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Enhanced glucocorticoid sensitivity of cytokine release from circulating leukocytes stimulated with lipopolysaccharide in healthy male smokers

Petra H. Wirtz,[a] Roland von Känel,[b] Sabine Kunz-Ebrecht,[c] Ulrike Ehlert,[a] and Joachim E. Fischer[b,*]

[a] *Department of Clinical Psychology II, University of Zurich, Zurich, Switzerland*
[b] *Institute for Behavioral Sciences, Swiss Federal Institute of Technology, Turnerstrasse 1, CH 8092, Zurich, Switzerland*
[c] *Department of Epidemiology and Public Health, University College, London, UK*

Abstract

Smoking is a strong cardiovascular risk factor that promotes inflammation. The source of elevated pro inflammatory cytokines in the circulation of smokers is not fully understood. We investigated the release of pro inflammatory cytokines from circulating leu kocytes stimulated with lipopolysaccharide (LPS) and its inhibition by glucocorticoids in smokers and non smokers. Ninety three middle aged apparently healthy men were categorized as smokers (>10 cigarettes/day; $n = 41$) or life long non smokers ($n = 52$). Peripheral cortisol was assessed from overnight urine. C reactive protein (CRP) and tumor necrosis factor (TNF) α were measured in plasma. LPS stimulated interleukin (IL) 6 and TNF α release from harvested circulating leukocytes were assessed using an in vitro whole blood assay with and without co incubation of increasing concentrations of either dexamethasone or hydrocortisone. Gluco corticoid sensitivity was defined as the concentration of glucocorticoids required that inhibits LPS stimulated cytokine release by 50%. Smokers had higher CRP levels ($p = .005$) and a trend for higher basal TNF α levels ($p < .07$), and they also showed lower IL 6 and TNF α release after LPS stimulation than non smokers (p's < .001). While peripheral cortisol concentration showed no significant group difference, inhibition of LPS stimulated leukocyte IL 6 and TNF α release by either glucocorticoid was enhanced in smokers as compared to non smokers (p's < .022). The finding suggests that, in spite of a low grade systemic inflammation, smokers have decreased LPS stimulated cytokine release from circulating leukocytes and greater glucocorticoid sensitivity of this cytokine release than non smokers. Circulating leukocytes unlikely contribute to the elevated pro inflammatory cytokine levels in the blood of smokers.

Keywords: Glucocorticoid sensitivity; Leukocytes; Cytokines; Smoking; Cardiovascular disease

1. Introduction

Smoking is a well-established risk factor for cardiovascular disease that exerts part of its cardiovascular risk by eliciting inflammation (Gensini et al., 1998; Villablanca et al., 2000). Elevated plasma levels of the inflammatory markers C-reactive protein (CRP), tumor necrosis factor (TNF)-α, and interleukin (IL)-6 have been prospectively associated with increased cardiovascular risk (Koenig et al., 1999; Ridker et al., 2000a,b; Ridker, 2001). These inflammatory markers are also elevated in the blood of smokers (Bostrom et al., 1998; Harris et al., 1999; Taaffe et al., 2000; Zoppini et al., 2001) suggesting a link between smoking and systemic up-regulation of inflammation.

The sources of pro-inflammatory cytokines in the circulation of smokers are not fully understood. While nicotine may induce production and release of TNF-α from cultured endothelial cells (Albaugh et al., 2001), the data on whether leukocyte hyperactivity might be involved is scare and ambiguous (Ohta et al., 1998; Zeidel et al., 2002). In addition, numerous studies found

* Corresponding author. Fax: +41 1 632 12 19.
E mail address: fischer@ifv.gess.ethz.ch (J.E. Fischer).

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decreased cytokine release from bronchoalveolar macrophages upon stimulation with lipopolysaccharide (LPS) suggesting an impaired pulmonary immune response (Higashimoto et al., 1992; McCrea et al., 1994; Mikuniya et al., 1999; Soliman and Twigg, 1992; Yamaguchi et al., 1993). In contrast, using IL-1β instead of LPS to stimulate lavage-derived alveolar macrophages, Ito et al. (2001) found more macrophage cytokine release in smokers than in non-smokers. To resolve this discrepancy, the authors suggested that smoking might downregulate the expression of the CD14/Toll-like receptor 4 that is involved in the recognition and binding of LPS (i.e., from gram-negative bacteria) or other aspects of the CD14/Toll signaling pathway (Ito et al., 2001).

