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No role of DT-diaphorase (NQO1) in the protection against oxidized quercetin

AW Boots, A Bast, GRMM Haenen

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Abstract

Background

Quercetin is one of the most studied alimentary antioxidants. During its antioxidant activity quercetin becomes oxidized into its ortho-quinone/quinone methide, denoted as QQ. QQ is toxic since it is highly reactive towards thiols. DT-diaphorase (NQO1) might protect against QQ toxicity by reducing QQ to quercetin. However, conflicting data have been reported. The aim of the present study is to elucidate the role of DT-diaphorase in the protection against QQ-mediated thiol reactivity.

Methods

Several competition experiments have been performed with isolated compounds as well as with liver cytosol. The thiol reactivity of QQ, and the influence of various reactants, has been assessed using blood plasma. All experiments were monitored spectrophotometrically as well as by high performance liquid chromatography (HPLC).

Results

It was found that QQ is indeed a substrate for DT-diaphorase. However, compared to its reaction with the enzyme, QQ reacted much faster with either glutathione or protein thiols. Moreover, even ascorbate, known to react much slower with QQ than glutathione (GSH), reacted faster with the quinine than DT-diaphorase did.

Conclusions

The results of the present study indicate that QQ will primarily react with thiols, i.e. GSH or when GSH is depleted with protein thiols. Therefore, it is concluded that DT-diaphorase has no role in the protection against QQ.

Introduction

One of the most prominent dietary antioxidants is quercetin, a flavonoid that is nearly ubiquitous in foods, including vegetables, tea, fruit and wine (1,2) as well as in countless food supplements. Quercetin can scavenge highly reactive species such as peroxynitrite and the hydroxyl radical (3,4) and this activity is involved in the beneficial health effect of quercetin. During this antioxidant activity, quercetin becomes oxidized to its ortho-quinone/quinone methide, denoted as QQ (5,6). QQ can exert toxic effects since it is highly reactive towards thiols.

Protection against quinone-mediated toxicity might be provided by DT-diaphorase [NAD(P):H-(quinone acceptor) oxidoreductase NQO1, EC 1.6.99.2] (7). This is a cytosolic enzyme that acts as a two-electron reductase. It has a flavin-adenine dinucleotide (FAD) prosthetic group in the active site that directly donates two electrons to reduce a quinone to its corresponding hydroquinone (8). A tyrosine residue in the active site is involved in the reduction of para-quinones, the primary substrates of DT-diaphorase (9). The reduction by DT-diaphorase is often regarded as the first line of defence against quinone-mediated toxicity (7,10). Data on the role of DT-diaphorase in the protection against the ortho-quinone/quinone methide QQ that is generated when quercetin scavenges highly reactive pro-oxidants are conflicting, since DT-diaphorase is reported to enhance (11) or protect against (12) this toxicity.

The aim of the present study is to elucidate the role of DT-diaphorase in the regeneration of QQ. To this end the reaction rate of QQ with DT-diaphorase is compared to the reaction rate of QQ with ascorbate and glutathione (GSH), endogenous compounds known to react readily with QQ. Experiments are performed with isolated compounds as well as with human liver cytosol.

Materials and methods

Materials

Quercetin, reduced glutathione (GSH), ascorbate, tyrosinase, menadione and DT-diaphorase (E.C. 1.6.99.2, also called [NAD(P):H-(quinone acceptor) oxidoreductase or NQO1) were purchased from Sigma (St. Louis, MO, USA). Reduced β-Nicotinamide adenine dinucleotide disodium salt (NADH) and 5',5'-dithiobis (2-nitrobenzoic acid) (DTNB) were obtained from ICN Biomedicals (Ohio, USA). Human liver was obtained from liver tissue that was removed by surgical excision of a liver tumour from 40 to 60 years old

patients. A “healthy” part of the excised material that did not contain any tumour cells was used. The livers were removed according to the standard surgical procedure and the use of the material is in accordance with the medical and ethical guidelines of the academic hospital of Maastricht. Liver cytosol was prepared as described previously (13). Blood plasma was obtained from two healthy volunteers (age 27 and 30) again in accordance with the medical and ethical guidelines of the Academic Hospital of Maastricht.

