View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Respiratory Medicine (2009) **103**, 364—372

available at www.sciencedirect.com

journal homepage: www.elsevier.com/locate/rmed

Antioxidant status associated with inflammation in sarcoidosis: A potential role for antioxidants

Agnes W. Boots [a,b,*], Marjolein Drent [b,c], Els L.R. Swennen [a], Harald J.J. Moonen [d], Aalt Bast [a,b], Guido R.M.M. Haenen [a,b]

[a] *Department of Pharmacology and Toxicology, Faculty of Medicine, Maastricht University, 6200 MD Maastricht, The Netherlands*
[b] *ILD Care Center, University Hospital Maastricht, 6202 AZ Maastricht, The Netherlands*
[c] *Department of Respiratory Medicine, University Hospital Maastricht, 6202 AZ Maastricht, The Netherlands*
[d] *Department of Health Risk Analysis and Toxicology, Faculty of Health Sciences, Maastricht University, 6200 MD Maastricht, The Netherlands*

Received 21 July 2008; accepted 6 October 2008
Available online 17 November 2008

KEYWORDS
Antioxidants;
Oxidative stress;
Tumor necrosis factor alpha;
Quercetin

Summary

Rationale: Enhanced production of reactive oxygen species (ROS), capable of reducing endogenous defense levels and enhancing inflammation, is suggested to play a role in sarcoidosis. Antioxidant supplementation might offer protection against such ROS-mediated damage. A promising candidate for antioxidant supplementation is the flavonoid quercetin.
Aim: To determine the antioxidant and inflammatory status in sarcoidosis. Furthermore, the potential of quercetin to mitigate the occurring inflammation will be assessed.
Methods: Non-smoking sarcoidosis patients and healthy controls matched for age, gender and dietary behavior were enrolled (NCT-00512967). Measurements included assessment of total plasma antioxidant capacity, vitamin C, uric acid, glutathione, basal and LPS-induced levels of tumor necrosis factor alpha (TNFα), interleukin (IL)-8 and -10 as well as the effect of quercetin on these levels.
Results: Compared to their controls, the sarcoidosis patients displayed significantly lower total plasma antioxidant capacity, decreased levels of vitamin C, uric acid and glutathione and increased levels of basal TNFα and IL-8. Quercetin significantly decreased *ex vivo* LPS-induced TNFα- and IL-8 production in a concentration-dependent manner in both groups. Interestingly, this quercetin effect was more pronounced in sarcoidosis patients.
Discussion: The endogenous antioxidant defense was significantly reduced in sarcoidosis, indicating that oxidative stress underlies the pathology of this disease. Furthermore, the inflammatory status was significantly enhanced in sarcoidosis. Finally, our results regarding the effect of quercetin on cytokine production imply that sarcoidosis patients might benefit from

* Corresponding author. Department of Pharmacology and Toxicology, Faculty of Medicine, Maastricht University, Universiteitssingel 50, P.O. Box 616, 6200 MD Maastricht, The Netherlands. Tel.: +31 43 388 1340; fax: +31 43 388 4149.
E-mail address: a.boots@farmaco.unimaas.nl (A.W. Boots).

0954-6111/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.rmed.2008.10.007

antioxidant supplementation not only by empowering the relatively low protection against ROS but also by reducing inflammation.
© 2008 Elsevier Ltd. All rights reserved.

Introduction

Sarcoidosis is an interstitial lung disease which incidence varies among different countries over the world. In Scandinavian countries the incidence is higher compared to more southern countries.[1] In The Netherlands and Germany it is estimated that the incidence is approximately 20–25 per 100,000 inhabitants.[1]

Sarcoidosis is an antigen-driven, multisystem, granulomatous disorder of which the exact etiology is unknown.[2,3] Current evidence supports the concept that the pathogenesis of sarcoidosis involves a highly polarized T-helper 1 immune response to pathogenic tissue antigens or specific environmental factors.[1] Granuloma formation is regulated by a complex interaction between T-helper lymphocytes and macrophages, in which cytokines such as tumor necrosis factor (TNF)-α play an important role.[2]

Enhanced formation of reactive oxygen species (ROS) is suggested to play a role in the pathogenesis of sarcoidosis.[4–6] However, little is known about the endogenous defense levels present in sarcoidosis, i.e. the antioxidant levels that can offer protection against ROS-mediated damage. This prompted us to determine the endogenous antioxidant levels present in sarcoidosis patients. It is expected that the high production of ROS that occurs in sarcoidosis will consume antioxidants, thereby reducing their levels. As a result, antioxidant supplementation might be beneficial in sarcoidosis treatment.

ROS are also capable of initiating and mediating inflammation in the lung.[7,8] Besides enhanced ROS formation, inflammation plays a key-role in the occurrence and progression of sarcoidosis too.[9,10] Conventional treatment is focused on attenuating granuloma formation with drugs that inhibit antigen presentation or with non-specific anti-inflammatory agents such as glucocorticosteroids, methotrexate, or azathioprine.[2] However, all these therapies fail to be completely efficacious.[11,12] Strengthening the endogenous antioxidant defense with antioxidant supplementation, and thereby decreasing the levels of ROS production and ROS-mediated damage, might mitigate the elevated inflammation present.

The past few years, much attention has been given to the potential health-beneficial properties of flavonoids, natural occurring polyphenolic compounds, and to quercetin, the most commonly occurring flavonoid, in particular. Quercetin is an extremely powerful antioxidant[13] and therefore it is tempting to speculate that quercetin can exert positive effects in sarcoidosis.

The aim of the present study is to determine both the antioxidant and inflammatory status in sarcoidosis. Furthermore, the potential anti-inflammatory effects of antioxidants, exemplified with the flavonoid quercetin, will be assessed in this disease. To this extent, cytokine production will be evoked *ex vivo* by lipopolysaccharide (LPS), a patho-physiological relevant stimulator of monocytes, neutrophils and B lymphocytes.[14–17]

Materials and methods

Chemicals

Quercetin and lipopolysaccharide (LPS, *E. coli* 0.26:B6) were purchased from Sigma Chemical Co. (St. Louis, USA). RPMI 1640 medium containing L-glutamine was obtained from Gibco (UK). Human TNFα (7300 pg/ml), human IL-10 (4000 pg/ml) and human IL-8 (10 ng/ml) were acquired from CLB/Sanquin (Amsterdam, The Netherlands). All other chemicals were of analytical grade.