Endogenous glucocorticoids downregulate cytokine release (Munck et al., 1984; Sapolsky et al., 2000). Even though smokers appear to have unchanged glucocorticoid secretion (Kirschbaum et al., 1994; Pruessner et al., 1997), altered leukocyte sensitivity to glucocorticoids might also underlie differences in inflammatory activity between smokers and non-smokers. In the mentioned study by Ito et al. (2001), dexamethasone suppressed IL-1β stimulated cytokine release from alveolar macrophages to a greater extent in non-smokers than in smokers. In that study, cigarette smoke reduced the activity of the histone deacetylases resulting in enhanced expression of genes coding for inflammatory proteins; moreover, the reduced glucocorticoid responsiveness correlated with the activity of histone deacetylases. It has not been investigated whether smokers and non-smokers show a difference in glucocorticoid sensitivity of circulating leukocytes.

The aim of this study was to shed more light on a possible contribution of circulating leukocytes to blood cytokine levels in smokers. We compared the LPS-stimulated release of TNF-α and IL-6 from harvested circulating leukocytes of smokers to that of non-smokers and the effect of glucocorticoids on this cytokine release. To address these questions, we choose a whole blood assay allowing us to study leukocytes in vitro in an environment similar to in vivo conditions. This is important because subtle environmental changes such as the presence of either pro- or anti-inflammatory cytokines (Arzt et al., 2000; Costas et al., 1996; Wilder, 1995) or a preceding endogenous glucocorticoid release (DeRijk et al., 1996, 1997; Rohleder et al., 2002, 2001) may affect leukocyte sensitivity to glucocorticoids. To specifically study the monocyte-initiated inflammatory pathway involved in the early stages of atherosclerosis, we used LPS that selectively stimulates the cell surface protein CD14 on monocytes (Schletter et al., 1995; Wright et al., 1990, 1991). This whole blood assay has been widely used to assess the glucocorticoid sensitivity of pro-inflammatory cytokine production across a wide range of conditions, including sepsis, inflammatory disease, exercise, and psychosocial stress (DeRijk et al., 1996; Franchimont et al., 1999; Molijn et al., 1995; Rohleder et al., 2001).

2. Methods

2.1. Experimental subjects

The study was part of a project conducted in an airplane manufacturing plant in Southern Germany. The study complies with the Declaration of Helsinki. The institutional review board has formally approved the study protocol and informed consent was obtained from all subjects. From a total of 1760 employees, participation was offered to a representative sample of 532 men and women, who participated in a questionnaire-based assessment of working conditions. Medical examination and blood withdrawal were performed in 332 volunteers (280 men, 52 women). Exclusion criteria for the present study were self-reported psychiatric, endocrine, cardiovascular, and any other chronic diseases as well as being on a regular drug regimen, including psychoactive drugs, β-blockers, and glucocorticoids. We also excluded women to prevent confounding of inflammatory activity by intake of oral contraceptives or by the female cycle.

We excluded current non-smokers with a recent history of regular smoking ($n = 83$) as well as moderate smokers who reported to smoke a maximum of 10 cigarettes/day ($n = 11$). These exclusion criteria allowed us to define two distinct populations of smoking and non-smoking individuals. The final study sample comprised 93 apparently healthy Caucasian men (mean age $\pm$ SEM, 38 ± 0.9 years). Based on the self-reported smoking history and according to previous work (Bergmann et al., 1998), we termed life-long non-smokers as "non-smokers" ($n = 52$), and smokers who reported to smoke currently >10 cigarettes/day as "smokers" ($n = 41$). Table 1 provides characteristics of the two groups.

2.2. Experimental protocol

The medical work-up comprised completion of a 96-item questionnaire assessing medical history and health behavior (smoking, physical activity, and alcohol consumption). Questions were derived from the Nurses Health Study (Michael et al., 1999) and items concerning smoking behavior were from the MONICA study (Jonsson et al., 1999). After completing the questionnaire, subjects had a 15-min rest period while sitting. Thereafter, systolic and diastolic blood pressure (BP) were determined twice within 5 min by sphygmomanometry.