Methods

All incubations were performed at 37ºC in a 143 mM phosphatebuffer pH 257.4 and were monitored spectrophotometrically as well as by high performance liquid chromatography (HPLC).

QQ was formed by oxidizing 100 μM quercetin with 25 U/ml tyrosinase. The oxidation product that accumulated during 5 minutes oxidation of quercetin by tyrosinase appeared not to be a substrate for DT-diaphorase (data not shown). The product also did not react with GSH, indicating that the product formed is not QQ, since GSH is known to readily react with QQ (14,15). HPLC analysis revealed that two products accumulated after the oxidation of quercetin with tyrosinase (6-QOH and 8-QOH), denoted as QOH. It should be noted that in some studies (11) it was stated that preformed QQ is used, which actually is QOH.

Due to this relative instability of QQ, QQ had to be generated in situ for each experiment in the presence of the reactants studied. DT-diaphorase activity was studied at an enzyme concentration of 10 U/ml plus 0.2 mM NADH and quantified by determining the NADH decrease at 240 nm. Quercetin consumption was determined spectrophotometrically at 400 nm and ascorbate consumption at 270 nm. Spectra were recorded with a scanspeed of 480 nm/min.

HPLC of the incubation mixtures was performed using a Supelcosil LC318 column (25 cm x 4.6 mm). The column was eluted isocratically with water containing 0.1% (v/v) trifluoroacetic acid and 5% acetonitrile during 5 minutes, followed by a linear gradient to 20% acetonitrile from 5 to 10 minutes and to 30% acetonitrile from 10 to 16 minutes.

Quercetin was quantified using external calibrators (detection at 290 nm). To quantify the glutathionyl adducts, 50 μM quercetin was oxidized by 25 U/ml tyrosinase in the presence of 100 μM GSH. After approximately half of the quercetin was oxidized based on spectrophotometrical analysis (the exact degree of quercetin oxidation was determined using HPLC), the solution was directly injected on the HPLC system. The peak area of both glutathionyl adducts (6-GSQ and 8-GSQ) was determined (detection at 290 nm) and the concentration of the adducts was calculated assuming that

(i) all quercetin consumed is converted quantatively into GSQ under these conditions and (ii) the response factor of both glutathionyl adducts is identical. Comparable to the strategy used for the glutathionyl adducts, the decomposition products of QQ (QOH) were quantified using HPLC (detection at 290 nm). Attempts to synthesize QOH and GSQ failed, probably due to the relative instability of these compounds. QQ formation was quantified by trapping QQ with GSH and determination of GSQ.

In the experiments performed with liver cytosol and blood plasma, the DT-diaphorase activity was determined by adding 0.2 mM NADH and 50 μM of the substrate menadione to the cytosol and monitoring spectrophotometrically the NADH consumption due to DT-diaphorase activity at 340 nm. As reference the same incubation without menadione was used. The GSH concentration of the liver cytosol was measured by adding 0.3 mM DTNB and determining the absorption of the incubation mixture at 412 nm. In the reference no DTNB was added.

NADH consumption due to DT-diaphorase reduction of oxidized quercetin was quantified by monitoring spectrophotometrically the decrease at 340 nm in the incubation mixture containing liver cytosol, 0.2 mM NADH, 50 μM quercetin and 25 U/ml tyrosinase. In the reference quercetin was omitted. GSH consumption was measured by adding 0.3 mM DTNB to 1 ml of this incubation mixture after 1 minute incubation and the absorption at 412 nm was immediately determined. In the blanc DTNB was replaced by buffer and the results were compared to an incubation that did not contain quercetin. Quercetin and GSH consumption as well as QOH and GSQ formation were also determined using HPLC as described above. To this end, all incubation mixtures were deproteinated by adding an equal volume of 10% TCA to the sample before vortexing and centrifuging it for 1 minute at 14.000 rpm. The supernatant was injected on the HPLC system.