Methods

Participants

General information

All participating patients were recruited from the patients visiting the out-patient clinic of the university hospital Maastricht, a tertiary referral for The Netherlands. The sampling took place at the day they had to come in for a regular check-up. Based on food questionnaires, all patients had comparable dietary habits with an average daily intake of quercetin of approximately 15 mg. None of the participants used any vitamin or food supplementation.

The Medical Ethical Committee of Maastricht University and the University Hospital Maastricht had approved the protocol before the beginning of the study and the study was registered at http://www.clinicaltrials.gov (NCT-00512967). All participants were fully informed about the aim and details of the study and have given their written informed consent.

Sarcoidosis patients

Twenty-one non-smoking patients with symptomatic sarcoidosis (age 44 ± 1) were enrolled in this study. Patients had been diagnosed with sarcoidosis based on clinical features, together with bronchoalveolar lavage (BAL) fluid analysis results (data not shown).[18] Moreover, 17 out of the 20 sarcoidosis patients had a transbronchial biopsy confirmation of the disease. The clinical symptoms of all patients included respiratory symptoms, i.e. dyspnea, coughing and chest pain. The characteristics of the studies population are summarized in Table 1. According to their chest radiographic stage, patients were subdivided into two groups, i.e. mild (stage 0–1) and severe sarcoidosis (stage 2–4). In the sarcoidosis patients, both the DLCO ($80 \pm 3\%$) and the FEV_1 ($84 \pm 6\%$) were reduced. This reduction in FEV_1 and DLCO was even more pronounced in stage 4 sarcoidosis patients compared with the other sarcoidosis

Table 1 Characteristics of the participants.

	Sarcoidosis	Controls
Number (m/f)	20 (16/4)	11 (7/4)
Age	31–60 (44 ± 1)	40–58 (48 ± 3)
Length	158–194 (177 ± 2)	162–188 (175 ± 3)
Weight	52–107 (80 ± 4)	59–95 (77 ± 3)
Body mass index	18.5–31 (25 ± 1)	18.5–30 (26 ± 1)
Time since diagnosis	0–30 (5 ± 2)	–
Biopsy taken	Yes:17 No:3	–
DLCO	39–107 (80 ± 3)	90–110
FEV_1	42–135 (84 ± 6)	90–110
FVC	49–152 (92 ± 6)	90–110
Chest radiograph stage 0/I/II/III/IV (*n*)	1/4/9/2/4	–

Controls are matched on age and gender and do therefore not significantly differ from the patients regarding these parameters. Age is expressed in year, length in cm, weight in kg, and both DLCO (diffuse capacity of the lung for carbon monoxide) and FEV_1 (forced expiratory volume in 1 s) in % of the predicted value based on age and gender. Data are expressed as range (mean ± SEM).

patients (52 ± 10% ($n = 4$) vs 91 ± 5% ($n = 16$) and 60 ± 4% ($n = 4$) vs 83 ± 3% ($n = 16$) respectively). With regards to the FVC, only stage 4 patients displayed lower levels than the other sarcoidosis patients, but on average this lung function parameter did not differ considerably from the control range. This lack of major differences in FVC, a reliable indicator of disease progression and pulmonary function loss, suggests that lung function in the sarcoidosis group and the healthy controls is comparable.

At the moment this study was performed, none of the sarcoidosis patients used any medication.

Controls

The control group consisted of 11 non-smoking healthy volunteers, all with dietary habits comparable to those of the patients and a quercetin intake of approximately 15 mg per day. All controls did not use any medication or vitamin/food supplementation.

Lung function measurement

Lung function measurements included FEV_1, DLCO and FVC. FEV_1 and FVC were measured with a pneumotachograph, DLCO by the single-breath method (Masterlab, Jaeger, Würzburg, Germany). Values were expressed as a percentage of those predicted based on age and gender.[19]

Preparation of blood samples

Blood was collected from all participants in EDTA-containing vacutainer tubes (Vacutainer, Becton-Dickinson) and kept on ice prior to processing which occurred within 1 h after blood collection. Blood was aliquoted into eppendorfs for both the ascorbic acid and the GSH/GSSG analysis: for the former 10% TCA was added to the whole blood, whereas 1.3% SSA in 10 mM HCl was used to preserve the samples for the latter. Another aliquot of blood was used for the incubations required for the blood-based cytokine production assay as described in that section below. The remaining blood was centrifuged (3000 rpm, 5′ at 4 °C) to obtain plasma. Deproteinization of an ailquot of this plasma, using 10% TCA (1:1) followed by centrifugation (13,000 rpm, 5′ at 4 °C), was carried for the trolox equivalent antioxidant capacity measurement. All samples were stored at −80 °C prior to analysis.

Antioxidant status

The antioxidant status was evaluated by measuring the total plasma antioxidant status as well as the plasma levels of the individual antioxidants ascorbic acid, uric acid and glutathione.

Trolox equivalent antioxidant capacity

The trolox equivalent antioxidant capacity (TEAC value) is a measurement for the total antioxidant status, relating the free radical scavenging properties of a solution or a compound to that of the synthetic antioxidant trolox. The assay is performed as previously described[20] with minor modifications. In short, an ABTS·− solution was prepared freshly by mixing 23 mM ABAP with 2.24 mM ABTS[2]− stock solution in 100 mM phosphate buffer (pH 7.4). This solution was heated at 70 °C for approximately 10 min. To evaluate the ABTS·− formation, the absorbance at 734 nm was determined every 2–3 min. At a absorbance between 0.6 and 0.7 the reaction was stopped by cooling the ABTS·− solution on ice.