Fasting blood samples were obtained within the ensuing 3 weeks between 7:00 am and 8:45 am (i.e., within

Table 1
Characteristics of the two groups

	Non smokers (n 52)	Regular smokers (n 41)	p value
Age (years)	38.1 ± 1.1	38.5 ± 1.4	.84
Body mass index (kg/m^2)	25.2 ± 0.3	25.3 ± 0.4	.78
Waist/hip ratio	0.9 ± 0.0	0.9 ± 0.0	.37
Total cholesterol (mg/dl)	214 ± 5.6	215 ± 7.0	.93
LDL cholesterol (mg/dl)	118 ± 4.4	114 ± 5.5	.57
HDL cholesterol (mg/dl)	46.6 ± 1.5	40.9 ± 1.5	.01
Physical activity (kcal/day)	540 ± 53	551 ± 74	.91
Alcohol/day (g)	17.8 ± 2.5	18.8 ± 2.7	.78
Systolic blood pressure (mmHg)	127 ± 1.5	128 ± 2.1	.69
Diastolic blood pressure (mmHg)	80.1 ± 1.0	79.2 ± 1.3	.61
Cigarettes/day	0.0 ± 0.0	20.88 ± 1.1	<.001
Hemoglobin A1c (%)	5.08 ± 0.1	5.26 ± 0.1	.02
Urinary cortisol (μg/L)	43.6 ± 3.3	51.0 ± 9.5	.42
Hematocrit (%)	45.7 ± 0.4	47.5 ± 0.4	.004
Leukocytes (× 10^6/ml)	5.91 ± 0.18	8.93 ± 0.36	<.001
Neutrophils (× 10^6/ml)	3.28 ± 0.13	5.48 ± 0.30	<.001
Lymphocytes (× 10^6/ml)	2.08 ± 0.08	2.75 ± 0.14	<.001
Monocytes (× 10^6/ml)	0.55 ± 0.02	0.71 ± 0.03	<.001

Values given are means ± SEM; LDL, low density lipoprotein; HDL, high density lipoprotein.

2 h from awakening), and before subjects began a morning shift. Glucocorticoid sensitivity assays were started within 5 min of blood collection in a cell culture facility adjacent to the blood collection room. For the TNF-α assay, venous blood was processed by standard techniques using cooled (4 °C) citrate tubes. Overnight urine collection for cortisol determination started at 9:00 pm the night before blood sampling, and it ended in the morning with the first void after awakening (Seeman et al., 1997).

2.3. Glucocorticoid sensitivity assay

The whole blood assay minimizes post-collection artifacts arising from cell preparation and maintains the individual's natural environment (i.e., plasma compounds, hormone levels) as much as possible during in vitro assays (De Groote et al., 1992, 1993). To assess glucocorticoid sensitivity, whole blood was stimulated with LPS and co-incubated with increasing amounts of either dexamethasone or hydrocortisone (Breuninger et al., 1993; DeRijk et al., 1997; Rohleder et al., 2001, 2002).

Assay procedures. The LPS stock solution (*Escherichia coli*, 055:B5, No. L2880, Sigma Aldrich Chemie GmbH, Steinheim, Germany) was prepared by dissolving LPS in pyrogene-free sterile saline (NaCl 0.9%, Fresenius Kabi, Stans, Switzerland) to achieve a final concentration of 15 ng/ml LPS during the culture. Dexamethasone (No. D8893, Sigma Aldrich Chemie GmbH, Steinheim, Germany) or hydrocortisone (No. H0396, Sigma Aldrich Chemie GmbH, Steinheim, Germany) stock solutions were prepared using pyrogene-free sterile saline solution (NaCl 0.9%, Fresenius Kabi, Stans, Switzerland) to achieve equipotent final culture concentrations: 0, 10 ^{10}, 10 ^{9}, 10 ^{8}, and 10 ^{7} mol/L for dexamethasone and 0, 3 × 10 ^{9}, 3 × 10 ^{8}, 3 × 10 ^{7}, and 3 × 10 ^{6} mol/L for hydrocortisone.