Protein thiol arylation by QQ was assessed in blood plasma. The plasma was incubated in a 10-fold dilution with 50 μM quercetin with or without 25 U/ml tyrosinase in either in the presence or the absence of 10 U/ml DT-diaphorase plus 0.2 mM NADH. After 5 minutes incubation 0.6 mM DTNB was added to this reaction mixture. The reduction of DTNB was measured spectrophotometrically at 412 nm. In the blanc DTNB was replaced by buffer.

Formation of glutathionyl adducts and NADH or ascorbate consumption showed that addition of ascorbate or DT-diaphorase plus NADH results in a small inhibition of the formation of quercetin by tyrosinase as also reported previously for ascorbate (14). DT-diaphorase mediated NADH reduction is constant in time in the experiments. This indicates that there is no relevant inhibition by the compounds under our experimental conditions.

All experiments were performed, at least, in triplicate. Data are given as mean or as a typical example. The SD of all results was usually less than 5%

and always less than 10%. Statistical analysis was performed using Student's t-test.

Results

The ability of DT-diaphorase to reduce the quinone/quinone methide of quercetin (QQ) was investigated. QQ was generated by the oxidation of 50 μM quercetin with 25 U/ml tyrosinase.

Due to the reactivity of QQ, QQ was generated in situ in the presence of the reactants studied. It appeared that QQ is a substrate for DT-diaphorase (10 U/ml) based on the NADH consumption (Figure 4.1C). Menadione, a para-quinone and one of the most studied substrates for DT-diaphorase, was used as a control (Figure 4.1A). Ascorbate (100 μM) had no effect on the reaction of DT-diaphorase (10 U/ml) with menadione (50 μM) (Figure 4.1B). Ascorbate almost completely prevented the DT-diaphorase catalysed consumption of NADH by QQ, indicating that ascorbate reacts faster with QQ than DT-diaphorase does (Figure 4.1D).

GSH (100 μM) reacts with QQ forming glutathionyl adducts, as is seen in the spectral changes that occur in the incubation mixture containing quercetin, tyrosinase and GSH (Figure 4.2C). The increase of the absorption at 340 nm and the isosbestic points at 310 nm and 355 nm can be attributed to the formation of the glutathionyl adducts (14). When DT-diaphorase is also present, again the spectral changes indicative for the formation of glutathionyl adducts occur (Figure 4.2D). The formation of GSQ was confirmed using HPLC analysis, which also shows that DT-diaphorase could not compete with GSH for QQ (Figure 4.3).

Addition of GSH to the incubation mixture containing menadione, DT-diaphorase and NADH had no effect on the DT-diaphorase mediated reduction of menadione (data not shown). These results made us conclude that the reaction of QQ with GSH exceeds that of QQ with DT-diaphorase by far (Figure 4.2).