To measure the antioxidant capacity of the samples, 50 μl of the deproteinated sample was mixed with 950 μl of 37 °C ABTS·− solution and pre-incubated for 20 s at 37 °C. Absorbance was then measured at 734 nm and related to the absorbance of trolox to calculate the trolox equivalent of the samples.[20]

Ascorbic acid measurement

Calibrators were prepared freshly, containing the same amount of TCA as the samples. Samples and calibrators were processed identically.[21]

All samples were centrifuged (5000 rpm, 10′ at 4 °C) after which the supernatant was added to a 4.5 M sodium-acetate buffer (pH 6.2), containing an ascorbate oxidase spatula, and incubated for 5 min at 37 °C. Subsequently, the spatula was removed and 0.1% (w:v) OPDA was added. After mixture the samples were incubated for 30 min at 37 °C. Afterwards, the samples were injected in the HPLC system. HPLC analysis of the samples was performed using a Hypersil BDS column (125 mm × 2 mm). The column was eluted isocratically with eluens containing 20% methanol and 80% potassium phosphate buffer using 355 nm as excitation and 425 nm as emission wavelength. The ascorbic acid content of each sample was quantified using the calibrators.[21]

Uric acid measurement

Uric acid was measured in the plasma of all samples as described previously.[22] To this extent an aliquot of

deproteinated plasma was injected in the HPLC system with fluorescent detection. HPLC analysis of the samples was performed using a Hypersil BDS column (125 mm × 4 mm). The column was eluted isocratically with a 5 mM sodium phosphate buffer (pH 3.3) using 250 nm as excitation and 410 nm as emission wavelength. The uric acid content of each sample was quantified using calibrators with known levels of uric acid.

GSH, GSSG and hemoglobin measurement

Both GSH and GSSG calibrators were prepared freshly and contained the same concentrations of SSA as the samples. Samples and calibrators were treated identically.[23]

All samples were centrifuged (5000 rpm, 10′ at 4 °C) after which the supernatant was collected. For the GSH measurement, an aliquot of the supernatant of each sample was added (1:1) to pre-heated 0.6 mM DTNB (37 °C) and incubated for 15 s (37 °C), after which the absorbance of the sample was measured at 412 nm. For the GSSG measurement, an aliquot of the supernatant of each sample was added to 2-vinyl-pyridine (10:1) and incubated for 1 h at room temperature. Subsequently, the supernatant was added (1:9) to a mixture of 0.6 mM DTNB and 0.8 mM NADPH (37 °C) and the reaction was started by adding the enzyme glutathione reductase (1:20). The absorbance of the sample was measured for 3 min at 412 nm. Both the GSH and GSSG content of each sample were quantified using calibrators and related to the hemoglobin content. Hemoglobin content was determined by preparing hemolysates of each sample by adding blood to milliQ (1:1). After diluting these hemolysates 20 times, each sample was added to an equal volume of Drabkins reagens, vortexed thoroughly and incubated 15 min at room temperature. Subsequently, the absorbance of each sample was measured at 540 nm and hemoglobin contents were quantified using appropriate standards.[23]

Inflammatory status

Both basal and LPS-induced levels of the selected cytokines were quantified using PeliKine Compact human ELISA kits (CLB/Sanquin, The Netherlands). Consequently, the LPS-induced cytokine production assay was performed priory.

LPS-induced cytokine production assay

Within 1 h after blood collection, the blood-based cytokine production assay was performed as described previously.[15,24] To this end, blood was aliquoted into 24-well sterile plates and diluted 1:4 with RPMI 1640 (supplementend with L-glutamine). Subsequently, pre-incubation of the diluted blood with quercetin (0, 1, 3 and 30 μM dissolved in 0.5% ethanol) was performed for 30 min. Afterwards, 0.1 ng/ml LPS was added to induce cytokine production. After this stimulation, the plates were incubated for 24 h at 37 °C in 5% CO_2. Cell-free supernatant fluids were then collected by centrifugation (6000 rpm, 10′ at 4 °C) and stored at −20 °C prior to analysis. All incubations were performed in triplicate.

ELISA measurement

All cytokines were quantified using PeliKine Compact human ELISA kits (CLB/Sanquin, The Netherlands) based on appropriate and validated sets of monoclonal antibodies. Assays were performed as described in the manufacturer's instructions. Cytokine production was related to that of the control incubation without quercetin. The ethanol (0.5%) used to dissolve quercetin did not show any influence on the *ex vivo* LPS-induced cytokine production (data not shown).

Statistics

The data of both the patient groups and their matched controls were compared using a Wilcoxon's signed rank test. A two-tailed probability value (*P*-value) equal to or less than 0.05 was considered to be statistically significant.

Results

All antioxidant parameters measured were decreased in the blood from sarcoidosis patients (Fig. 1) compared to the antioxidant-levels of their age-, gender- and dietary behavior matched controls.

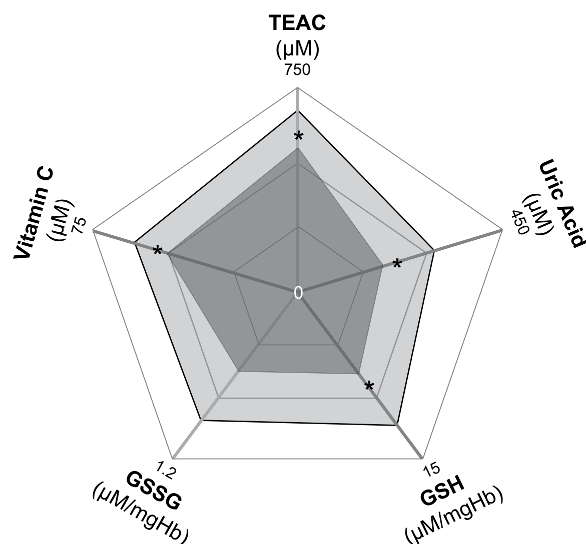

Figure 1 Antioxidant status in sarcoidosis patients ($n = 20$) compared to that in their matched controls ($n = 11$). The values representing the levels of the various parameters in the patient group are connected to form the dark gray area whereas the light gray area reflects the same in the control group. Total plasma antioxidant status (TEAC) is expressed in Trolox equivalent (μM), vitamin C and uric acid in μM and both GSH and GSSG in μM/mg Hb. All axes have a linear scale. The origin is zero. The value at the end of the axes is indicated by the number depicted there. Data are expressed as mean ± SEM; * = $P < 0.03$ vs the matched controls. The TEAC value of sarcoidosis patients is significantly lower than that of their matched controls (535 ± 21 μM vs 652 ± 21 μM). The levels of individual antioxidants, i.e. uric acid, glutathione and vitamin C, are also significantly reduced in the patient group compared to the controls (respectively 185 ± 16 μM vs 324 ± 15 μM; 8.2 ± 0.6 μM vs 13 ± 1.9 μM; 52 ± 4 μM vs 65 ± 4 μM).

