9 µg·well^{-1}, as determined by the method of Lowry using commercially available reagents (Mikro Protein Determination Kit, Sigma).

Buffers and chemicals

The buffer used in the superoxide anion assay was HBSS containing 136 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.3 mM Na_2HPO_4, 0.9 mM K_2HPO_4, 5.6 mM glucose, and 4.2 mM $NaHCO_3$ at pH 7.3–7.4. Cytochrome C (type VI, horse heart), superoxide dismutase (SOD), phorbol 12-myristate 13 acetate (PMA), platelet-activating factor (1-0-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine), lyso-PAF (1-0-hexadecyl-sn-glycero-3-phosphocholine), and staining dyes were purchased from Sigma Chemicals (Deisenhofen/FRG). WEB 2086 [15] was a generous gift from Boehringer Ingelheim, Ingelheim, Germany. All buffers and reagents used in these experiments were tested for the presence of endotoxin by monitoring the gelation of Limulus amoebocyte lysate (E-Toxate-Test, Sigma) and were found to have less than 1 ng·ml^{-1} as assessed by this technique. Cytochrome C (160 µM) and cytochrome C/SOD solutions were freshly prepared in HBSS. For SOD stock solution, SOD was dissolved in saline to a concentration of 5 mg·ml^{-1} and stored at -20°C. PMA stock solution was prepared in dimethyl sulphoxide (DMSO) (2.5 mg·ml^{-1}) and stored at -80°C. PAF and lyso-PAF solutions were prepared in 0.9% saline containing 2.0 mg bovine serum albumin·ml^{-1}. Aliquots were stored at -80°C. PMA, PAF and lyso-PAF were diluted in cytochrome C solution (final DMSO concentration <0.01%) immediately before the assay. The final concentrations used in the experiments were between 10^{-12} and 10^{-5} M for PAF and PMA. Fresh WEB 2086 solution was prepared daily in cytochrome C solution with a short ultrasonic exposure (30 s) at 30°C and diluted with cytochrome C solution between 10^{-10} and 10^{-5} M. Preincubation with WEB 2086 was always done 10 min before adding the stimuli to the $O_2^{\cdot}$ assay.

Superoxide anion assay

$O_2^{\cdot}$ release was quantified by assaying the capacity of $O_2^{\cdot}$ to reduce ferricytochrome C to ferrocytochrome C [16, 17]. To 96-multiwell plates, 100 µl of cytochrome C solution (160 µM in HBSS), with or without stimuli, were added to each well, containing 10^5 AM. All preparations were done in triplicate or quadruplet. Incubation conditions were 37°C, 95% H_2O and 5% CO_2. After incubation for 10, 20, 30, 60, 90 or 120 min, the plates were measured in an enzyme-linked immunosorbent assay (ELISA) microplate reader (Dynatech, Denkendorf, Germany) at 550 nm. Control measurements to ensure the optimal wave length were done at 545 and 555 nm. Since the macrophage monolayers were controlled in all plates, the cells influence on the readings was homogeneous [17]. Reduction of cytochrome C in the presence of SOD (1 mg·ml^{-1}) was substracted from the values without SOD and only the SOD-inhibitable reduction of cytochrome C was used to calculate the amount of $O_2^{\cdot}$ released. The reduction of ferricytochrome C was calculated using the molecular extinction coefficient for cytochrome C of 21.1 mM^{-1}·cm^{-1}. Since the vertical light path passing through 100 µl cytochrome C solution added to each well of a Nunc flat bottom microtitre plate is 3 mm,

nmol O_2^- can be calculated as absorbance at 550 nm × 15.87 [17]. Results were adjusted to the actual cell number per well of each measured plate and expressed as nM $O_2^-\cdot 10^{-6}$ cells per time indicated. The interassay coefficient of variation for nmol O_2^- was 8%, and the intra-assay variation in one subject was <12%.

Statistics

Statistical analysis was carried out on a Macintosh SE-computer (Apple Inc. Cupertino, CA, USA) using a commercial statistic program (Statworks®, Cricket Software, Inc., Philadelphia, PA, USA). All values were expressed as mean±SEM. Differences in mean values were analysed using the two-tailed, unpaired Student's t-test or a paired t-test, as required. Values of $p<0.05$ were considered significant.