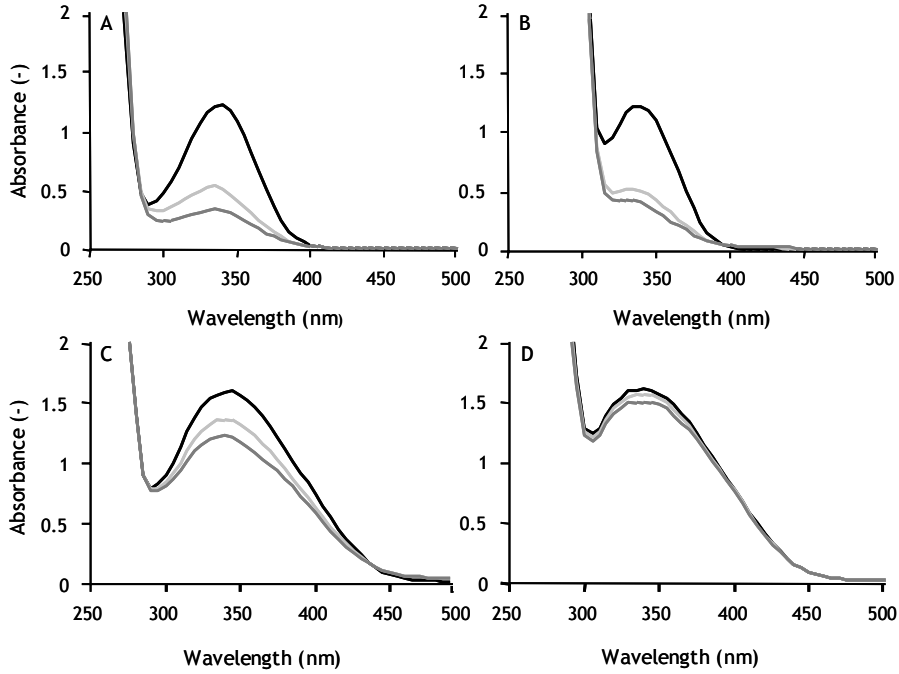

Figure 4.1 Repetitive UV scans of the incubation mixture containing 0.2 mM NADH, 10 U/ml DT-diaphorase and 50 μM menadione in the absence (A) or presence (B) of 100 μM ascorbate. The same experiment was also performed with 50 μM quercetin, instead of menadione, in the presence of 25 U/ml tyrosinase and in the absence (C) or presence (D) of 100 μM ascorbate. The reactions were started by the addition of DT-diaphorase (A and B) or a mixture of DT-diaphorase and tyrosinase (C and D). The UV scans were measured 0 (black), 3 (light grey) or 5 minutes (dark grey) after the addition of the enzyme(s). Scanspeed was 480 nm/min. A typical example is shown.

To assess the relative contribution of the reaction of QQ with each reactant studied, competition experiments were performed (Figure 4.3). In these experiments, QQ formation by tyrosinase was quantified by trapping QQ with GSH and determination of the amount of glutathionyl adducts formed.