Nine milliliters of venous blood was collected into sterile pyrogene-free syringes containing 1 ml of heparinized 0.9% saline solution. Heparinized whole blood (400 μl) was added to 50 μl of LPS-stock solution and to 50 μl of the stock solutions of the various concentrations of either dexamethasone or hydrocortisone on a 24-well cell culture plate (No. 3047 Becton Dickinson, San Diego, California). Following a 6 h incubation period at 37 °C in 5% CO_2, plates were centrifuged for 10 min at 2000*g* at 4 °C. The supernatant was collected and stored at −80 °C until assayed. All preparations were performed under strictly aseptic conditions. Cell cultures were started within 5 10 min from sample collection in a room ultimately adjacent to the blood collection facility in order to minimize post-collection artifacts.

2.4. Biochemical analyses

Stimulated plasma levels of TNF-α and IL-6 were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (BD Pharmingen, San Diego, CA). High sensitivity ELISAs were chosen to measure plasma concentrations of TNF-α (Quantikine HS, R&D Systems Europe, Abington, UK) and of CRP (Immunolite, DPC Biermann GmbH, Germany; detection limit 0.1 mg/L). Total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, glycosylated hemoglobin A1c (HbA1c), and urinary cortisol were determined by a commercial laboratory (Synlab, Augsburg, Germany). To obtain a complete blood count including a differential, blood was collected into 2.7-ml EDTA tubes

(Sarstedt, Rommelshof, Germany) and processed on a Sysmex SE-9000 cell counter (Sysmex, Norderstedt, Germany) within 3 h from collection (Synlab, Augsburg, Germany).

2.5. Statistical analyses

As an index for glucocorticoid sensitivity, we calculated the IC_{50} of the dose response curve for dexamethasone or hydrocortisone inhibition of LPS-induced cytokine release. The IC_{50} is the dexamethasone or hydrocortisone concentration that achieves a 50%-inhibition of LPS-stimulated leukocyte cytokine release. The IC_{50} is inversely related to the glucocorticoid sensitivity; i.e., a higher IC_{50} indicates lower sensitivity, and a lower IC_{50} indicates higher sensitivity. The IC_{50} is calculated intra-individually using a logistic curve fit function (mean determination coefficient of $r^2 = 0.99$). Following previous methods (Rohleder et al., 2001, 2002), cytokine production was standardized for the monocyte count. The rationale for this correction is the fact that monocytes are the main source of proinflammatory cytokine production upon stimulation of whole blood with LPS (Berczi, 1998; Wright et al., 1990).

To approximate a normal distribution, values for CRP, unstimulated TNF-α, and IC_{50} were tested for normality and values for CRP and unstimulated TNF-α were log transformed. For clarity, all data are presented in original units. In case of missing data, cases were excluded listwise. Across the two subject groups (smokers and non-smokers), univariate ANOVAs were calculated for absolute numbers of monocytes, CRP, unstimulated TNF-α, LPS-stimulated cytokine production, and IC_{50} values. Repeated measure ANOVAs were calculated for inhibition of LPS-induced cytokine production by both dexamethasone and hydrocortisone. To correct for non-sphericity of the repeated measures, the Huynh Feldt correction was applied (Huynh and Feldt, 1976). All calculations were performed using SPSS Inc. (10.0) and Curve Expert (1.3) software packages. Data are presented as means $\pm$ SEM. Results were considered statistically significant at the $p \leqslant .05$ level; all tests were two-tailed.

3. Results

3.1. Characteristics of the two groups

The 52 non-smokers and the 41 regular smokers differed in several health-related variables As expected, smokers had higher hematocrit, lower HDL cholesterol, and higher HbA1c levels than non-smokers. Smokers also had higher absolute counts of total leukocytes, neutrophils, lymphocytes, and monocytes (Table 1). The mean urinary cortisol secretion showed no statistically significant difference between smokers and non-smokers.

3.2. Basal inflammatory measures

Fig. 1 shows that smokers had a higher basal plasma CRP concentration than non-smokers ($p < .01$). Similarly, there is a statistical trend toward a higher TNF-α concentration in smokers ($p < .07$).