Results

We investigated 18 nonsmoking and 30 smoking subjects with a wide variety of diseases (table 1). All of them showed a clearly localized lung disease, with the exception of eight patients without any sign of pulmonary disease after intensive diagnostic procedures. The basic clinical data of the two groups showed no differences other than their smoking habits (table 2). Fluid recovery of the total BAL volume was similar in the two groups (smokers: 55±15% = 110±38 ml; nonsmokers: 62±7% = 124±14 ml). The total number of cells·ml^{-1} BAL fluid was $35.4\pm5.8\times10^4$ in smokers compared to $10.8\pm2.9\times10^4$ in nonsmokers ($p<0.01$). The differential cell counts were not different between smokers and nonsmokers (table 2).

Table 1. – Final diagnosis of all patients included in the study

Final diagnosis	Nonsmoker	Smoker
Exclusion of stenosis	3	4
Bronchial cancer or solitary metastasis	2	4
Hamartoma	1	2
Pleural lipoma	1	2
Mediastinal mass	1	1
Pleural disease	2	4
Inactive minor tuberculosis	3	4
Smear negative minor tuberculosis	1	2
Minor lung embolism	1	2
No pathological finding	3	5

Final diagnoses were determined after the clinical reports and all available results from laboratory investigations, including the results of mycobacteriological cultures.

After 2 h of adhesion to a plastic surface, alveolar macrophages were enriched to >96±2%. The mean cell number after adherence was $0.9\pm0.2\times10^5$·well^{-1}. Viability of the macrophages after adhesion was >95% without differences between smoker and nonsmoker cells.

Stimulating the cells with PAF resulted in a dose-dependent increase of O_2^- production by both nonsmokers and smoker cells (fig. 1). PAF concentrations between 10^{-12} and 10^{-10} M did not increase O_2^- production significantly compared to control cells without stimulation. Between 10^{-9} M and 10^{-5} M PAF, O_2^- production was significantly higher than in control ($p<0.01$) both in smokers and in nonsmokers.

Table 2. – Basic clinical data and differential cell counts of 48 subjects from whom alveolar macrophages were obtained by bronchoalveolar lavage (BAL)

	Nonsmokers	Smokers
Clinical data		
Sex M/F	11/7	17/13
Age yrs	39±17	43±19
Pack years#		23±8
FEV_1/VC %	88±7	85±6
Rawt hPa·l^{-1}·s^{-1}	2.7±0.2	2.9±0.6
Differential cells counts		
Total cells·ml^{-1} BAL	$10.8\pm2.9\times10^4$	$35.4\pm5.8\times10^4$
Macrophages %	86±2	89±4
Lymphocytes %	11±2	7±3
Neutrophil granulocytes %	2±1	3±1
Eosinophil granulocytes %	0.5±0.05	0.5±0.05

Values are mean±SEM. Differential cell counts were made using cytocentrifuge preparations of BAL cell pellets stained according to Pappenheim. #: nonsmokers stopped smoking at least 5 yrs before bronchoscopy and have a smoking history of <8 yrs (4±4 yrs); FEV_1/VC: forced expiratory volume in one second expressed as % vital capacity; Rawt: total airway resistance as determined by body-plethysmographic spirometry.

