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The anti-inflammatory activity of
quercetin

AW Boots, LC Wilms, ELR Swennen, JCS Kleinjans, A Bast,
GRMM Haenen

Submitted

Abstract

Background

Quercetin, the most commonly occurring flavonoid, is an excellent antioxidant that is also suggested to possess other beneficial activities. The present study has been designed to investigate the possible anti-inflammatory effects of physiologically attainable quercetin concentrations.

Methods

Quercetin was added to blood in the test tube, or blood taken after four weeks quercetin supplementation. Primary pro-inflammatory cytokine measured in this study was tumour necrosis factor alpha ($\text{TNF}\alpha$), of which the production has been evoked using lipopolysaccharide (LPS).

Results

It was found that quercetin dose-dependently inhibited *in vitro* LPS-induced $\text{TNF}\alpha$ production in the blood of healthy volunteers. A concentration of 1 μM of the flavonoid already reduced the cytokine production to 77%. The *in vitro* LPS-induced IL-10 production remained unaffected by quercetin. Four weeks quercetin supplementation resulted in a significant increase in plasma quercetin concentration. The supplementation also increased the total plasma antioxidant status, but did not affect the glutathione, vitamin C and uric acid plasma concentrations. Basal as well as *ex vivo* LPS-induced $\text{TNF}\alpha$ levels were also not altered by the supplementation.

Conclusions

The present study shows that quercetin increases the antioxidant capacity *in vivo* and displays anti-inflammatory effects *in vitro*, but not *in vivo* or *ex vivo*, in the blood of healthy volunteers. The lack of effect in the healthy volunteers is probably due to their low cytokine and high antioxidant levels at baseline, indicating that neither inflammation nor oxidative stress is present. Only in people with increased levels of inflammation and oxidative stress, e.g. patients suffering from a disease of which the pathology is associated with these two processes, antioxidant supplementation is expected to be fruitful.

Introduction

Flavonoids are a class of naturally occurring polyphenolic compounds that are ubiquitously present in fruits, vegetables, nuts, plant-derived beverages such as tea and wine as well as in some traditional herbal-containing medicines (1). The total amount of flavonoids consumed in the Netherlands is estimated at several hundreds of mg per day (2). The Dutch intake of flavones and flavonols, two important subgroups of the flavonoids, is determined as 23-24 mg per day and 70% of this amount is quercetin (3). Higher estimates, reporting an average daily flavonoid intake of 1 gram including about 50 mg of quercetin, have been made in other Western countries (4).

Much attention has been given to the potential health-promoting properties of flavonoids in general and of quercetin in particular. Several epidemiological studies have reported an inverse relation between flavonoid intake and both the risk for cardiovascular diseases and the incidence of lung and colorectal cancer (5). These beneficial effects have been attributed to the anti-oxidative capacities of flavonoids which have already been determined both *in vitro* (6,7) and *in vivo* (8,9). However, these findings are not conclusive since various other studies have failed to demonstrate such health-promoting effects of flavonoids (5).

Recently, some studies have also reported that, *in vitro*, quercetin can inhibit various cytokines, including tumour necrosis factor α (TNF α) (10,11). Extrapolation of these findings to a physiological relevant effect of *in vivo* quercetin supplementation is difficult, since most studies have been performed in immortalized cancer cell lines with relatively high concentrations of the flavonoid. This prompted us to investigate the anti-inflammatory effects of physiologically attainable quercetin concentrations in whole blood from healthy subjects, a model more closely resembling the *in vivo* situation. The effects of quercetin are tested both *in vitro*, i.e. added to blood in the test tube, and *ex vivo*, i.e. in blood taken after the administration of quercetin in a supplementation study. Moreover, the direct *in vivo* effect of the quercetin supplementation on basal cytokine levels is also assessed. The primary cytokine measured in this study is TNF α since this cytokine is an important mediator of inflammation and reported to be elevated in various chronic diseases such as sarcoidosis and idiopathic pulmonary fibrosis (12). Lipopolysaccharide (LPS), a patho-physiological relevant stimulator of monocytes, neutrophils and B lymphocytes, is used to evoke TNF α production *ex vivo* (13-16).