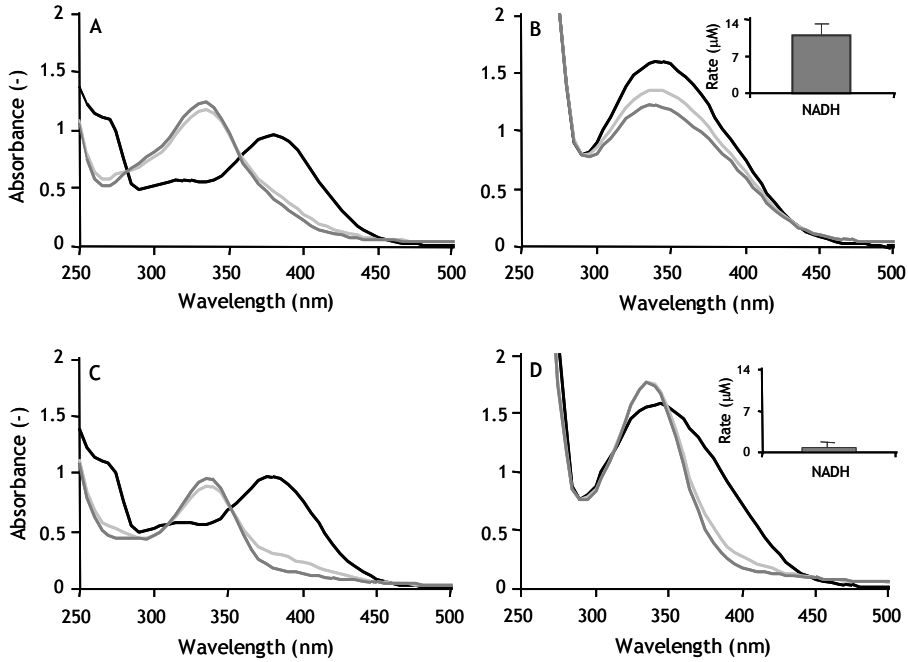

Figure 4.2 Repetitive UV scans of the incubation mixture containing 50 μM quercetin and 25 U/ml tyrosinase (A). Further additions were 10 U/ml DT-diaphorase plus 0.2 mM NADH (B), 100 μM glutathione (C) and both 10 U/ml DT-diaphorase plus 0.2 mM NADH and 100 μM GSH (D). All reactions were started by the addition of a mixture of DT-diaphorase and tyrosinase. The UV scans were measured 0 (black), 3 (light grey) or 5 minutes (dark grey) after the addition of the enzyme mixture. Scanspeed was 480 nm/min. A typical example is shown.
When DT-diaphorase and NADH were added to the incubations (i.e. panel B and D), inserts are added to show the time dependent decrease of NADH (rate).

In the control experiment containing none of the reactants, all the QQ generated was converted into the decomposition products, QOH. Under the conditions used, addition of DT-diaphorase resulted in approximately 60% regeneration of quercetin out of QQ. With ascorbate, this regeneration was approximately 90%. By the combination of DT-diaphorase and ascorbate, all QQ was converted back into quercetin. In the presence of GSH, all the QQ was transformed into the glutathionyl adducts, irrespective of the presence of DT-diaphorase or ascorbate (Figure 4.3).

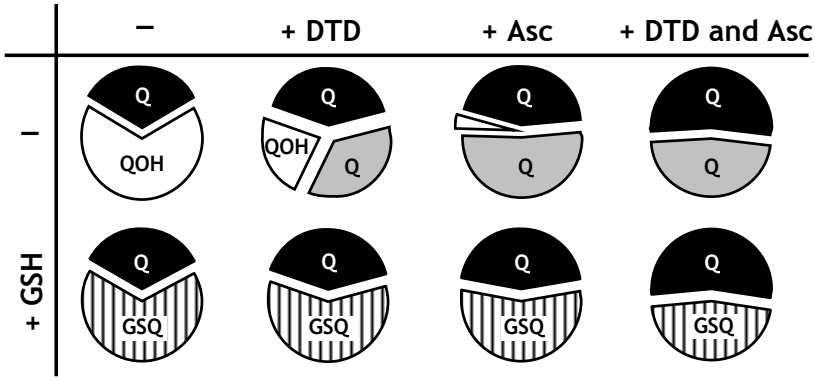

Figure 4.3 Influence of DT-diaphorase, ascorbate and glutathione on the products formed during the oxidation of quercetin by tyrosinase. Quercetin at a concentration of 50 μM was oxidized by 25 U/ml tyrosinase in the absence (upper row) or presence (lower row) of 100 μM glutathione. Further additions were 10 U/ml DT-diaphorase plus 0.2 mM NADH (+ DTD), 100 μM ascorbate (+ Asc) or the combination of 10 U/ml DT-diaphorase plus 0.2 mM NADH and 100 μM ascorbate (+ DTD and Asc). In all incubations, the fraction of quercetin that either did not react (Q black) or after oxidation was regenerated (Q grey) was determined, as well as the formation of decomposition products (QOH) and of the glutathionyl adducts (GSQ). The incubation mixture was analysed 1 min after the incubation was started by the addition of tyrosinase. Quantification was performed using HPLC. Experiments were carried out, at least, in triplicate and expressed as means. The SD for all values is less than 3 μM (not depicted). The extent of regeneration of all incubations was significantly different ($P < 0.05$).

In human liver cytosol, DT-diaphorase activity is 9.4±1.1 U/ml and the GSH concentration is 4.7±0.5 mM. The spectral changes observed in the incubation mixture containing quercetin, tyrosinase and cytosol in the absence (Figure 4.4A) or presence of NADH (Figure 4.4B) are indicative for the formation of glutathionyl adducts. During these incubations, the quercetin consumed (30±1 μM/min in the absence and 32±2 μM/min in the presence of NADH) equalled the GSH consumption (33±2 μM/min in the absence and 31±1 μM in the presence of NADH) while the NADH consumption was negligible (1±0.1 μM/min) (Figure 4.4, insert). This indicates that GSH is the primary target of QQ in the liver and that the role of DT-diaphorase in the reaction with oxidized quercetin is negligible.