3.3. Stimulation of cytokine production by LPS

There were significant differences between groups in terms of stimulated TNF-α ($p < .001$) and IL-6 secretion ($p < .001$) without co-incubation with glucocorticoids. Smokers had less LPS-stimulated TNF-α release than non-smokers per 10^5 monocytes (Fig. 2A). Smokers also showed lower LPS-stimulated IL-6 release as compared to non-smokers per 10^5 monocytes (Fig. 2B). Differences between groups were retained when cytokine release was corrected for total leukocyte counts (all p's < .001).

3.4. Inhibition and glucocorticoid sensitivity of stimulated cytokine production

Dexamethasone and hydrocortisone provoked a similar inhibitory pattern with increasing concentrations resulting in increasing inhibition of cytokine release (Figs. 2A and B). The glucocorticoid sensitivity (IC_{50}) indicating the concentration required to inhibit 50% of the LPS stimulated cytokine release without glucocorticoid co-incubation showed a similar pattern for both cytokines and for both glucocorticoids (Figs. 3A and B). Smokers had significantly lower IC_{50} for TNF-α and for IL-6 than non-smokers (dexamethasone TNF-α: $F(1,78) = 8.2$, $p = .005$; hydrocortisone TNF-α: $F(1,77) = 5.5$, $p = .021$; dexamethasone IL-6: $F(1,74) = 5.8$, $p = .019$; hydrocortisone IL-6: $F(1,77) = 15.2$, $p < .001$). In other words, lower glucocorticoid concentrations were required to achieve a 50%

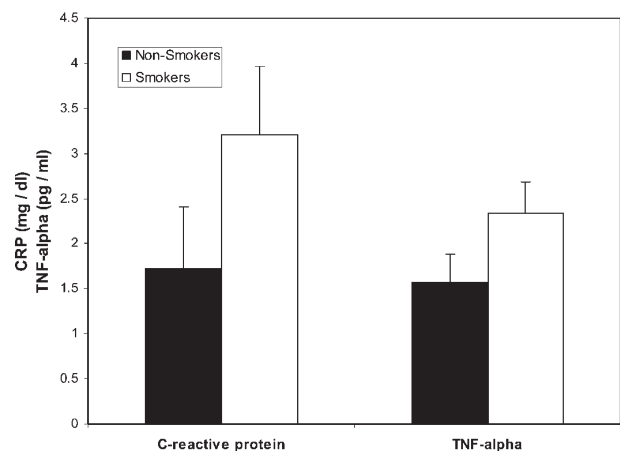

Fig. 1. Plasma levels of C reactive protein (CRP) and of unstimulated tumor necrosis factor (TNF) α in the two groups (means $\pm$ SEM). CRP was significantly higher in smokers ($F(1,88)$ 8.2; p .005), and TNF α showed a similar trend towards significance p .066.