Materials and Methods

Chemicals

Quercetin, reduced glutathione (GSH), oxidized glutathione (GSSG), GSSG reductase, sulfosalicylic acid (SSA), 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB), O-phenyleendiamin (OPDA), ascorbic acid 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 (Paisley, UK). Ascorbate oxidase spatula were purchased by Roche Diagnostics (Basel, Switzerland). Human TNF α (7300 pg/ml) and human IL-10 (4000 pg/ml) were acquired from CLB/Sanquin (Amsterdam, The Netherlands). All other chemicals were of analytical grade. The quercetin-rich blueberry-apple juice mixture was produced specifically for this study by Riedel Drinks (Riedel, Ede, The Netherlands).

Methods

Participants

The *in vitro* experiments, of which the data are shown in Figure 7.1 and 7.2, are performed with the blood of 3 healthy volunteers (2 male, 1 female) between the age of 27 and 45 years.

All other experiments are performed with the blood of 7 healthy volunteers (3 male, 4 female) between the age of 20 and 40 years. In a comparable study, it has been shown that for a biomarker of oxidative stress, i.e. the total plasma antioxidant capacity (TEAC), a 10% difference between treatments ($\alpha=0.05$; two-sided and a power of 80%) can be demonstrated with such a small number of volunteers (17).

All healthy volunteers were recruited through advertisements in local newspapers. Volunteers were included if they were non-smoking and did not use any medication or vitamin supplementation at the moment of the intervention. All participants filled in a questionnaire regarding their dietary habits and self-experienced health status and were, based on that information, considered healthy. No large differences in dietary habits were found. Mean quercetin intake of all volunteers was approximately 15 mg per day.

The Medical Ethical Committee of Maastricht University and the Academic Hospital Maastricht had approved the protocol before the beginning of the study. All participants were fully informed about the aim and details of the study and have given their written informed consent.

Supplementation study

Prior to the actual supplementation period, volunteers were subjected to a five-day during “washout”-period. During this period they were not allowed to consume food ingredients rich in flavonoids in general and in quercetin in particular. These food ingredients included onions, apples, red wine, tea, biological and freshly pressed fruit juices, berries (e.g. blueberries and elderberries), grapes, cherries, raisins, parsley, broccoli, cabbage, beans and tomatoes (8). Subjects also had to minimise the use of spices and herbs during this period.

The paired design of this supplementation study comprises that each subject acts as his or her own control. Based on the results from an earlier pilot study (8), it was concluded that best results would be obtained after 4 weeks of supplementation. The five-day flavonoid washout period was therefore followed by a four-week supplementation period with a large increase in quercetin intake, established by consuming a blueberry-apple juice mixture. This juice contains about 97 mg of quercetin per litre, most of which is bound to a glucoside or a galactoside at the 3-position, making it well biologically available (18). After the washout-period as well as after the supplementation study venous blood samples were drawn into EDTA-containing vacutainer tubes (Vacutainer, Becton-Dickinson) and kept on ice prior to processing which occurred within 1 hour after blood collection.

The design of this supplementation as well as the efficacy of the washout period is based on a pilot study, described earlier (8).

Preparation of the blood samples

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 aliquot 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.

Determination of total plasma quercetin concentration

Total quercetin concentration was analysed in plasma by means of high performance liquid chromatography (HPLC) with coulometric array-detection after enzymatic hydrolysis as described previously (19).

Trolox 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). The relative contribution of uric acid, vitamin C and quercetin to the total TEAC value is calculated using the TEAC value described for each individual antioxidant, i.e. 1, 1 and 6.24 respectively (21).

Ascorbic acid measurement

Ascorbic acid has been included in the present study since it is known to be an important contributor to the TEAC. Calibrators were prepared freshly, containing the same amount of TCA as the samples. Samples and calibrators were processed identically as described previously (22).

Uric acid measurement

Uric acid has been included in the present study since it is known to be an important contributor to the TEAC. Uric acid was measured in the plasma of all samples as described previously (23).

GSH, GSSG and haemoglobin 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 and GSH and GSSG levels measured were related to the haemoglobin content as described previously (24).

Blood-based cytokine production assay

Within one hour after blood collection, the blood-based cytokine production assay was performed as described previously (14). Care was taken that handling of the blood prior to LPS-stimulation did not influence the cytokine release.