Reduced glutathione (GSH) was significantly declined in the blood of sarcoidosis patients compared to the GSH-level in matched controls (25% decline). Glutathione disulphide (GSSG) levels, that are relatively low because of the efficient reduction of GSSG by endogenous glutathione reductase, did not significantly differ between the patient and control group.

The total plasma antioxidant capacity, i.e. the total sum of all plasma antioxidants that is expressed as Trolox equivalent, was in sarcoidosis patients approximately 75% of that of the matched controls.

Two endogenous antioxidants that are known to contribute substantially to the total plasma antioxidant status are uric acid and vitamin C. Uric acid levels as well as vitamin C levels were significantly declined in the plasma of sarcoidosis patients (to respectively 60% and 79% of the control levels) compared to their matched controls.

Basal levels of both TNFα and IL-8 (Fig. 2A), two pro-inflammatory cytokines, were significantly increased in patients suffering from sarcoidosis compared to their controls. Basal levels of the anti-inflammatory cytokine IL-10 were not different between the sarcoidosis patients and the matched controls (Fig. 2A). As a result, the ratios of the pro- vs the anti-inflammatory cytokines TNFα/IL-10 and IL-8/IL-10 were also significantly increased in the sarcoidosis patients.

The LPS-induced TNFα and IL-8 levels (Fig. 2B) found in the sarcoidosis group were significantly higher compared to the controls, whereas IL-10 production was similar in both groups after LPS stimulation (Fig. 2B).

The flavonoid quercetin significantly reduced the TNFα (Fig. 3A) and IL-8 production (Fig. 3B), induced in blood by LPS, in the sarcoidosis group as well as in their matched controls. The extent of this reduction depended on: (i) the quercetin concentration (Fig. 3); and (ii) the cytokine production induced by LPS without quercetin (Fig. 4). The slopes of the fits depicted in Fig. 4 indicate that both cytokines responded more sensitive (50% for IL-8 and 80% for TNFα) to quercetin in the sarcoidosis group compared to the matched controls (Figs. 4A,B). The IL-10 production, induced in blood by LPS, of both patients and controls was not affected by quercetin (Fig. 3C). As a result, the ratios of the pro- vs the ant-inflammatory cytokines TNFα/IL-10 and IL-8/IL-10 were also significantly reduced by the flavonoid.

Antioxidant as well as cytokine levels did not display significant differences between the two sexes. No difference was found between the mild (stage 0–1, $n = 5$) and severe (stage 2–4, $n = 15$) form of sarcoidosis regarding the residual plasma antioxidant capacity or the GSSG, uric acid, vitamin C, basal IL-8 and basal IL-10 levels. Compared to the mild form of sarcoidosis, the severe form showed a trend towards decreased GSH levels ($6.8 \pm 1.1\ \mu M$ ($n = 15$) vs $7.6 \pm 1.3\ \mu M$ ($n = 5$); $P < 0.35$), a decreased total plasma antioxidant capacity ($519 \pm 19\ \mu M$ ($n = 13$) vs $533 \pm 30\ \mu M$ ($n = 5$); $P < 0.25$) and increased TNFα levels (24 ± 3 pg/ml ($n = 15$) vs 19 ± 3 pg/ml ($n = 5$); $P < 0.28$). Stage 4 sarcoidosis patients displayed a significant lower total plasma antioxidant capacity compared to the other sarcoidosis patients ($473 \pm 15\ \mu M$ vs $543 \pm 23\ \mu M$; $P \leq 0.05$).

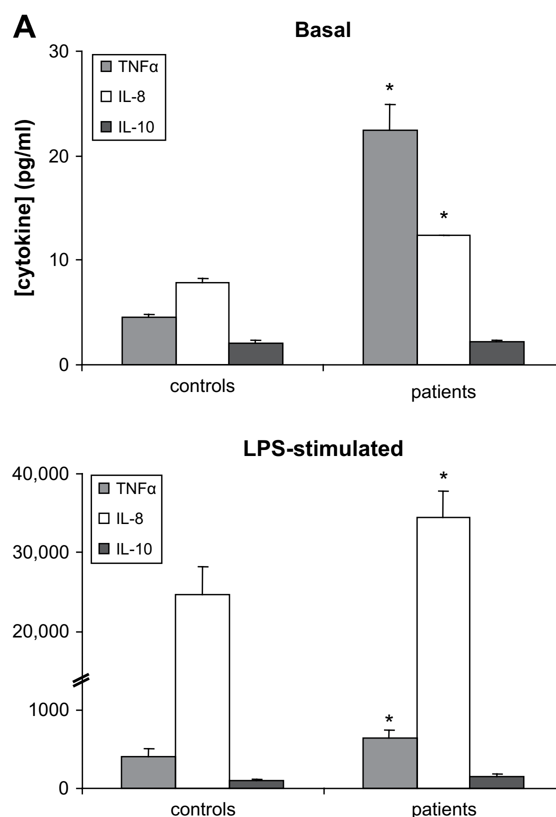

Figure 2 Basal (panel A) as well as LPS-induced levels (panel B) of TNFα, IL-8 and IL-10 in sarcoidosis patients ($n = 20$) compared to their matched controls ($n = 11$). Basal TNFα and IL-8 levels are both significantly higher in the patient group compared to their matched controls (TNFα: 22 ± 2 pg/ml vs 5 ± 0.3 pg/ml; IL-8: 12.3 ± 1.7 pg/ml vs 7.9 ± 0.4 pg/ml). Basal IL-10 levels are unaltered in the patients compared to their matched controls (2.2 ± 0.2 pg/ml vs 2.1 ± 0.2 pg/ml). LPS-induced TNFα and IL-8 levels are both significantly higher in the patients than in the controls. LPS-induced IL-8 levels are approximately eight times higher than the LPS-induced TNFα and IL-10 levels. Data are expressed as mean $\pm$ SEM; $^*P \leq 0.01$ vs the matched controls for panel A; $^*P < 0.04$ vs the matched controls for panel B.