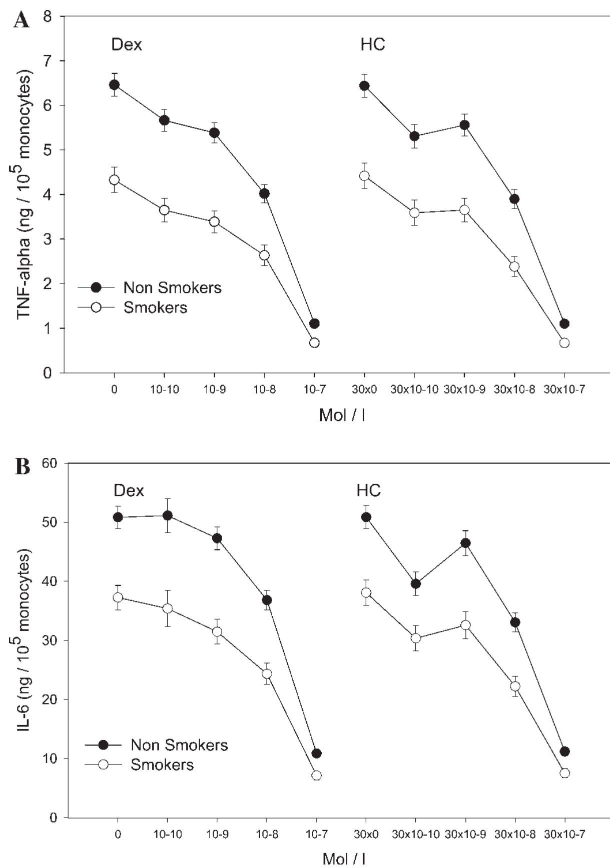

Fig. 2. (A) Lipopolysaccharide stimulated release of tumor necrosis factor (TNF) α and inhibition of TNF α release by glucocorticoids (dexamethasone, DEX; hydrocortisone, HC) in the two groups (mean s ± SEM). Note that the starting points of the individual inhibition curves refer to the uninhibited release of TNF α in response to LPS. TNF α release after LPS stimulation was lower in smokers ($F(1, 79)$ 32.8; $p < .001$). TNF α release was significantly inhibited by incremental concentrations of dexamethasone ($F(2.3, 179.5)$ 578.1, $p < .001$) and of hydrocortisone ($F(2.7, 197.2)$ 15.0, $p < .001$). (B) Lipopolysaccharide stimulated release of interleukin 6 (IL 6) and inhi bition of IL 6 release by glucocorticoids (dexamethasone, DEX; hy drocortisone, HC) in the two groups (means ± SEM). Note that the starting points of the individual inhibition curves refer to the uninhibited release of IL 6 in response to LPS. IL 6 release after LPS stimulation was significantly lower in smokers ($F(1, 79)$ 25.9; $p < .001$), and IL 6 release was significantly inhibited by incremental concentrations of dexamethasone ($F(2.2, 170.2)$ 274.5, $p < .001$) and of hydrocortisone ($F(3.3, 239.5)$ 355.3, $p < .001$).

inhibition in smokers as compared to non-smokers, indicating that smokers had a higher glucocorticoid sensitivity than non-smokers.

4. Discussion

Corroborating previous studies (Bostrom et al., 1998; Harris et al., 1999; Taaffe et al., 2000), we found significantly higher plasma levels of CRP and a statistical trend for higher plasma levels of TNF-α in smokers, compatible with a systemic inflammatory state.

Our data further suggest that, alike alveolar macrophages (Higashimoto et al., 1992; McCrea et al., 1994; Mikuniya et al., 1999; Soliman and Twigg, 1992; Yamaguchi et al., 1993), leukocytes harvested from the circulation of smokers release less cytokines upon LPS stimulation. There may be two explanations for this observation. First, Ito et al. (2001) have suggested that the finding of decreased cytokine release from alveolar macrophages may specifically relate to the LPS stimulus because these authors found increased cytokine release if alveolar macrophages were stimulated with IL-1β. Even though this has not been investigated, the CD14/Toll-like receptor 4 and its signaling pathway might be less responsive to LPS in smokers than in non-smokers. A similar mechanism might be at work in circulating leukocytes. Thus, our finding does not contradict the study by Ito et al. but rather suggests that interpretation of findings on impaired immune responses both in the lung and in the circulation must consider the particular leukocyte stimulant used. The assay applied might also be an issue. A previous study found increased TNF-α and IL-6 secretion from isolated peripheral blood mononuclear cells upon stimulation with LPS in smokers (Zeidel et al., 2002). Isolated mononuclear cells might behave differently from leukocytes stimulated in a more physiological milieu of whole blood.

Second, Bergmann et al. (1998) showed that smokers have a lower absolute count of activated monocytes and monocyte macrophages and a higher count of non-activated monocytes than non-smokers. As in our study, that study found a higher total leukocyte and monocyte count in smokers than in non-smokers. We assume that, in spite of a greater number of circulating leukocytes, the less active white cells of smokers released a lower amount of cytokines in response to LPS than the more active white cells of non-smokers.

We found that glucocorticoid inhibition of stimulated cytokine release from leukocytes was stronger in smokers as compared to non-smokers. This observation of an enhanced glucocorticoid sensitivity of leukocytes of smokers has not been reported; how could it be explained? Differences in the activity of the hypothalamic pituitary adrenal axis were unlikely responsible for this finding. In line with previous work (Kirschbaum et al., 1994; Pruessner et al., 1997), our smokers and non-smokers showed no significant difference in overnight urinary cortisol excretion. Also, it is unlikely that basal inflammatory activity led to increased glucocorticoid receptor function in smokers. Rather, basal pro-inflammatory activity should lead to an acquired glucocorticoid resistance (Miller et al., 1999).