The advantage of the current *ex vivo* blood assay is that this model, unlike models using isolated cells or cell lines grown in medium, represents a more physiological and well reproducible model to measure cytokine production *ex vivo*. 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 (14).

Enzyme linked immune sorbent assay (ELISA) measurement

Both TNF α and IL-10 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 *in vitro* data as well as the before versus the after data were compared using the Wilcoxon's signed rank test. A one-tailed probability value (P-value) of less than 0.05 was considered to be statistically significant.

Results

To determine the possible anti-inflammatory effects of the antioxidant quercetin, inflammation has been evoked *ex vivo*, using LPS, in the blood of healthy volunteers. To optimize this assay, the TNF α -inducing ability of various concentrations of LPS, as well as the effect of 30 μ M quercetin on this production, have been measured. Figure 7.1 shows that LPS, starting at the very low concentration of 0.005 ng/ml, dose-dependently induced TNF α -production. Quercetin (30 μ M) inhibited this LPS-induced TNF α -production, but the percentage of inhibition caused by the flavonoid depended on the amount of TNF α present. The most pronounced inhibitory effect of quercetin (61 \pm 4%) was found when 0.1 ng/ml LPS was used to induce TNF α -production. This relatively low LPS-concentration has been used to further evaluate the anti-inflammatory effect of quercetin.

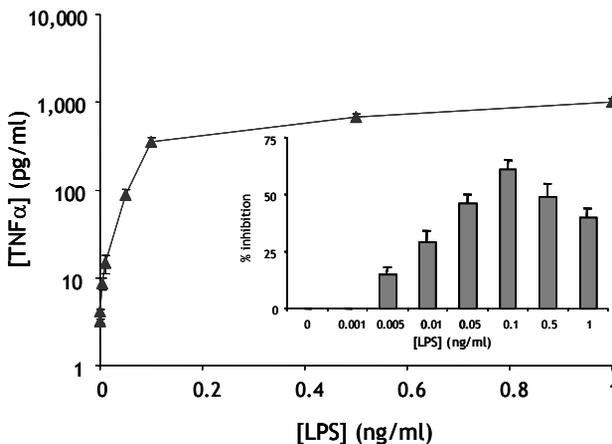


Figure 7.1 LPS-induced TNF α production *in vitro* after 24 hours, without or with (insert) 30 μ M quercetin (30 min pre-incubation) in the blood of healthy volunteers. In the insert, results are expressed as % inhibition of the LPS-induced TNF α release. Data are expressed as mean \pm SEM (n=3).

Quercetin dose-dependently inhibited the LPS-induced $\text{TNF}\alpha$ -production as is depicted in Figure 7.2.

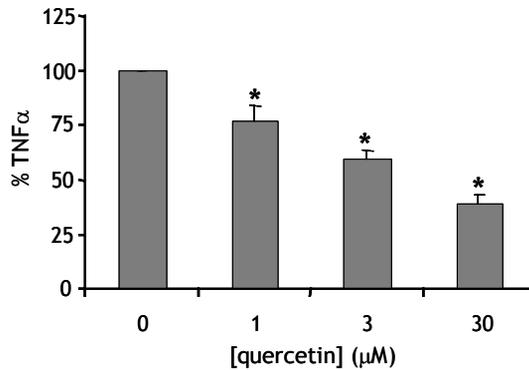


Figure 7.2 The inhibitory effect of quercetin on *ex vivo* LPS-induced $\text{TNF}\alpha$ production in the blood of healthy volunteers. Blood was incubated with increasing quercetin concentrations for 30 minutes and subsequently stimulated with 0.1 ng/ml LPS for 24 hours. Results are expressed in percentage with 100% representing $\text{TNF}\alpha$ under stimulation by LPS in the absence of quercetin. Data are expressed as mean \pm SEM (n=7). * = $P < 0.01$ compared to the control incubation without quercetin

At a concentration of 1 μM , the flavonoid already reduced the cytokine production to $77 \pm 7\%$. The LPS-induced IL-10 production remained unaffected by the flavonoid. As a result, quercetin was also capable of significantly reducing the ratio of pro- versus anti-inflammatory marker $\text{TNF}\alpha/\text{IL-10}$, a frequently used diagnostic parameter of inflammation. It was found that this *in vitro* effect of quercetin greatly depends on the level of $\text{TNF}\alpha$ induced by LPS (Figure 7.3).

