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Summary and general discussion

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Aerobic life forms are associated with the formation of reaction oxygen species (ROS) that can cause damage to macromolecules like lipids, proteins, DNA and RNA. Fortunately, the human body comprises an elaborated defense system against ROS-induced damage that consists of antioxidants, i.e. molecules that can neutralize ROS and other reactive species. To offer maximum protection, antioxidants work together in an intricate network. Failure of this network, either by an overload of ROS or by a decline of sufficient antioxidants, results in a situation called oxidative stress, i.e. an imbalance between the production of and the protection against ROS. Oxidative stress is implicated in the pathophysiology of various chronic inflammatory diseases like sarcoidosis. To reduce the occurring oxidative stress and thereby hopefully also the symptoms of these diseases, treatment with exogenous antioxidants might be useful. Such an antioxidant therapy might by of special interest since the treatment of several of these diseases with e.g. glucocorticoids sometimes fail to be completely efficacious. A suitable candidate for antioxidant therapy might be the flavonoid quercetin that is known for its excellent antioxidative properties. This thesis focuses on the health effects of quercetin from the mechanism of action of the flavonoid to its use as nutraceutical.

In **chapter 2** it is shown that catechol-containing antioxidants such as quercetin are indeed excellent scavengers that can offer protection against lipid peroxidation, i.e. oxidative damage to lipids. During this antioxidant activity, catechol oxidation products such as semiquinone radicals and quinones are formed. These oxidation products are capable of inactivating the GSH-dependent protection against lipid peroxidation and the calcium sequestration in liver microsomes, probably by arylating free thiol groups of the enzymes responsible for these two processes, i.e. the free radical reductase and calcium ATPase. It is therefore concluded that a catechol-containing antioxidant such as quercetin might shift radical damage from lipid peroxidation to sulfhydryl arylation.

The oxidation products of quercetin are studied in more detail in **chapter 3**. The primary oxidation product is an *ortho*-quinone, denoted as QQ, which has four tautomeric forms, i.e. the *ortho*-quinone and three quinone-methides. The reaction of QQ with two of the most important and abundant endogenous antioxidants has been investigated, i.e. its reaction with ascorbate (vitamin C) and glutathione (GSH). It appears that ascorbate is capable of recycling QQ into its parent compound quercetin. With, GSH, QQ forms two glutathionylquercetin adducts (GSQ), i.e. 6-GSQ and 8-GSQ. When both GSH and ascorbate are present, QQ is converted exclusively into GSQ. In

the absence of GSH, protein sulfhydryl groups can be arylated by QQ. This protein arylation is also not prevented by ascorbate.

Since one of the most important and abundant endogenous antioxidants, ascorbate, could not offer protection against the strong thiol-reactivity of QQ, another line of defense against this QQ-induced toxicity has been examined in **chapter 4**. The enzyme DT-diaphorase (NQO1) is reported to protect against quinone-induced toxicity by reducing quinones to hydroquinones. For QQ this would mean that DT-diaphorase converts the quinone into quercetin, thereby not only preventing QQ toxicity but also regenerating the consumed antioxidant and thus boosting the antioxidant effect. It was found that QQ is indeed a substrate for DT-diaphorase. However, DT-diaphorase cannot compete with thiols; QQ reacted much faster with glutathione or protein sulfhydryl groups (>1000 times) than with the enzyme in experiments with isolated compounds as well as with human liver cytosol or blood plasma. This indicates that DT-diaphorase plays no substantial role in the protection against QQ.

Based on our studies described in chapter 2-4, it can be concluded that GSH is the main reactant of QQ. This reaction gives rise to the formation of an adduct between QQ and GSH, i.e. GSQ. As described in **chapter 5**, we have found that GSQ is not stable; it rapidly dissociates into GSH and QQ with a half life of 2 minutes. Surprisingly, GSQ incubated with 2-mercapto-ethanol (MSH), a far less reactive thiol, results in the conversion of GSQ into the MSH-adduct MSQ. MSQ appeared to be much more stable than GSQ. Incubation of GSQ with the dithiol dihydrolipoic acid ($L(SH)_2$) results in the formation of quercetin out of GSQ.