Because monocytes are the main source of cytokine release in the early phase following LPS stimulation, these differences were most likely accounted for by a different glucocorticoid sensitivity of monocytes. Indeed,

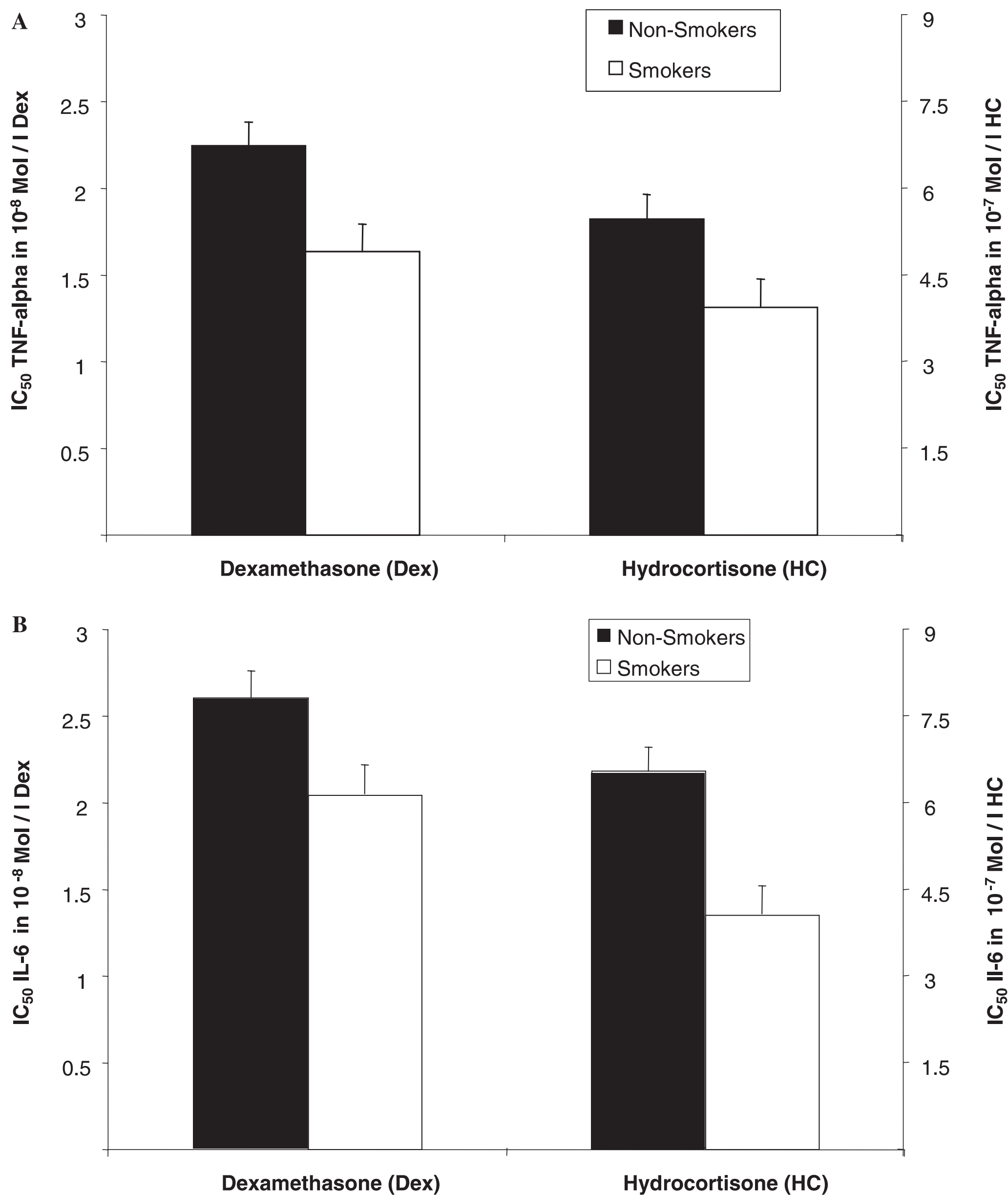

Fig. 3. (A) Glucocorticoid (dexamethasone or hydrocortisone) sensitivity of lipopolysaccharide stimulated release of tumor necrosis factor (TNF) α in the two groups expressed as the quantity of glucocorticoids required to suppress 50% of the LPS stimulated cytokine release (IC_{50}). The IC_{50} is inversely related to glucocorticoid sensitivity; hence, higher IC_{50} indicates lower sensitivity. In smokers, the TNF α IC_{50} was significantly lower for dexamethasone ($F(1,78)$ 8.2; p .005) and for hydrocortisone ($F(1,77)$ 5.5; p .021). Error bars indicated standard error of the mean. (B) Glucocorticoid (dexamethasone or hydrocortisone) sensitivity of lipopolysaccharide stimulated release of interleukin (IL) 6 in the two groups. Across all subjects, smokers had lower IL 6 IC_{50} for dexamethasone ($F(1,74)$ 5.8; p .019) and for hydrocortisone ($F(1,77)$ 15.2; $p < .001$).

controlling for the total leukocyte count did not change the significance of this observation. Thus, circulating monocytes from smokers did not only secrete lower quantities of cytokines after LPS stimulation, this cytokine release was also more readily curtailed by the presence of glucocorticoids in our in vitro assay. The reduced responsiveness to LPS in cells from smokers might contribute to the altered responsiveness to glucocorticoids in this group by lowering the relative level of cellular excitation to be inhibited.

Our observation of enhanced glucocorticoid sensitivity in circulating leukocytes and monocytes, respectively, does not necessarily contradict the previous finding of reduced glucocorticoid sensitivity of cytokine release from alveolar macrophages of smokers (Ito et al., 2001). In fact, circulating monocytes are immature cells as opposed to the more differentiated macrophages. Maturation along the developmental steps from the circulating juvenile monocyte to the matured macrophage trafficked in the bronchoalveolar space might

confer changes in the pattern of glucocorticoid sensitivity such as related to the activity of histone acteylases (Ito et al., 2001).