In human blood plasma GSH is practically absent and DT-diaphorase activity was below 0.1 U/ml. Incubation of blood plasma with QQ resulted in a 61±8% reduction of the protein thiol content (Figure 4.5). This thiol consumption did not change significantly when extra DT-diaphorase (10 U/ml)

and NADH (0.2 mM) were added to the incubation and was 58±5%. Apparently, protein thiol consumption is not prevented by the addition of DT-diaphorase, implying that DT-diaphorase cannot protect protein thiols against QQ.

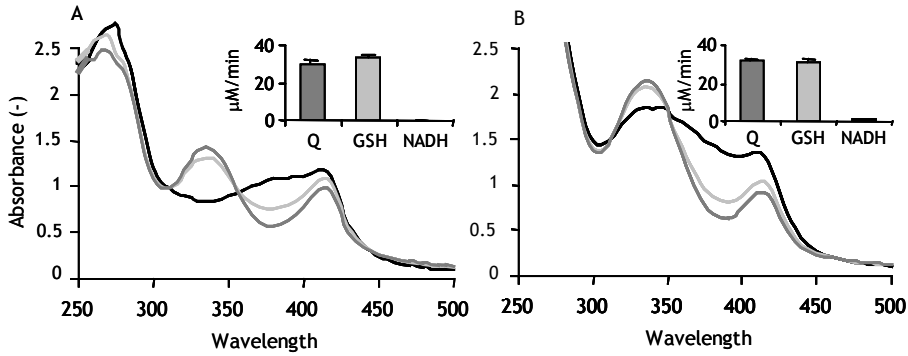

Figure 4.4 UV-scans of the reaction of QQ in human liver cytosol. The cytosol was diluted to give a final GSH concentration of 100 μM. QQ was generated in situ by adding 50 μM quercetin and 40 U/ml tyrosinase. In panel B, 0.2 mM NADH was added to promote DT-diaphorase activity. The reactions were started by the addition of tyrosinase. The UV scans were measured 0 (black), 3 (light grey) or 5 minutes (dark grey) after the addition of the enzyme. Scanspeed was 480 nm/min. A typical example is shown.
The inserts show the quercetin (Q), GSH and NADH consumption during these incubations in μM/min. No substantial NADH consumption (measured at 340 nm) is observed, indicating that, in the liver, DT-diaphorase cannot compete with GSH for oxidized quercetin (QQ).

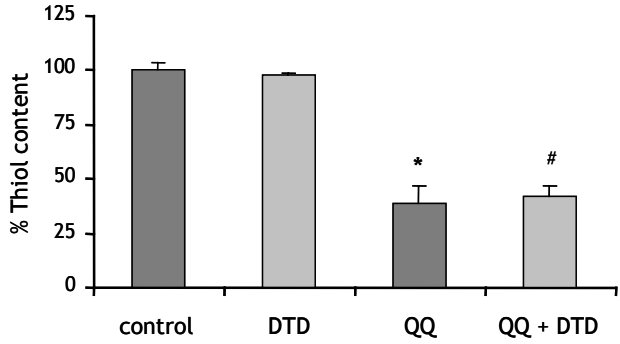

Figure 4.5 Thiol content of blood plasma after the following incubations: 10 U/ml DT-diaphorase plus 0.2 mM NADH (DTD), 50 μM quercetin plus 25 U/ml tyrosinase (QQ) or the combination of 10 U/ml DT-diaphorase plus 0.2 mM NADH and 50 μM quercetin plus 25 U/ml tyrosinase (QQ + DTD). Thiol content is expressed as percentage and relative to that of the control incubation. * $P<0.01$ compared to the control; # $P<0.01$ compared to the control and not significantly different from QQ.