These results indicate that as long as the GSH concentration is high, QQ will be trapped as GSQ. In this way GSH will protect against QQ. However, when the GSH concentration is low, the dissociated QQ will react with other thiol groups, e.g. protein sulfhydryls. It is proposed that the kind of QQ-adduct that eventually will accumulate depends on (i) the reactivity of the thiols present (ii) the characteristics of the leaving group of the adducts (iii) the relative concentrations of the various thiols and (iv) the time span of the incubation period. The poor leaving group characteristics of most protein sulfhydryls indicate that, at the end, QQ might be transferred from a GSQ to a protein-thiol-Q. These findings imply that the “protection” offered by GSH against QQ might only be transient; GSH protects against QQ by scavenging QQ at the site of formation, but on the long run GSH will transfer QQ to other thiols. In that way, the initially highly focussed toxicity of QQ might be dispersed by the formation of GSQ that finally spreads QQ-induced toxicity, possible even over cells.

In order to define the delicate balance between the protective effects of quercetin and the potential toxic effects of its oxidation products, the effect of the flavonoid has been investigated in a more integrated system, *viz*. rat lung epithelial cells (RLEs) (**chapter 6**). Quercetin was capable of efficiently protecting RLE cells against H_2O_2-induced DNA damage, but concurrently markers of cell toxicity (i.e. GSH loss and LDH leakage) and cell function loss (i.e. free cytosolic calcium concentration) were increased. It is therefore concluded that even in living lung cells, quercetin swaps one kind of toxicity for another. This quercetin paradox, i.e. the fact that in the process of offering protection, quercetin is converted into an oxidation product with possible toxic effects, has not been investigated in cultured cells before. Following depletion of GSH in the cells by BSO pre-treatment, this quercetin paradox becomes more pronounced, confirming that the formation of thiol reactive quercetin metabolites is involved in the quercetin paradox. Therefore, it is stated that the potential toxicity of metabolites formed during the actual antioxidant activity of free radical scavengers such as quercetin should be considered in the use of antioxidant supplementation.

Interestingly, quercetin supplementation in either healthy volunteers (**chapter 7**) or sarcoidosis patients (**chapter 9**) did not affect the GSH levels. This indicates a limited impact of potentially thiol-reactive oxidation products in the applied dosing regimes, as could be anticipated based on the relative low plasma quercetin concentrations achieved in both supplementations (respectively 0.05 and 0.27 μM) compared to the high cellular concentrations of GSH (1 to 10 mM). Apparently, quercetin supplementation will only lead to QQ-induced toxicity when, as a result of elevated oxidative stress, substantial amounts of QQ are formed and GSH levels are remarkably compromised. This might indicate that QQ-induced toxicity as a result of quercetin supplementation can be especially expected when relatively high dosages are applied to people suffering from severe oxidative stress during a relatively long period.

Because of the association between ROS and inflammation, it is often suggested that an antioxidant such as quercetin might, by scavenging ROS, also exert anti-inflammatory capacities. Therefore, in **chapter 7** the possible anti-inflammatory effects of physiologically attainable quercetin concentrations have been investigated in whole blood from healthy subjects, i.e. a model closely resembling the *in vivo* situation. Cytokine production was *ex vivo* evoked by lipopolysaccharide (LPS), a patho-physiological relevant stimulator of monocytes, neutrophils and B lymphocytes. It was found that quercetin dose-dependently inhibited *ex vivo* LPS-induced production of tumour necrosis factor α (TNFα), an important mediator of inflammation. This anti-TNFα effect of quercetin already occurred at the physiological achievable concentration of 1 μM. The *ex vivo* LPS-induced IL-10 production

remained unaffected by quercetin. Consequently, quercetin also caused a significant reduction of the ratio of pro- versus anti-inflammatory cytokines TNFα/IL-10, a frequently used diagnostic parameter of inflammation.

The *in vitro* anti-TNFα effect of quercetin showed a large dependency on the level of TNFα induced by LPS; the higher the LPS-induced TNFα level, the higher the inhibiting effect of quercetin on this cytokine level is. This finding indicates that the anti-inflammatory effect of quercetin is more pronounced at a higher level of inflammation.

To further elucidate the *ex vivo* anti-inflammatory effects of the flavonoid, a quercetin supplementation study for four weeks, by means of a quercetin-rich fruit juice comprising 97 mg per day, has been conducted in healthy volunteers (**chapter 7**). This supplementation increased the plasma quercetin concentration as well as the total plasma antioxidant capacity in healthy volunteers, but did not affect basal plasma (*in vivo*) or LPS-induced (*ex vivo*) TNFα levels. This lack of effect is probably due to the low cytokine and high antioxidant levels of the healthy volunteers at baseline, indicating that neither inflammation nor oxidative stress are present. This means that no quercetin supplementation is needed in these subjects, simply because there is no damage that needs to be reduced. Consequently, antioxidant supplementation is expected to be more fruitful 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.