Our data suggest that circulating leukocytes unlikely contribute to elevated blood cytokine levels in smokers. However, in addition to endothelial cells (Albaugh et al., 2001), macrophages might represent one important source of elevated cytokines in the circulation of smokers. Previous studies (Bergmann et al., 1998; Dovgan et al., 1994) suggest that monocytes of smokers more readily adhere to the endothelial layer with a consecutive transmigration to the subendothelial space, in particular the alveolar space and the vessel intima. Once becoming resident, matured macrophages might become more activated and might exhibit decreased glucocorticoid sensitivity, and, thereby, might contribute to increased basal inflammatory activity in smokers. In contrast, the remainder of the circulating monocytes represent a pool of relatively immature cells, which produce reduced quantities of cytokines upon stimulation, and which are more easily downregulated in their cytokine response by glucocorticoids.

To confirm such a hypothesis, a study that investigates glucocorticoid sensitivity in circulating monocytes along with that in alveolar macrophages in the same population of smoking and non-smoking individuals is clearly needed. Also, some of the difference in the glucocorticoid sensitivity between circulating leukocytes and alveolar macrophages might relate to the aforementioned different effects of LPS and IL-1β on leukocytes. Ideally, future studies should include different stimulants to test for their unique effects on leukocyte cytokine release and its inhibition by glucocorticoids.

Several caveats of our study need to be addressed. First, it is arguable whether overnight urinary cortisol excretion provides the best proxy measure to assess the effects of endogenous cortisol on leukocyte activity. Preferably, we should have obtained diurnal cortisol profiles including the awakening response. Unfortunately, the representatives of the company refuted such a sampling procedure. Second, our interpretation of the data would have gained support from flow cytometry based assessment of monocyte maturity. Third, we were unable to unequivocally delineate the precise source of measured cytokines. While LPS is a highly monocyte-specific stimulus, we cannot rule out that other white blood cells became activated and released cytokines during the entire 6-h incubation period. Fourth, our findings may not be generalizable to populations with atherosclerotic diseases, and, it also remains to be seen, whether they are of any value in predicting clinical endpoints.

Taken together, the present study suggests that circulating leukocytes of smokers show an increased glucocorticoid sensitivity of pro-inflammatory cytokine release in response to LPS stimulation. Circulating leukocytes are most likely not a source of increased blood cytokine levels observed in smokers.

Acknowledgments

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