Discussion

Quercetin is an alimentary antioxidant with an alleged positive health effect. Quercetin is able to scavenge all sorts of reactive species. This results in protection against the damage by these reactive species, but the price that is being paid is the accumulation of oxidized quercetin, e.g. QQ. Although the general reactivity of QQ, compared to e.g. $OH^{\bullet}$ or ONOOH, is relatively low, QQ displays a selective toxicity since it is highly reactive towards thiols, including GSH and protein thiols. So by quercetin administration, the general damage inflicted by oxidative stress tends to become focussed on cellular thiols (6).

DT-diaphorase can protect against quinone toxicity by converting a quinone into the corresponding hydroquinone. For QQ this would mean that DT-diaphorase converts it into quercetin, thereby not only preventing QQ toxicity but also regenerating the consumed antioxidant and thus boosting the antioxidant effect.

In the present study it was found that 100 μM ascorbate reacted faster with QQ than 10 U/ml DT-diaphorase. GSH (100 μM) reacts at least 1000 times as fast as ascorbate (100 μM) with QQ (14). Combining both findings made us conclude that 100 μM GSH reacts at least 1000 times faster with QQ than 10 U/ml DT-diaphorase does. This indicates that DT-diaphorase cannot compete with GSH in the reaction with QQ.

The activity of DT-diaphorase used in the present in vitro study (10 U/ml, i.e. an activity of 10.000 μM substrate/min) is in the same range as the enzyme activity we measured in human liver, i.e. 9.4 U/ml, and corresponds nicely with the enzyme activity reported in literature, i.e. 4-6 U/ml or 4.000-6.000 μM/min in liver and lung (10,16). The GSH level is in most cells much higher than the 100 μM used in this in vitro study, e.g. the GSH concentration in the lung is ~ 1 mM (17) and in liver cytosol even 4.7 mM (this study). This means that in the liver GSH reacts at least 47.000 times as fast as DT-diaphorase with QQ. This strengthens the conclusion that the role of DT-diaphorase in the protection against QQ is negligible. Indeed, no DT-diaphorase activity was observed when QQ was formed in human liver cytosol (Figure 4.4). In blood plasma, where practically no GSH is present (18) and no DT-diaphorase activity could be detected, addition of DT-diaphorase (10 U/ml) did also not protect against protein thiol loss in blood plasma caused by QQ (Figure 4.5). This indicates that DT-diaphorase also cannot compete with protein thiols for QQ.

Gliszczynska-Swiglo et al. found that quercetin gave a similar reduction in cell proliferation (~ 20%) in wild type chinese hamster ovary (CHO) cells (DT-diaphorase activity 11 $\mu M \cdot min^{-1}$) as in CHO cells over-expressing DT-diaphorase (244 $\mu M \cdot min^{-1}$) (12). They observed that addition of

dicoumerol, an inhibitor of DT-diaphorase, increased the quercetin toxicity in both cell types (cell proliferation was reduced by ~ 70%). The observed lack of difference in quercetin toxicity between wild type cells and cells overexpressing DT-diaphorase made the authors conclude that even in wild type cells DT-diaphorase activity is sufficient for optimal protection against QQ toxicity. An alternative explanation, not considered by Gliszczynska-Swiglo et al., is that DT-diaphorase plays no role in quercetin toxicity. Opposite to the results of Gliszczynska-Swiglo et al., Metodiewa and co-workers observed a significant lower toxicity of quercetin in wild type CHO cells compared to CHO cells over-expressing DT-diaphorase (11). Also opposite to Gliszczynska-Swiglo et al., they found that dicoumerol reduced quercetin-induced toxicity. These conflicting results once again indicate that experiments in cells using "specific" inhibitors are not conclusive and fraught with artefacts.

Our results show that DT-diaphorase efficiently reacts with menadione and that both GSH and ascorbate do not compete with DT-diaphorase for menadione. Similarly, Gliszczynska-Swiglo et al. have shown that menadione toxicity was less in DT-diaphorase enriched cells compared to wild type cells (12). Dicoumerol abolished the protection observed in DT-diaphorase enriched cells against menadione toxicity. This indicates that for menadione toxicity DT-diaphorase might play a role.