To examine whether sarcoidosis patients are more likely candidates for quercetin supplementation than healthy volunteers, the antioxidant and inflammatory status of these patients has been determined. As shown in **chapter 8**, the total plasma antioxidant capacity was decreased and the levels of the individual endogenous antioxidants GSH, vitamin C and uric acid were declined in the blood of sarcoidosis patients. Moreover, sarcoidosis patients also displayed elevated levels of the pro-inflammatory cytokines TNFα and interleukin-8 (IL-8). No difference in the anti-inflammatory cytokine IL-10 is observed between sarcoidosis patients versus healthy controls. As a result, the ratios of the pro- versus the anti-inflammatory cytokines TNFα/IL-10 and IL-8/IL-10, frequently used diagnostic parameters of inflammation, were also significantly increased in sarcoidosis.

Determining the *in vitro* anti-inflammatory effects of quercetin on *ex vivo* LPS-induced TNFα and IL-8 production in both the sarcoidosis and control group showed that the inhibiting effects of the flavonoid regarding both cytokines were much more pronounced in the patients than in the matched controls. Since the LPS-induced cytokine levels were significantly higher in the patient group, this finding confirms the earlier *in vitro* result that the

anti-inflammatory effect of quercetin increases with the level of occurring inflammation.

The *in vivo* anti-oxidative and anti-inflammatory effects of quercetin in people, suffering from both oxidative stress and inflammation, have been investigated in a double blinded quercetin supplementation study (4x500 mg orally within 24 hours), conducted with 2 groups of non-smoking, non-treated sarcoidosis patients (**chapter 9**). This quercetin supplementation caused an increase of the antioxidant defence, as can be deduced from the observed increase in plasma quercetin concentration as well as in the total plasma antioxidant capacity. Additionally, this quercetin supplementation resulted in a decrease of malondialdehyde (MDA), a marker of oxidative damage to lipids. This suggests that strengthening the endogenous antioxidant shield with quercetin supplementation indeed results in more protection against oxidative damage, caused by reactive species such as ROS. Furthermore, both basal (*in vivo*) and LPS-induced (*ex vivo*) ratios of the pro- versus the anti-inflammatory cytokines, i.e. TNFα/IL-10 and IL-8/IL-10, were reduced by the quercetin supplementation.

Interestingly, both the anti-oxidative and anti-inflammatory effects of quercetin appeared to be more pronounced when the level of respectively the occurring oxidative stress or inflammation is higher at baseline. This observed correlation indicates that beneficial effects of antioxidant supplementation can especially be expected in people with elevated oxidative stress, e.g. patients suffering from a disease of which the pathology is associated with this process.

Conclusion

The flavonoid quercetin is an excellent antioxidant that also possesses anti-inflammatory capacities. The excellent anti-oxidative properties of quercetin can be deduced form the fact that the flavonoid offers protection against ROS-induced oxidative damage to lipids both in vitro and in vivo. The anti-inflammatory capacities of the flavonoid are indicated by its reducing effect on the inflammatory markers TNFα/IL-10 and IL-8/IL-10 both in vitro and in vivo.

Interestingly, both the anti-oxidative and anti-inflammatory effects of quercetin are more pronounced when the basal levels of respectively the occurring oxidative stress and inflammation are high. This indicates that the use of quercetin supplementation is especially fruitful in people suffering from a disease that is associated with both processes, such as sarcoidosis. Moreover, the correlation between the effects of quercetin and the basal levels of both oxidative damage and inflammation may explain why, after supplementation, no anti-inflammatory effects of the flavonoid could be detected in healthy volunteers.

Up to date, many health-benefit studies on antioxidant supplementation are conducted in healthy volunteers. These volunteers will display only low levels of inflammation and oxidative stress and have high levels of endogenous antioxidants. Consequently, additional supplementation with extra antioxidants will most likely not result in any significant beneficial health effects, because there is no need for an extra defense against oxidative stress or inflammation. The design of this type of study resembles the testing of a new remedy against migraine in healthy volunteers. Inevitable nonspecific side-effects such as headaches will wrongly disqualify the new drug. This metaphor explains the disappointing, but foreseeable, negative outcome of numerous antioxidant intervention studies.