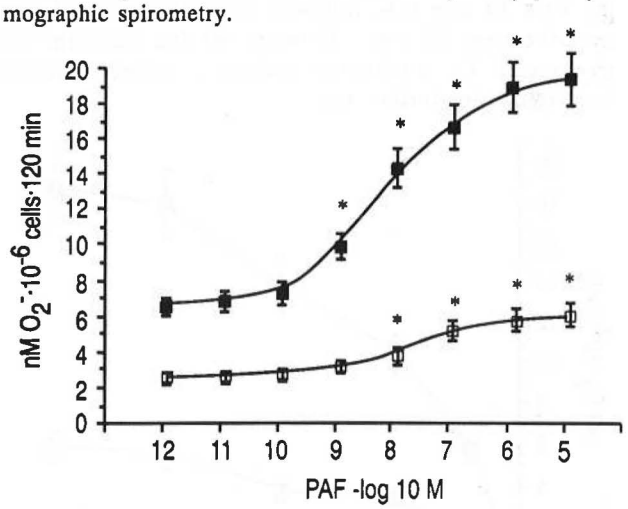

Fig. 1. – Dose-response curve for the effect of different platelet-activating factor (PAF) concentrations on O_2^- production by human alveolar macrophages (AM) obtained by bronchoalveolar lavage. After separation of AM, cells were stimulated with PAF, and O_2^- production was monitored by measurement of ferricytochrome C reduction (see Material and Methods). Values are mean±SEM expressed as nM $O_2^-\cdot10^{-6}$ cells per 120 min. Closed squares denote the results of smoker AM, open squares of nonsmoker AM. *: indicates values significantly above control values ($p<0.01$).

PAF concentrations higher than 10^{-5} M induced no further increase of O_2^- production in AM from both groups. The dose-response curve for PAF on the O_2^- production of AM from smokers showed a leftward shift compared to the cells from nonsmokers.

The EC_{50} for PAF action on O_2^- production of AM was 1.0×10^{-7} M for nonsmoker cells and 0.5×10^{-8} M for smoker cells. The inactive precursor/metabolite substance lyso-PAF had no influence on the release of O_2^- from nonsmoker or smoker AM at concentrations between 10^{-9} M and 10^{-6} M (table 3).

Table 3. – Spontaneous superoxide anion (O_2^-) production by human AM from nonsmoking (NS) and smoking (S) subjects and effects of lyso-PAF

Time min	O_2^- production* Control NS	Control S	lyso-PAF NS	lyso-PAF S
10	0.9±0.2	2.9±0.4	1.1±0.1	2.6±0.3
30	1.3±0.3	4.1±0.3	1.2±0.4	4.5±0.6
60	1.5±0.1	4.4±0.5	1.4±0.3	4.6±0.5
120	2.2±0.3	6.15±0.5	2.3±0.2	6.3±0.7

O_2^- production was measured in unstimulated cells (control) and lyso-PAF- (10^{-6} M) stimulated cells at indicated time points. *: data are expressed as nM $O_2^-\cdot10^{-6}$ cells (mean±SEM). AM: alveolar macrophages; PAF: platelet-activating factor.

At all time points measured, spontaneous O_2^- production by AM was significantly higher in smoker than in nonsmoker cells ($p<0.01$) (fig. 2). The time course of O_2^- production from smoker and nonsmoker cells showed only differences looking on the absolute values of O_2^-, but were similar regarding the shape of the curve. An initial steep rise of O_2^- release over the first 30 min was followed by a less marked rise over the next 60 min. Between 90 and 120 min the increase of O_2^- production reached a plateau without any significant further rise.

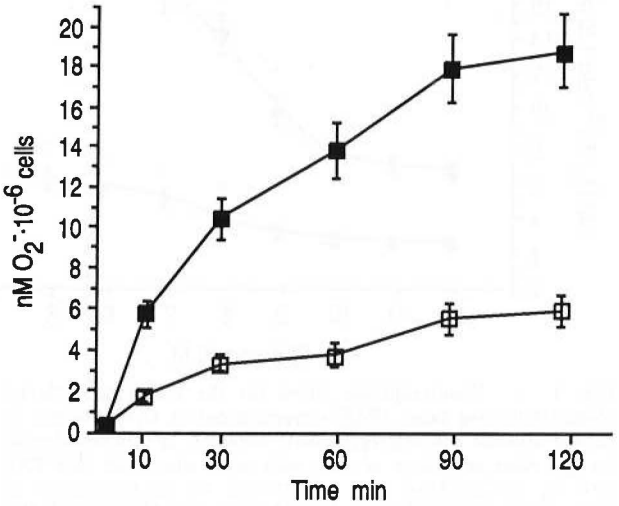

Fig. 2. – Time course of O_2^- production by human alveolar macrophages (AM) after stimulation with 10^{-6} M platelet-activating factor (PAF). Values are mean±SEM expressed as nM $O_2^-\cdot10^{-6}$ cells. Closed squares denote the results of smoker AM, open squares of nonsmoker AM.