The present study demonstrates that GSH reacts much faster than DT-diaphorase with QQ. Moreover, in most body compartments GSH levels are relatively high and DT-diaphorase activities are in the same order of magnitude as the levels used in our experiments. In blood plasma, where GSH is practically absent, protein thiols are the primary reactants of QQ and DT-diaphorase (10 U/ml added) could not protect against the arylation of these protein thiols by QQ (Figure 4.5). Apparently, DT-diaphorase reacts sluggishly with QQ compared to the fast reaction of QQ with protein thiols. Besides DT-diaphorase, also ascorbate cannot protect against protein thiol arylation by QQ [12].

It has been reported that treatment of lymphocytes with quercetin reduces the protein thiol content of the cells (19). Moreover, Walle et al. (2003) has shown that quercetin covalently binds to cellular DNA and proteins in human cell lines (20). As also proposed by these authors (20), conversion of quercetin into QQ is the primary step involved in the covalent binding to proteins (20).

Based on literature and the data of the present study the scheme depicted in Figure 4.6 has been constructed. During its antioxidant activity quercetin becomes oxidized into QQ, which will react with thiols, i.e. with GSH or, when GSH is depleted, with protein thiols. The results of the present study show that DT-diaphorase has no role in the protection against QQ.

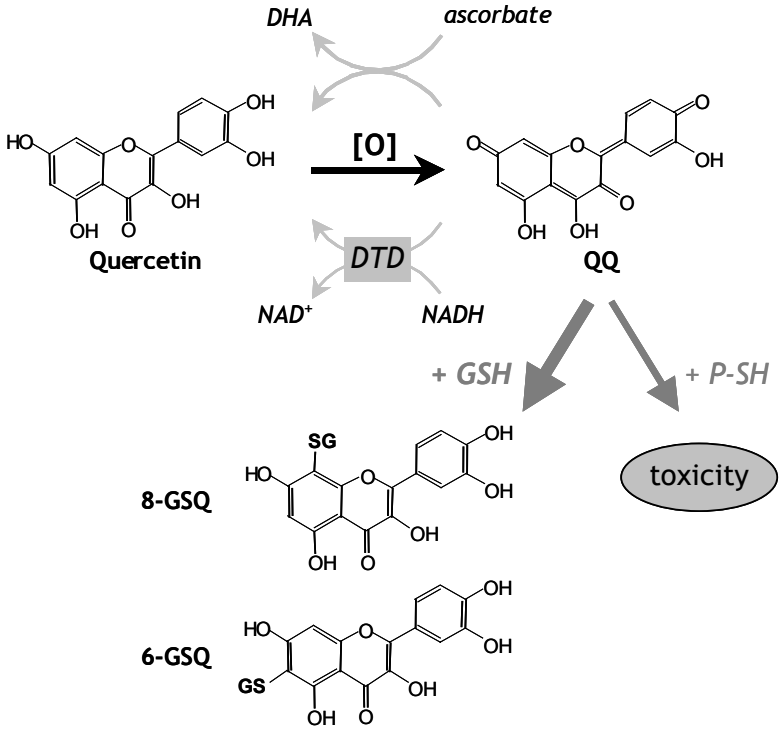

Figure 4.6 Reactions of the oxidation product of quercetin. When quercetin is oxidized, an *o*-quinone/quinonmethide (referred to as QQ) is formed. In the figure only one of the four tautomeric forms of QQ is given. Quercetin can be regenerated from QQ by ascorbate, resulting in the formation of dehydroascorbate (DHA), or by DT-diaphorase (DTD). The latter regenerates quercetin using NADH as cofactor. QQ can also react with glutathione (GSH), thereby forming 6-glutathionylquercetin (6-GSQ) and 8-glutathionylquercetin (8-GSQ).

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