During its scavenging activities, quercetin becomes oxidized and possible toxic oxidation products are formed. The most important oxidation product of quercetin is its *ortho*-quinone, denoted as QQ. QQ is highly thiol reactive and reacts almost instantaneously with GSH or, in the absence of this thiol, with protein sulfhydryl groups, thereby impairing the function of several critical enzymes. Consequently, during *in vivo* quercetin supplementation care should be taken of the possible toxicity of its metabolites. During both our supplementation studies, no signs of this toxicity are observed. However, in a chronic disorder such as sarcoidosis, supplementation has to proceed over a much longer time period. The safety, tolerability and efficacy of the long term use of quercetin in sarcoidosis has to be established.

Implications and further research

The research conducted in the present thesis clearly indicates that *in vivo* quercetin supplementation may exert anti-oxidative and anti-inflammatory properties in people suffering from a condition that is associated with both processes such as sarcoidosis. However, only a relatively small number of patients has been included in our study and measurements comprised only biomarkers of oxidative stress and inflammation. Moreover, it is still unclear whether these anti-oxidative and anti-inflammatory properties of the flavonoid will actually lead to a reduction of the clinical symptoms present in sarcoidosis patients. Therefore, a larger quercetin supplementation study should be conducted in sarcoidosis patients to (i) confirm our results and to (ii) expand our data by measuring the effect of the supplementation on clinical parameters of the disease. In sarcoidosis, besides reduced lung function, the main symptom is an extreme fatigue. Consequently, it would be interesting to examine whether long term quercetin supplementation would improve both the lung function and and the physical condition in sarcoidosis patients.

Another point that should be addressed in a larger supplementation study with quercetin is the safety of the use of this flavonoid. Since sarcoidosis is a chronic disease, it can be expected that, in order to improve the health status of patients suffering from this disease, chronic use of quercetin supplementation is required. Up to date, there are no data available regarding the safety of long term use of high dosages of antioxidants in general and quercetin in particular. As is described in the current thesis, quercetin becomes oxidized while exerting its anti-oxidative capacities and potentially toxic oxidation products are formed. The long term cytotoxic effects of these oxidation products should be monitored in detail in order to evaluate the safety of long term quercetin supplementation. This can be achieved, for example, by screening the health status of cells that are expected to be exposed to the flavonoid the most, i.e. blood and lung cells. Alternatively, the thiol reactivity of these oxidation products can be quantified by, for example, measuring the activity of various enzymes of which it is known that they can be affected by the oxidation products of quercetin, i.e. calcium ATPase of glutathione transferases. Prevention of toxicity, induced by the thiol reactive oxidation products, might be achieved by supplementing quercetin together with a dithiol that is capable of capturing, and thus detoxifying, these products.

Optimal effects of any supplementation can only be expected when the compound that needs to be supplemented is administered in the most appropriate way. For quercetin, various ways of supplementing are possible.

In the current thesis, two variants are used, i.e. a pure supplement and a diet intervention using a juice with a high quercetin content. The supplement only contains the aglycone form of quercetin, whereas the juice comprises high amounts of various quercetin derivatives that might have a better biological availability than the aglycone itself. Another advantage of a dietary supplementation versus a 'conventional' supplement might be a better compliance, especially in long term use. A good compliance is mandatory in chronic diseases such as sarcoidosis. Conversely, a disadvantage of using the diet is that the intake of alimentary antioxidants is relatively low, i.e. ~100 mg a day versus up to several grams a day using an antioxidant supplement. As a result, the plasma quercetin concentrations achieved by the quercetin supplement were higher compared with the plasma levels obtained by the dietary intervention in our studies. Consequently, larger beneficial health effects might be expected when supplementation is applied via a supplement instead of via the diet. However, in order to make a balanced overall comparison between both ways of supplementation, more research is necessary.

Finally, it should be noted that sarcoidosis is a multi-system disorder that can manifest throughout the body by influencing various pathways. It is, therefore, not surprising that the current treatment of sarcoidosis with only anti-inflammatory agents, such as glucocorticoids, fails to be completely efficacious. Instead, better results can be expected from a multi-factorial treatment that could tackle the various aspects of sarcoidosis. The flavonoid quercetin displays not only the anti-inflammatory and anti-oxidative properties investigated in the current thesis. Additionally, immuno-suppressive, anti-fibrotic, anti-coagulative and anti-proliferative capacities of quercetin have been reported as well. Interestingly, all these activities have also been suggested for the treatment of sarcoidosis. This complimantory activity appears to make quercetin an even more promising candidate for the treatment of sarcoidosis than could be expected based on the results presented in the current thesis.