The increased response of smoker AM to PAF was not dependent on the higher spontaneous O_2^- release from smoker cells compared to nonsmoker cells (6.15 *versus* 2.2 nM·10^{-6} cells per 120 min). When we compared the effect of PAF on O_2^- production in relation to this spontaneous release (% of control), we observed that, between 10^{-9} M and 10^{-5} M PAF, the increase in O_2^- production by smoker cells was significantly higher ($p<0.01$) than that by nonsmoker cells (fig. 3A). These findings indicate a higher sensitivity of smoker AM to PAF, which is not dependent on the higher spontaneous release of O_2^-.

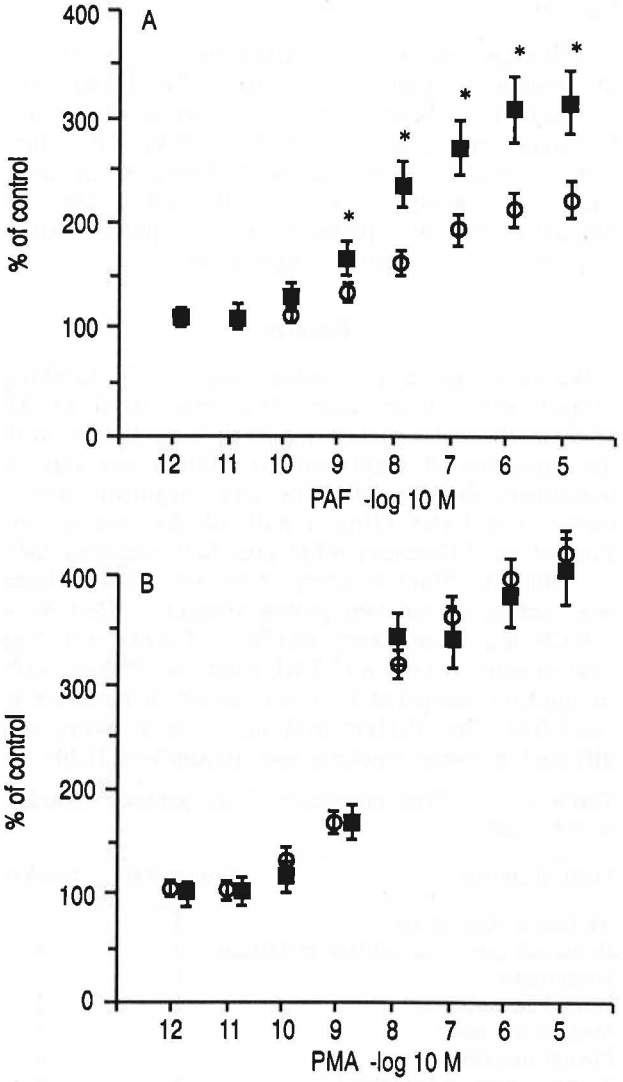

Fig. 3. – A) Increase of O_2^- production by human alveolar macrophages (AM) after stimulation with different platelet-activating factor (PAF)-concentrations for 120 min. Results are expressed as % of unstimulated cells (control): ((O_2^- $PAF_{Induced}/O_2^-$ unstimulated) × 100). *: significant differences ($p<0.01$). B) Increase of O_2^- production by human AM after stimulation with different phorbol 12-myristate 13 acetate (PMA)-concentrations for 120 min. Results are expressed as % of unstimulated cells (control): ((O_2^- $PMA_{Induced}/O_2^-$ unstimulated) × 100). Data are mean±SEM. Closed squares denote the results of smokers AM, open circles of nonsmoker AM.

To study whether the increased sensitivity of smoker cells to PAF is a nonspecific or specific PAF-related effect, we compared the relative increase of O_2^- production after PMA-stimulation (10^{-6} M) in nonsmokers and smokers. After incubation with 10^{-6} M PMA for 120 min, O_2^- release from 10^6 nonsmoker AM increased from 2.2±0.2 to 8.5±0.6 nM. Under the

same experimental conditions, the increase of O_2^- production by smoker AM was significantly higher (6.15±0.5 to 24.00±2.1 nM) ($p<0.01$). PMA was able to increase the generation of O_2^- at all measured concentrations between 10^{-6} and 10^{-10} M and at all measured time points both in nonsmoker and smoker cells (data not shown). However, when calculating relative values as % of control, we demonstrated that the increase of O_2^- generation from 10^6 nonsmoker AM rose to 390±40% and from 10^6 smoker AM to 370±29% after 120 min ($p>0.1$). For all PMA concentrations between 10^{-12} and 10^{-6} M, we found no significant differences between nonsmoker and smoker cells regarding the relative increase of O_2^- production (fig. 3B). Therefore, in contrast to the effects seen with PAF, the sensitivity of smoker and nonsmoker cells to PMA does not seem different.