Four weeks quercetin supplementation resulted in a significant increase in both the plasma quercetin concentration and the total plasma antioxidant capacity, i.e. the total sum of all plasma antioxidants that is expressed as Trolox equivalent, in the blood of the healthy volunteers (Figure 7.4). The glutathione (GSH) levels and basal $\text{TNF}\alpha$ levels were unaffected by this quercetin supplementation (Figure 7.5). No significant changes were found between the uric acid and vitamin C levels before and after the supplementation (data not shown).

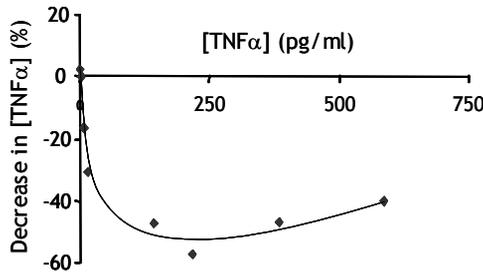


Figure 7.3 The relation between the amount of $TNF\alpha$ present and the effect of quercetin on these $TNF\alpha$ levels *in vitro*. Blood was pre-treated with $30\ \mu M$ quercetin prior to 24-hours incubation with various concentrations of LPS ($37^{\circ}C$, $5\% CO_2$). Afterwards, the $TNF\alpha$ released into the supernatants was analyzed by ELISA and expressed on the x-axis. The decrease in $TNF\alpha$ production caused by quercetin is expressed, as percentage, on the y-axis. Data are expressed as mean \pm SEM ($n=3$).

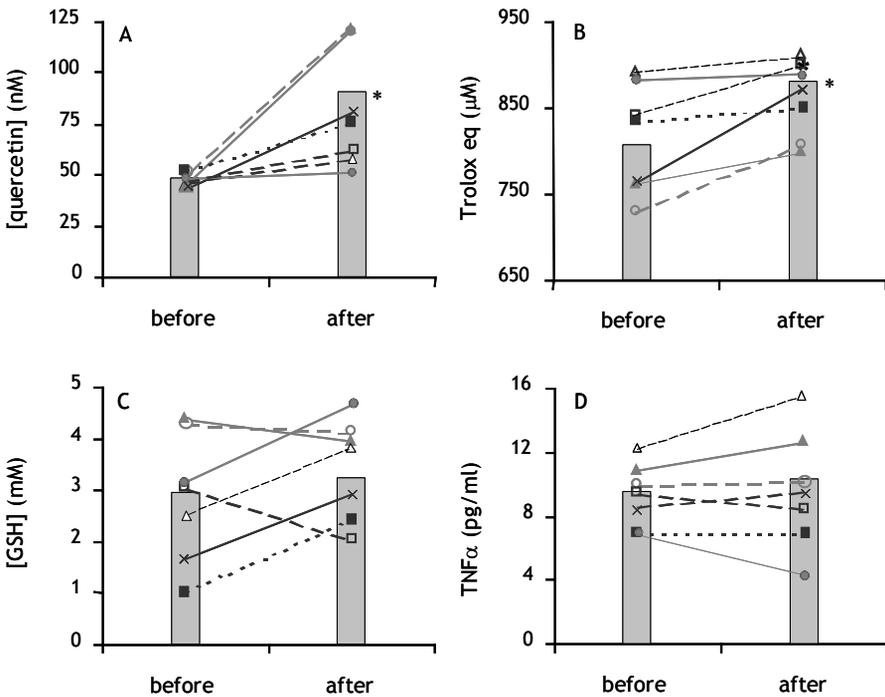


Figure 7.4. The effect of four weeks quercetin-supplementation in healthy volunteers regarding the plasma quercetin concentration (panel A), the total plasma antioxidant capacity (expressed as Trolox equivalents, panel B), the GSH concentration (panel C) and the basal $TNF\alpha$ level (panel D). Blood was drawn before and after the quercetin-intervention and all data are individually expressed ($n=7$); the light grey bars represent the mean.
 *= $P < 0.05$ compared to the “before” measurement prior to the intervention