Discussion

The role of enhanced ROS production has been implicated in the pathology of sarcoidosis,[4,5] but the influence of this increased ROS formation on the endogenous antioxidant levels in sarcoidosis have not yet been reported. To the best of our knowledge, this study is the first that demonstrates a significant decrease in the total plasma antioxidant capacity as well as in the blood levels of the important endogenous antioxidants glutathione, vitamin C and uric acid in sarcoidosis patients compared to healthy controls matched for age, gender and dietary behavior. The severe form of sarcoidosis displays a trend towards a lower total plasma antioxidant capacity as well as a lower GSH level, indicating that the severity of the disease might be related to the level of oxidative stress occurring.

The patients and controls were matched for their dietary behavior, indicating that the observed decreased

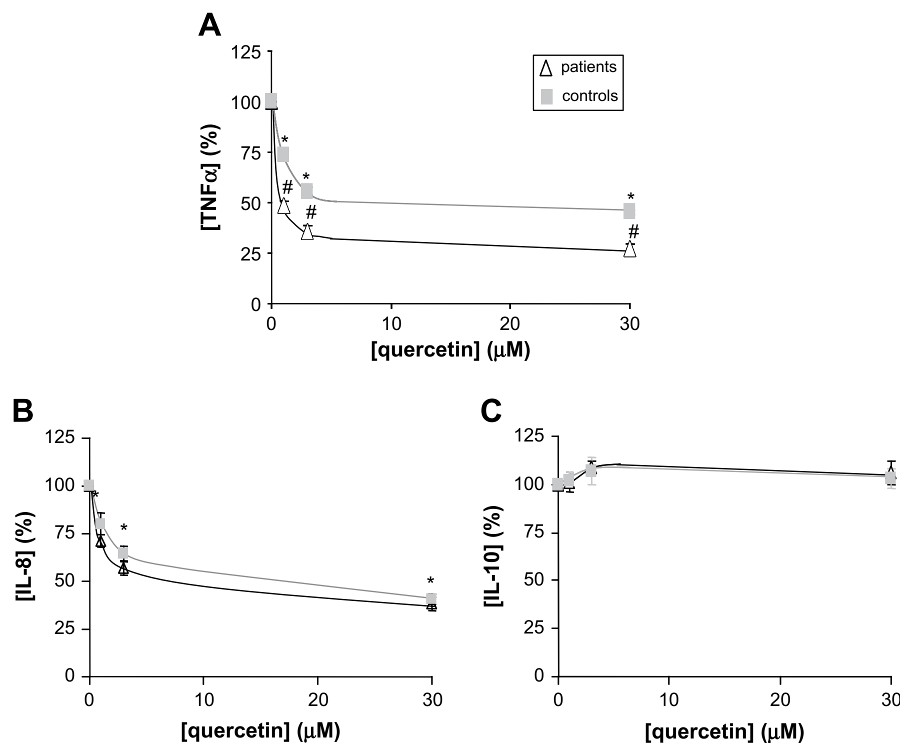

Figure 3 Modulating effect of quercetin on the LPS-induced TNFα, IL-8 and IL-10 production in sarcoidosis ($n = 20$) (panel A, B and C respectively) compared to their matched controls ($n = 11$). In both patients and controls quercetin significantly reduces the LPS-induced TNFα and IL-8 production in a concentration-dependent manner. This effect is more pronounced in the sarcoidosis patients. Results are expressed in percentage, with 100% representing the cytokine-release under stimulation of LPS in the absence of quercetin. Data are expressed as mean ± SEM; * = $P < 0.01$ vs the incubation containing no quercetin; # = $P < 0.05$ vs controls and vs the incubation containing no quercetin.

antioxidant levels are not the result of a different dietary intake. The lower endogenous antioxidant defense found, therefore, confirms the elevated production of ROS present in sarcoidosis. Low endogenous antioxidant levels combined with enhanced ROS formation is defined as oxidative stress. Oxidative stress can cause serious oxidative damage to biological macromolecules like DNA, lipids and proteins.[25]

Several studies have shown that the levels of biomarkers of oxidative damage, i.e. exhaled ethane[4] and both 8-iso-prostane[26] and oxidized proteins[27] in the bronchoalveolar lavage fluid (BALF), are indeed increased in sarcoidosis patients of different clinical stages. Furthermore, the transcription factor NF-κB, of which it is known that it is activated by radical damage, is increased in alveolar macrophages[28] and mononuclear blood cells[29] of active sarcoidosis patients compared to those of healthy controls.

To strengthen the endogenous antioxidant defense and thus offer more protection against ROS, antioxidant supplementation seems a logical strategy in the treatment of sarcoidosis. Moreover, antioxidant supplementation might not only protect against ROS-mediated damage, it might also mitigate elevated inflammation since ROS can enhance inflammation. ROS are capable of promoting inflammation in the lung by activating transcription factors like NF-κB and activator protein-1 that induce pro-inflammatory cytokines and chemokines.[7,8] *In vitro* studies, using both macrophages and alveolar and bronchial epithelial cells, have demonstrated that oxidants can indeed initiate the production of inflammatory mediators like IL-8 and NO·.[30] Because of this relation between ROS and inflammatory processes, the inflammatory status was also evaluated in sarcoidosis patients. To this end, basal levels of the pro-inflammatory cytokines TNFα and IL-8 and the anti-inflammatory cytokine IL-10 were measured.