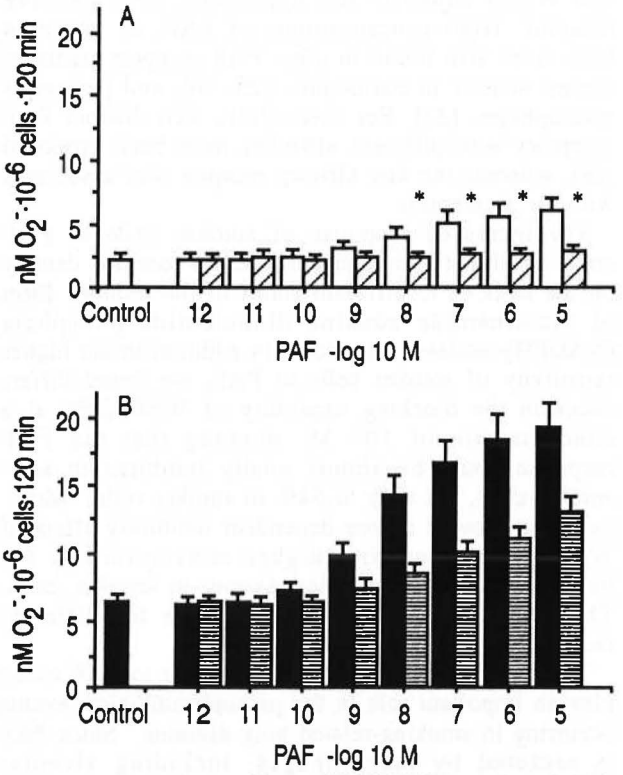

Fig. 4. – Inhibitory effect of WEB 2086 on the platelet-activating factor (PAF)-induced increase of O_2^- production by human alveolar macrophages (AM). Cells were preincubated without (control) or with 10^{-6} M WEB 2086 for 10 min and then stimulated with different PAF-concentrations. O_2^- production was monitored over 120 min. A) displays the data for nonsmoker AM (□: without WEB 2086; ▨: with WEB 2086). B) displays the data for smoker AM (■: without WEB 2086; ▤: with WEB 2086). Data are expressed in nM O_2^-·10^{-6} cells per 120 min as mean±SEM. *: significantly higher ($p<0.01$) inhibitory effect of WEB 2086 on O_2^- production of nonsmoker cells compared to smoker cells.

To investigate whether the effects of PAF on human alveolar macrophages were nonspecific or receptor-mediated, we preincubated the cells with a specific PAF antagonist, the triazole derivative WEB 2086 (10^{-6} M), 10 min before stimulation with PAF. Preincubation with 10^{-6} M WEB 2086 did not alter spontaneous O_2^- production of the control cells from nonsmokers or smokers (nonsmokers AM: control = 2.2±0.2 nM, control + 10^{-6} M WEB 2086 = 2.0±0.3 nM; smoker AM: control = 6.15±0.7 nM, control + 10^{-6} M WEB 2086 = 6.5±0.4 nM (all values for 10^6 cells per 120 min)). For the nonsmoker cells preincubated with WEB 2086 (10^{-6} M) we found a nearly complete reduction (≥90%) of O_2^- release of cells stimulated with PAF-concentrations between 10^{-12} to 10^{-5} M (fig. 4A). In smoker cells WEB 2086 was also able to inhibit the PAF-mediated increase of O_2^- production at concentrations above 10^{-9} M PAF, however, the inhibition between 10^{-7} M and 10^{-5} M PAF was not complete (≤60%) (fig. 4B).