The relative contribution of endogenous antioxidants uric acid and vitamin C, as well as that of the exogenous quercetin, to the total plasma antioxidant status is depicted in Figure 7.5. This reveals that the relative contribution of quercetin to the total plasma antioxidant capacity was rather small (0.04% before vs 0.06% after). A major part of this capacity was due to plasma anti-oxidants other than uric acid and vitamin C, such as low molecular protein thiols (Figure 7.5). This residual plasma antioxidant capacity was significantly increased after the four weeks of quercetin supplementation.

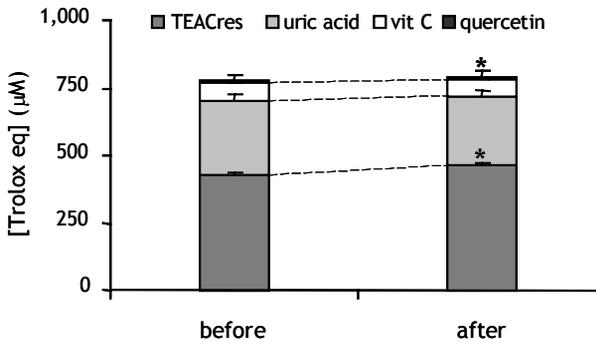


Figure 7.5 Total antioxidant capacity (expressed as Trolox equivalents) and the relative contribution of uric acid, vitamin C and quercetin in healthy volunteers before and after four weeks quercetin intervention. Subtraction of these three contributions results in a residual plasma antioxidant capacity (TEACres) that is significantly higher after the intervention compared to the control values before the intervention ($563 \pm 14 \mu\text{M}$ vs $590 \pm 19 \mu\text{M}$). Data are expressed as mean \pm SEM ($n=7$).
* = $P < 0.05$ compared to the “before” measurement prior to the intervention

Ex vivo LPS-induced $\text{TNF}\alpha$ production did not show a significant decrease after four weeks quercetin supplementation ($P \leq 0.1$, Figure 7.6). Also no clear correlation was found between the changes in the *ex vivo* LPS-induced $\text{TNF}\alpha$ production of the individual volunteers after the four weeks of quercetin supplementation and the increase in either the quercetin plasma concentration (Figure 7.7) or the total plasma antioxidant capacity (data not shown).

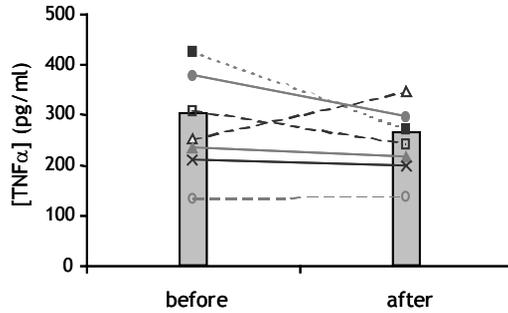


Figure 7.6 The effect of four weeks quercetin-intervention in healthy volunteers on *ex vivo* LPS-induced $\text{TNF}\alpha$ production. Data are individually expressed ($n=7$); the light grey bars represent the mean.

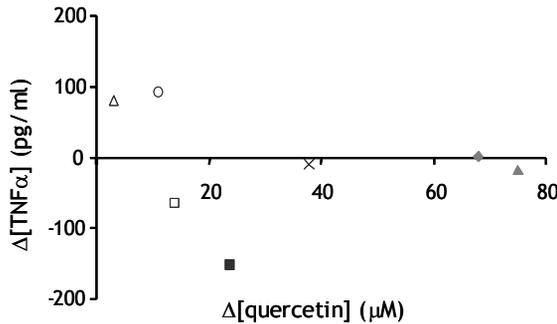


Figure 7.7 The relation between the increase in quercetin concentration ($\Delta[\text{quercetin}]_{\text{plasma}}$) and the decrease in the *ex vivo* LPS-induced $\text{TNF}\alpha$ production ($\Delta[\text{TNF}\alpha]$) in healthy volunteers after four weeks quercetin intervention. Each point represents the individual values of one healthy volunteer ($n=7$).