TNFα was chosen since it is suggested to be the most prominent cytokine present in sarcoidosis.[2,31] TNFα has been shown to play a pivotal role in the granuloma formation occurring in sarcoidosis.[2] Furthermore, the individual capability of a patient to release TNFα is suggested to be linked to the progression of the disease, thereby linking this cytokine to the pathogenesis of sarcoidosis.[32] TNFα is capable of activating transcription factors NF-κB and AP-1 that subsequently will further enhance other inflammatory mediators such as IL-8.[8,33] and TNFα itself, thereby amplifying the TNFα-mediated inflammatory effects.

Our study shows that basal TNFα-levels are significantly enhanced in sarcoidosis patients compared to healthy controls. This finding is in line with previous studies.[34,35] The severe form of sarcoidosis tends to display higher TNFα levels, indicating that the severity of the disease might be related to the level of inflammation occurring. It has been reported that conventional therapy with glucocorticoids fails to be completely efficacious in all sarcoidosis patients and that this therapy, for instance, does not reduce the enhanced NF-κB activity found in sarcoidosis patients.[29] Recently, alternative strategies using anti-TNFα agents

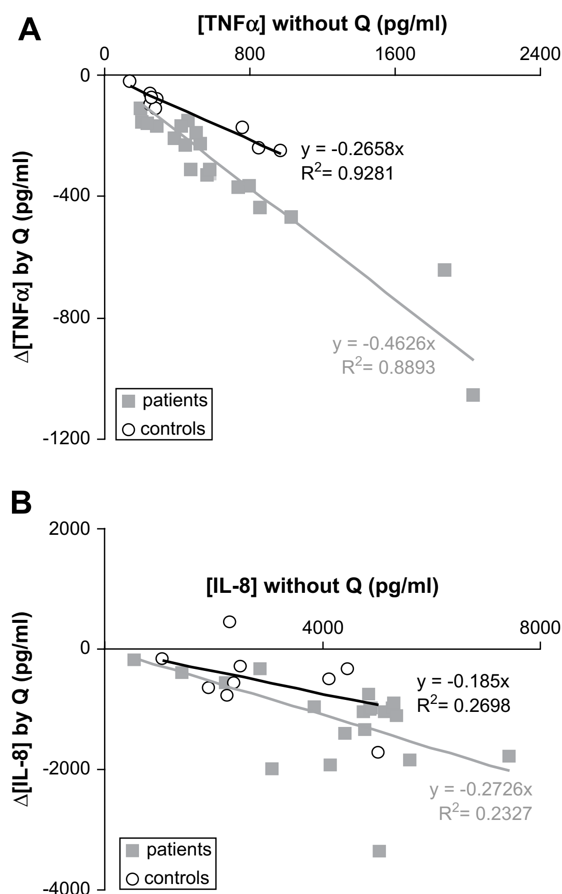

Figure 4 The effect of quercetin (1 μM) on LPS-induced TNFα (panel A) and IL-8 production (panel B) in both sarcoidosis patients ($n = 20$) and their matched controls ($n = 11$). The decrease in LPS-induced cytokine production caused by quercetin (Q) (y-axis) is plotted against the LPS-induced cytokine production in the absence of the flavonoid (x-axis). The slopes of panel A indicate that on average, 1 μM quercetin reduces the TNFα production in controls with 27% and in patients with 46%. For IL-8, this reduction is on average 19% in controls and 27% in patients (panel B). Data are expressed as individual values ($n = 20$ for the patients; $n = 11$ for the controls).

have been reported to have some clinical beneficial effects.[36] For example, Baughman et al have recently shown not only that anti-TNFα therapy significantly improves the predicted FVC of sarcoidosis patients after 24 weeks, but also that patients with more severe disease tend to benefit more from this treatment.[37]

The finding that basal IL-8 levels are significantly increased in sarcoidosis indicates that this cytokine is also involved in the enhanced inflammatory processes present in this disease. This is again in line with previous studies, which show an increased expression of IL-8 in sarcoidosis.[2,38,39] It should be noted that IL-8 can also be induced by TNFα and that the higher IL-8 level might reflect the higher TNFα level.

No changes were found in the basal levels of anti-inflammatory cytokine IL-10 between the sarcoidosis and control group. The results of previous studies on this cytokine are conflicting. Some studies report enhanced IL-10 levels in the broncho-alveolar lavage fluid (BALF) of sarcoidosis patients as a compensatory mechanism,[38,39] whereas other studies fail to demonstrate this increase.[40,41]

To determine the anti-inflammatory potential of antioxidants, the effect of quercetin on LPS-induced production of TNFα, IL-8 and IL-10 was quantified in blood. The employed model, unlike models using isolated cells or cultured cells grown in medium, represents a more physiological and well reproducible model to measure cytokine production *ex vivo*.[15,42] The natural cell-to-cell interactions are preserved and all blood components are present in *in vivo* ratios with non-cellular components, resulting in a system that reflects the *in vivo* condition well.[15,42]

In both the sarcoidosis and the control group, the antioxidant quercetin significantly reduces the LPS-induced TNFα and IL-8 production, whereas the LPS-induced IL-10 production is not significantly altered by the flavonoid. The inhibitory effect of quercetin on both anti-inflammatory cytokines is dose-dependent and could already be achieved at an *in vivo* attainable concentration of 1 μM.[43] Interestingly, the reduction in cytokine levels by quercetin is more pronounced in sarcoidosis patients compared with their matched controls. This finding is in agreement with our previous study which showed that quercetin did not display major anti-inflammatory effects *in vivo* or *ex vivo* in the blood of healthy volunteers.[24]

The anti-inflammatory actions of quercetin observed in the present study could very well be related to its antioxidative activity. This association between the antioxidative and anti-inflammatory capabilities of flavonoids has also been suggested in several *in vitro*[44,45] and *in vivo* studies.[46,47] The reduced antioxidant status may also be involved in the differences observed between the sarcoidosis patients and their matched controls regarding both: (i) the significantly higher TNFα and IL-8 production induced by LPS; and (ii) the more pronounced effect of quercetin herein. Therefore, sarcoidosis patients may benefit from the use of antioxidant supplementation. The fact that quercetin is a dietary antioxidant indicates that the diet is an important factor in sarcoidosis. However, the toxicity of quercetin should be carefully evaluated especially when it is chronically administered.[48,49] Clinical studies are needed to substantiate the efficacy as well as the safety of quercetin supplementation.