The increased sensitivity of smoker AM towards PAF was also demonstrated by a difference in the inhibitory effect of WEB 2086. For this, we measured the release of O_2^- after preincubation with different WEB 2086 concentrations (fig. 5). For nonsmoker AM, we found that concentrations between 10^{-8} to 10^{-5} M WEB 2086 were able to suppress PAF-stimulated O_2^- release, whereas, in smoker cells, nearly complete inhibition was found only at a concentration of 10^{-5} M. The PMA-induced increase in O_2^- production was not affected by the PAF-antagonist WEB 2086 (nonsmoker AM: 10^{-6} M PMA: 8.5±0.6 nM O_2^-, 10^{-6} M PMA + 10^{-6} M WEB 2086: 8.9±0.8 nM O_2^-; smoker AM: 10^{-6} M PMA: 24.0±2.1 nM O_2^-, 10^{-6} M PMA + 10^{-6} M WEB 2086: 23.2±2.3 nM O_2^- (all values for 10^6 cells per 120 min).

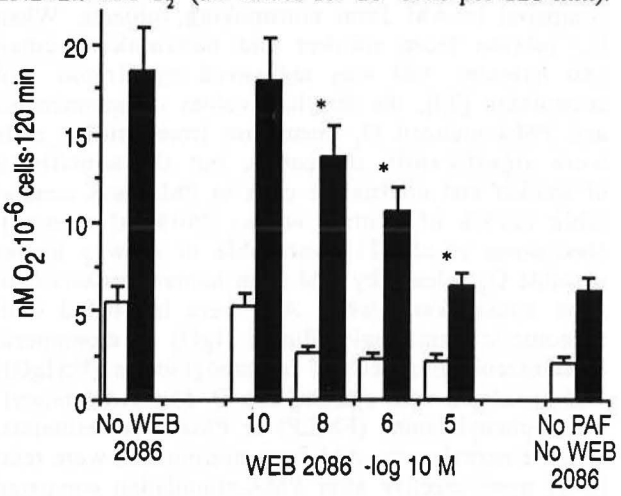

Fig. 5. – Dose-related effects of WEB 2086 on the platelet-activating factor (PAF)-induced increase of O_2^- production of human alveolar macrophages (AM). Cells were preincubated with different WEB 2086 concentrations 10 min before stimulation with 10^{-6} M PAF. Data are expressed in nM O_2^-·10^{-6} cells per 120 min as mean±SEM. Light bars denote the results for nonsmoker AM and dark bars for smoker AM. The first two columns represent the response to 10^{-6} M PAF without WEB 2086, whereas the last two columns represent the spontaneous O_2^- production without PAF stimulation. *: significant differences ($p<0.01$) of the inhibitory effects of WEB 2086 on O_2^- production of nonsmoker cells compared to smoker cells.

Discussion

In the present study, we have investigated the effects of PAF on O_2^- production by human alveolar macrophages in nonsmoking and smoking subjects. We

observed that smoker AM were significantly more sensitive to PAF than nonsmoker AM. This effect seems to be PAF-specific, since the relative increase after stimulation with PMA, unlike PAF-induced secretion, was not different in smoker or nonsmoker cells. The increased sensitivity of smoker AM was further suggested by the finding of a different dose-response of smoker AM to the PAF-specific antagonist WEB 2086. Compared to nonsmoker AM, higher WEB 2086 concentrations were needed for smoker AM to block O_2^- production completely. We therefore conclude that smoker AM are significantly more sensitive to PAF.

We are aware of the difficulty in studying patients instead of normal volunteers, but our two groups were very similar regarding their final diagnosis and their basic clinical data. Since we only differentiated between smoking subjects and nonsmokers including long-time ex-smokers, we think there has been appropriate internal control in our experiments.