Discussion

The present study shows that quercetin dose-dependently reduces $\text{TNF}\alpha$ production *in vitro* in the blood of healthy volunteers by LPS.

LPS is a pro-inflammatory glycolipid component of the gram-negative bacterial cell wall. LPS acts as a polyclonal mitogen for B lymphocytes (16) and as an activator of macrophages and neutrophils via the LPS-binding protein (LBP)/CD14/Toll like receptor (TLR)-4-dependent pathway, resulting in the production of specific cytokines (25). Short-term exposure to LPS induces an inflammatory reaction in the lung mediated primarily by human

blood monocytes and alveolar macrophages, which release an array of inflammatory cytokines including $\text{TNF}\alpha$ (26).

Under normal circumstances, ambient air contains low amounts of LPS (approximately 0.36 ng/m^3) that can easily be eliminated by the human endogenous immune defense system (27). However, LPS is present in larger amounts in airborne particles in polluted air (28) and cigarette smoke (29). Moreover, a variety of infections may provide a pathological burden of mediators like LPS (30). Various studies have shown a relation between LPS exposure and airway inflammation or obstruction (31-33). Moreover, long-term exposure to LPS in mice results in inflammatory and pathological changes that mimic changes observed in human subjects suffering from chronic lung diseases such as chronic obstructive pulmonary disease (COPD) (25). Altogether, this suggests that long-term exposure to LPS might play a large role in the pathogenesis of chronic lung diseases.

The *ex vivo* model applied in the present study uses a relatively low LPS concentration, i.e. 0.1 ng/ml , that could well be achieved *in vivo* when taken into consideration that smoking 1 cigarette delivers a local LPS dose of 120 ng (29). Moreover, the $\text{TNF}\alpha$ production evoked by the employed low LPS concentration shows a significant higher sensitivity towards quercetin than that induced by increasing amounts of LPS. This suggests that especially during exposure to relatively low LPS doses, antioxidants such as quercetin will have a relatively more pronounced effect on cytokine production.

LPS induces the reactive oxygen species (ROS)-producing enzymes inducible nitric oxide synthase and NADPH-oxidase in monocytes and macrophages, leading to the extensive production of NO^\cdot , $\text{O}_2^{\cdot-}$, peroxynitrite and other reactive oxygen (ROS) or nitrogen species (RNS) (34,35). It is known that ROS are capable of promoting inflammation by activating transcription factors like nuclear factor kappa-B (NF- κ B) and activator protein-1 that induce not only more ROS but also pro-inflammatory cytokines like $\text{TNF}\alpha$ (36,37). Since $\text{TNF}\alpha$ can also activate NF- κ B (36,38), a feed-forward mechanism, resulting in increased production of both cytokines and ROS, will be set in motion upon LPS exposure.

Quercetin is an excellent scavenger of both ROS and RNS. Consequently, the flavonoid might be used to reduce both oxidative stress, i.e. an imbalance between the production of and the protection against reactive species, and inflammation. Moreover, quercetin can also inhibit NF- κ B activation, thereby directly reducing the cytokine production via this transcription factor (10). Both these capacities of the flavonoid may contribute to the counteracting effect of quercetin on the LPS-induced $\text{TNF}\alpha$ production, as we observed *in vitro* in the present study. This is in line with the inhibiting effect of the antioxidant β -carotene found in alveolar macrophages on LPS-induced NF- κ B activation and ROS generation (39).

The total antioxidant status shows a small, but significant, increase after the quercetin supplementation. Interestingly, this increase significantly surpasses the increase of the plasma quercetin concentration. Vitamin C and uric acid levels, important determinants of the total antioxidant capacity, are not affected by the supplementation. This indicates that various metabolites of quercetin, such as small phenolic compounds, might have a substantial contribution to the rise in total antioxidant capacity. The residual antioxidant capacity, also corrected for the contribution of quercetin in the plasma, is still significantly enlarged after four weeks quercetin supplementation. Apparently, the supplementation also has a small, but persistent, effect on the residual antioxidant capacity.