In conclusion, our study shows for the first time that the endogenous antioxidant levels as well as the total plasma antioxidant capacity are significantly reduced in sarcoidosis, emphasizing that severe oxidative stress underlies the pathology of this disease. Moreover, inflammation is enhanced in sarcoidosis as shown by the increased basal levels of the pro-inflammatory cytokines TNFα and IL-8. The antioxidant quercetin shows *ex vivo* significant anti-inflammatory effects in both sarcoidosis patients and controls that are, interestingly, more pronounced in the first group. Probably, this larger anti-inflammatory effect of the flavonoid in sarcoidosis is associated with the compromised antioxidant status present in these patients. This suggests that particularly in sarcoidosis patients empowering the antioxidant defense system with alimentary antioxidants, such as quercetin, might be fruitful. This is especially important since it has been reported that conventional treatment with glucocorticoids is not efficacious in all sarcoidosis patients.

Conflict of interest statement

None of the authors have a conflict of interest to declare in relation to this work.

Acknowledgements

The authors wish to thank Mrs. G. Janssen-van Kan en Mrs. E. ten Hoor for their kind assistance during the recruitment and sampling and Mr. M.A.J.G. Fischer for his kind assistance during the measurements of the various parameters.

The Dutch Organization for Health Research and Development has financially funded this research (project no. 014-12-012).

References

1. Thomeer M, Demedts M, Wuyts W. Epidemiology of sarcoidosis. *Eur Respir Mon* 2005;**32**:1—10.
2. Baughman RP. Lower EE, du Bois RM. *Sarcoidosis. Lancet* 2003; **361**:1111—8.
3. Newman LS, Rose CS, Maier LA. Sarcoidosis. *N Engl J Med* 1997; **336**:1224—34.
4. Kanoh S, Kobayashi H, Motoyoshi K. Exhaled ethane: an in vivo biomarker of lipid peroxidation in interstitial lung diseases. *Chest* 2005;**128**:2387—92.
5. Halliwell B, Gutteridge JM, Cross CE. Free radicals, antioxidants, and human disease: where are we now? *J Lab Clin Med* 1992;**119**:598—620.
6. Uzun H, Yanardag H, Gelisgen R, et al. Levels of paraoxonase, an index of antioxidant defense, in patients with active sarcoidosis. *Curr Med Res Opin* 2008;**24**:1651—7.
7. MacNee W. Oxidative stress and lung inflammation in airways disease. *Eur J Pharmacol* 2001;**429**:195—207.
8. Rahman I. Oxidative stress, transcription factors and chromatin remodelling in lung inflammation. *Biochem Pharmacol* 2002; **64**:935—42.
9. Papakosta D, Kyriazis G, Gioulekas D, et al. Variations in alveolar cell populations, lymphocyte subsets and NK-cells in different stages of active pulmonary sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 2005;**22**:21—6.
10. Katchar K, Soderstrom K, Wahlstrom J, Eklund A, Grunewald J. Characterisation of natural killer cells and CD56+T-cells in sarcoidosis patients. *Eur Respir J* 2005;**26**:77—85.
11. Baughman RP, Lower EE. Therapy for sarcoidosis. In: Wouters EFM, editor. *Sarcoidosis*. Wakefield: The Charlesworth Group; 2005.
12. Raghu G, Brown KK, Bradford WZ, et al. A placebo-controlled trial of interferon gamma-1b in patients with idiopathic pulmonary fibrosis. *N Engl J Med* 2004;**350**:125—33.
13. Boots AW, Kubben N, Haenen GR, Bast A. Oxidized quercetin reacts with thiols rather than with ascorbate: implication for quercetin supplementation. *Biochem Biophys Res Commun* 2003;**308**:560—5.
14. Thorn J. The inflammatory response in humans after inhalation of bacterial endotoxin: a review. *Inflamm Res* 2001;**50**: 254—61.
15. Swennen EL, Bast A, Dagnelie PC. Immunoregulatory effects of adenosine 5′-triphosphate on cytokine release from stimulated whole blood. *Eur J Immunol* 2005;**35**:852—8.
16. Fischer KD, Tedford K, Wirth T. New roles for Bcl10 in B-cell development and LPS response. *Trends Immunol* 2004;**25**: 113—6.
17. De Groote D, Zangerle PF, Gevaert Y, et al. Direct stimulation of cytokines (IL-1 beta, TNF-alpha, IL-6, IL-2, IFN-gamma and GM-CSF) in whole blood. I. Comparison with isolated PBMC stimulation. *Cytokine* 1992;**4**:239—48.
18. Drent M, Nierop MAMF, van Gerritsen FA, Wouters EFM, Mulder PGH. Computer program using BALF analysis results as diagnostic tool in interstitial lung diseases. *Am J Respir Crit Care Med* 1996;**153**:736—41.
19. Quanjer PH, Tammeling GJ, Cotes JE, Pedersom OF, Peslin R, Yernaut JC. Lung volumes and forced ventilatory flows. Report working party. Standardization of lung functions tests. European Commission for Steel and Coal. Official statement of the European Respiratory Society. *Eur Respir J* 1993;**16**:5—40.
20. Fischer MA, Gransier TJ, Beckers LM, Bekers O, Bast A, Haenen GR. Determination of the antioxidant capacity in blood. *Clin Chem Lab Med* 2005;**43**:735—40.
21. Speek AJ, Schrijver J, Schreurs WHP. Fluoremetric determination of total vitamin C in whole blood by high performance liquid chromatography with pre-column derivatization. *J Chrom Biomed Appl* 1984;**305**:53—60.
22. Benzie IF, Chung W, Tomlinson B. Simultaneous measurement of allantoin and urate in plasma: analytical evaluation and potential clinical application in oxidant:antioxidant balance studies. *Clin Chem* 1999;**45**:901—4.
23. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem* 1969;**27**:502—22.
24. Boots AW, Wilms LC, Swennen EL, Kleinjans JC, Bast A, Haenen GR. In vitro and ex vivo anti-inflammatory activity of quercetin in healthy volunteers. *Nutrition* 2008;**24**:703—10.
25. Boots AW, Haenen GR, den Hartog GJ, Bast A. Oxidative damage shifts from lipid peroxidation to thiol arylation by catechol-containing antioxidants. *Biochim Biophys Acta* 2002; **1583**:279—84.
26. Montuschi P, Ciabattoni G, Paredi P, et al. 8-Isoprostane as a biomarker of oxidative stress in interstitial lung diseases. *Am J Respir Crit Care Med* 1998;**158**:1524—7.
27. Lenz AG, Costabel U, Maier KL. Oxidized BAL fluid proteins in patients with interstitial lung diseases. *Eur Respir J* 1996;**9**: 307—12.
28. Culver DA, Barna BP, Raychaudhuri B, et al. Peroxisome proliferator-activated receptor gamma activity is deficient in alveolar macrophages in pulmonary sarcoidosis. *Am J Respir Cell Mol Biol* 2004;**30**:1—5.
29. Drent M, van den Berg R, Haenen GR, van den Berg H, Wouters EF, Bast A. NF-kappaB activation in sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 2001;**18**:50—6.
30. Antonicelli F, Parmentier M, Hirani N, et al. LPS stimulation of IL-8 release is inhibited by thiol antioxidant at the transcriptional level in THP-1 macrophage cells. *Am J Respir Crit Care Med* 2000;**161**:A738.
31. Moller DR. Cells and cytokines involved in the pathogenesis of sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 1999;**16**:24—31.
32. Seitzer U, Swider C, Stuber F, et al. Tumour necrosis factor alpha promoter gene polymorphism in sarcoidosis. *Cytokine* 1997;**9**:787—90.
33. Rahman I, MacNee W. Role of transcription factors in inflammatory lung diseases. *Thorax* 1998;**53**:601—12.
34. Baughman RP, Strohofer SA, Buchsbaum J, Lower EE. Release of tumor necrosis factor by alveolar macrophages of patients with sarcoidosis. *J Lab Clin Med* 1990;**115**:36—42.
35. Ziegenhagen MW, Schrum S, Zissel G, Zipfel PF, Schlaak M, Muller-Quernheim J. Increased expression of proinflammatory chemokines in bronchoalveolar lavage cells of patients with progressing idiopathic pulmonary fibrosis and sarcoidosis. *J Investig Med* 1998;**46**:223—31.
36. Yee AM, Pochapin MB. Treatment of complicated sarcoidosis with infliximab anti-tumor necrosis factor-alpha therapy. *Ann Intern Med* 2001;**135**:27—31.