It is generally accepted that alveolar macrophages of subjects with a long history of smoking show alterations in cellular function and morphological characteristics [18]. In our study, AM of smokers showed a generally higher O_2^- production with a higher spontaneous and PMA-stimulated secretion of O_2^- than nonsmoker AM. HOIDAL and NIEWOEHNER [19] found a more pronounced, generalized enhancement of the oxidative metabolism of AM from smoking subjects compared to AM from nonsmoking subjects. When O_2^- release from smoker and nonsmoker human and hamster AM was measured by HOIDAL and co-workers [20], the absolute values of spontaneous and PMA-induced O_2^- formation from smoker cells were significantly increased, but the sensitivity of smoker and nonsmoker cells to PMA was comparable (295% of control *versus* 260% of control). NAKASHIMA *et al.* [21] were able to show a higher absolute O_2^- release by AM from human smokers than from nonsmokers, when AM were incubated with monomeric immunoglobulin G (IgG) or monomeric crystallizable fragment of immunoglobulin (Fc(IgG)) and stimulated with cytochalasin D, formyl-methionyl-leucyl-phenylalanine (FMLP) or PMA. Nevertheless, in these experiments, AM from nonsmokers were relatively more reactive after PMA-stimulation compared to smoker cells. Comparison of these results with our findings is difficult due to methodological differences. These authors measured O_2^- release over only a short period of time (5 min) and found higher variances as displayed by standard deviations of up to 37%. We investigated the cumulative O_2^- release of AM over 120 min, which showed a much lower variance. In a recent publication, THOMASSEN *et al.* [22] also found no significant differences between smoker and nonsmoker cells when investigating the relative PMA-induced O_2^- production. In contrast to our experiments, the absolute values of PMA-induced O_2^- production were not different between smoker and nonsmoker AM. A possible explanation for this difference, may be that THOMASSEN *et al.* [22] measured O_2^- production by AM after a four day culture period and not in freshly isolated cells. From other experiments investigating priming effects of cytokines on AM we know that prolonged culture time (≥48 h) will reduce both spontaneous and stimulated O_2^- production by human AM (unpublished observation).

Our experiments provide some evidence that the PAF-induced O_2^- release is a receptor-mediated event. We showed an effect of PAF on O_2^- production by AM at concentrations starting at 10^{-9} M. However, the EC_{50} of PAF for the induction of O_2^- production in smoker and nonsmoker cells (0.5×10^{-8} M and 1.0×10^{-7} M, respectively) was higher than for other biological effects. Tumour necrosis factor (TNF)-secretion by animal AM [23] and interleukin-1 (IL-1)-secretion by animal spleen monocytes [24] are known to be affected by PAF at nanomolar concentrations, a fact which supports the hypothesis of a putative receptor. High concentrations of PAF, as observed here, were also found in other PAF receptor-mediated events such as in eosinophils [25, 26], and peritoneal macrophages [27]. For eosinophils, two distinct PAF receptors with different affinities have been proposed [28], whereas the low affinity receptor was associated with O_2^- production.

The increased response of smoker cells to PAF could be due either to differences in receptor density on the cells or to different states of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase activation. In addition to the higher sensitivity of smoker cells to PAF, we found differences in the blocking capability of WEB 2086 at a concentration of 10^{-6} M, showing that the PAF response could be almost totally inhibited in nonsmoker cells, but only to 54% in smoker cells. Moreover, we showed a dose-dependent inhibitory effect of WEB 2086, requiring higher concentrations for blocking the PAF-mediated events in smoker cells. This finding provides some evidence for different receptor numbers on both cell types.

The higher sensitivity of smoker cells to PAF could play an important role in the pathophysiological events occurring in smoking-related lung diseases. Since PAF is secreted by macrophages, including alveolar macrophages [29], and has a very short half-life *in vivo* [6], any feedback effect of the substance on O_2^- production by AM themselves could contribute to the pathophysiological changes in the lungs of smokers. Increased O_2^- production is known to impair the antielastasis activity of $alpha_1$-antitrypsin, a mechanism which may contribute to the development of emphysema [30]. It could also increase peripheral bronchoconstriction of small airways [31] in smoking subjects, by increasing the formation of eicosanoids [32]. Since both mechanisms play a role in the peripheral lung, it is possible that PAF-activated AM act at this local level, where large amounts of these cells are distributed.

In summary, we have shown that PAF and PMA increase O_2^- formation by human alveolar macrophages in a dose-dependent manner. Despite the higher

spontaneous $O_2^{\cdot}$ release from smoker AM, the sensitivity of nonsmoker and smoker AM to PMA was comparable. However, AM from smokers are more sensitive to PAF than AM from nonsmokers. This effect of PAF on human AM seems to be receptor-mediated, since it could be blocked by a specific PAF antagonist. The possible pathophysiological effects of PAF interaction with human AM should be further investigated.

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