The *in vitro* experiments with quercetin and LPS added to the blood show that the inhibitory effect of quercetin was more potent at higher TNF α levels. This means that quercetin displays a more pronounced anti-TNF α effect when the production of this cytokine is elevated. Consequently, especially in people suffering from a disease of which the pathology is associated with elevated levels of inflammation, quercetin supplementation might exert a beneficial effect. The same probably applies for oxidative stress; only in people suffering from oxidative stress, strengthening the antioxidant defence through quercetin supplementation might be expected to be beneficial. This indicates that health-benefit studies regarding antioxidant supplementation should not be conducted in healthy volunteers that are expected not to display inflammation or oxidative stress during the intervention period.

In the *in vivo* experiment, the basal TNF α levels are not affected by the quercetin supplementation. As indicated by the *in vitro* experiment, this absence of an *in vivo* effect might be due to the baseline TNF α levels in the healthy subjects that were already very low and could therefore not further be reduced.

The effect of the quercetin supplementation has also been evaluated *ex vivo*. To the blood of the volunteers obtained both before and after supplementation, LPS was added to induce an inflammatory response. Still, no significant decrease of *ex vivo*-induced TNF α production could be observed after supplementation. Moreover, no correlation could be found between the increase in plasma quercetin concentration and the *ex vivo*-induced TNF α -levels. This absence of an effect of quercetin supplementation on the LPS-induced TNF α production *ex vivo* might be due to the small effect of the supplementation on the total plasma antioxidant capacity. Although the increase caused by the supplementation is statistically significant, it is relatively low compared to the fairly high total plasma antioxidant capacity already present at baseline in healthy volunteers. This is an extra argument that health benefit studies regarding antioxidants should not be conducted in

healthy volunteers not suffering from oxidative stress. When the intake of antioxidants, e.g. by a healthy diet, is adequate and there is no oxidative stress, there is no need to supply extra antioxidants. When the antioxidant capacity has declined, as for instance in diseases associated with increased production of ROS, empowering the weakened endogenous antioxidant shield is expected to be helpful. The rationale for antioxidant supplementation in healthy subjects is present when it is foreseen that a situation resulting in oxidative stress is likely to arise. The supplementation might then be used as a protective measure to prevent damage.

In the protection against free radicals, quercetin becomes converted into highly thiol-reactive and potential toxic oxidation products (40,41). In the present study, the level of glutathione (GSH), the most abundant and reactive endogenous thiol, is unaltered by the given quercetin supplementation. This indicates that the possible formation of reactive oxidation products has no major consequences in the applied dosing regime. Since this study concerns healthy volunteers with hardly any oxidative stress, the absence of quercetin-induced toxicity might be due to the limited formation of oxidized quercetin compared to the high levels of GSH. The expected limited formation of oxidized quercetin makes the information obtained with supplementation studies in healthy volunteers of limited value for predicting the toxicity of quercetin in patients with oxidative stress. In these patients, the formation of oxidized quercetin is higher and levels of GSH might be lower. This indicates that in patients with oxidative stress, the formation of reactive oxidation products of quercetin and the toxicity induced by these reactive metabolites might be more pronounced.

In conclusion, the results of the present study show anti-inflammatory effects of quercetin *in vitro* but not *ex vivo* or *in vivo* in healthy volunteers. The fact that no indication has been found for an anti-inflammatory effect in healthy volunteers after increasing the dietary intake of an antioxidant, is probably due to (i) the relatively small increase of the antioxidant capacity of healthy volunteers that is already high at baseline and (ii) the low inflammatory status of these subjects. In retrospect, the design of the present study is thus not optimal for demonstrating potential health effects of quercetin. Antioxidant supplementation in healthy volunteers that are not likely to have inflammation or oxidative stress is superfluous and, therefore, will not result in substantial beneficial health effects in these subjects. A healthy and diverse diet normally supplies sufficient antioxidants, provided that no enhanced ROS production is expected. There is only a rationale for any supplementation when on forehand a beneficial effect is foreseen. Actually, this has insufficiently been realised in the design of a great many antioxidant studies and may explain why various major trials on the preventive effect of antioxidant supplementation in healthy subjects had a

disappointing outcome. Particularly in people with increased levels of inflammation and oxidative stress, antioxidant supplementation is expected to be fruitful.

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