37. Baughman RP, Drent M, Kavuru M, et al. Infliximab therapy in patients with chronic sarcoidosis and pulmonary involvement. *Am J Respir Crit Care Med* 2006;**174**:795—802.
38. Meloni F, Caporali R, Marone Bianco A, et al. BAL cytokine profile in different interstitial lung diseases: a focus on systemic sclerosis. *Sarcoidosis Vasc Diffuse Lung Dis* 2004;**21**: 111—8.
39. Rutherford RM, Staedtler F, Kehren J, et al. Functional genomics and prognosis in sarcoidosis—the critical role of antigen presentation. *Sarcoidosis Vasc Diffuse Lung Dis* 2004; **21**:10—8.
40. Hauber HP, Gholami D, Meyer A, Pforte A. Increased interleukin-13 expression in patients with sarcoidosis. *Thorax* 2003; **58**:519—24.
41. Martinez JA, King Jr TE, Brown K, et al. Increased expression of the interleukin-10 gene by alveolar macrophages in interstitial lung disease. *Am J Physiol* 1997;**273**:L676—83.
42. Yaqoob P, Newsholme EA, Calder PC. Comparison of cytokine production in cultures of whole human blood and purified mononuclear cells. *Cytokine* 1999;**11**:600—5.
43. Conquer JA, Maiani G, Azzini E, Raguzzini A, Holub BJ. Supplementation with quercetin markedly increases plasma quercetin concentration without effect on selected risk factors for heart disease in healthy subjects. *J Nutr* 1998;**128**: 593—7.
44. Zern TL, Wood RJ, Greene C, et al. Grape polyphenols exert a cardioprotective effect in pre- and postmenopausal women by lowering plasma lipids and reducing oxidative stress. *J Nutr* 2005;**135**:1911—7.
45. Kostyuk VA, Potapovich AI, Vladykovskaya EN, Korkina LG, Afanas'ev IB. Influence of metal ions on flavonoid protection against asbestos-induced cell injury. *Arch Biochem Biophys* 2001;**385**:129—37.
46. Donnelly LE, Newton R, Kennedy GE, et al. Anti-inflammatory effects of resveratrol in lung epithelial cells: molecular mechanisms. *Am J Physiol Lung Cell Mol Physiol* 2004;**287**:L774—83.
47. Comalada M, Camuesco D, Sierra S, et al. In vivo quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF-kappaB pathway. *Eur J Immunol* 2005;**35**:584—92.
48. Boots AW, Balk JM, Bast A, Haenen GR. The reversibility of the glutathionyl-quercetin adduct spreads oxidized quercetin-induced toxicity. *Biochem Biophys Res Commun* 2005;**338**: 923—9.
49. Boots AW, Li H, Schins RP, et al. The quercetin paradox. *Toxicol Appl Pharmacol* 2007;**222**:89—96.