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

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Abstract 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. It was found that QQ is indeed a substrate for DT-diaphorase. However, QQ reacted much faster with glutathione or protein thiols than with DT-diaphorase in experiments with isolated compounds as well as with human liver cytosol or blood plasma. This indicates that DT-diaphorase has no role in the protection against QQ.

Keywords: Oxidative stress; Quercetin; Quinone; Ascorbate; Glutathione; DT-diaphorase

1. 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 defense 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 or blood plasma.

2. Materials and methods

2.1. Materials

Quercetin, reduced 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) were obtained from ICN Biomedicals (Ohio, USA). Human liver was obtained from liver tissue that was removed by surgical excision of a liver tumor from 40 to 60 years old patients. A ''healthy'' part of the excised material that did not contain any tumor 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.

2.2. Methods

All incubations were performed at 37 °C in a 143 mM phosphate buffer, pH 7.4, 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 min 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.

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Abbreviations: QQ, *o*-quinone/quinone methide of quercetin; GSH, glutathione; NADH, reduced β-nicotinamide adenine; 6-GSQ, 6-glutathionyl quercetin; 8-GSQ, 8-glutathionyl quercetin; DTNB, 5′,5′-dithiobis (2-nitrobenzoic acid); HPLC, high pressure liquid chromatography; $OH^{\cdot}$, hydroxyl radical; ONOOH, peroxynitrite

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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 scan speed of 480 nm/min.

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

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 M 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 min 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 min 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 the presence or the absence of 10 U/ml DT-diaphorase plus 0.2 mM NADH. After 5 min 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 (Fig. 4) 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 means or as a typical example. The S.D. of all results was usually less than 5% and always less than 10%. Statistical analysis was performed using Student's *t* test.

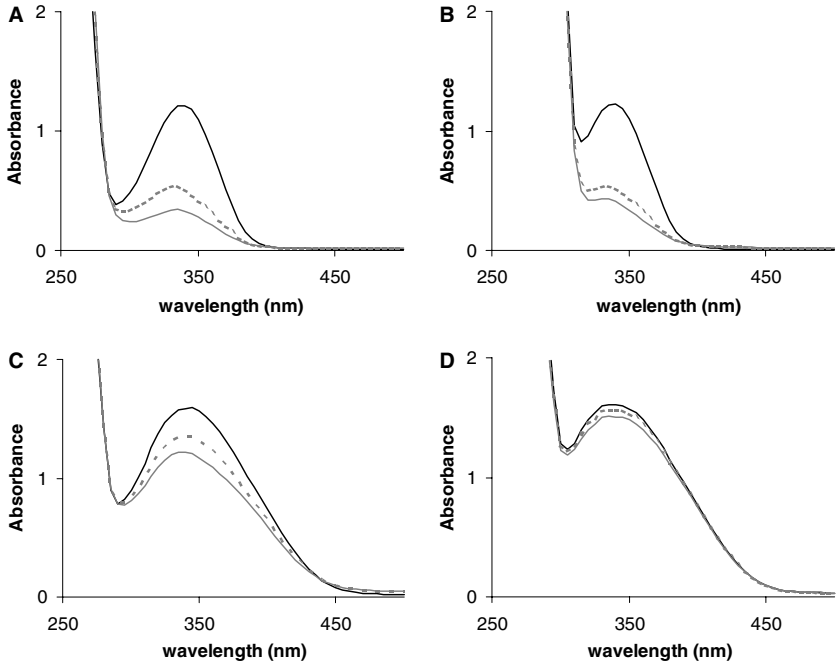

Fig. 1. Repetitive UV scans of the incubation mixture containing 0.2 mM NADH, 10 U/ml DT-diaphorase and 50 μM menadione in the absence (A) or presence (B) of 100 μM ascorbate. The same experiment was also performed with 50 μM quercetin, instead of menadione, in the presence of 25 U/ml tyrosinase and 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 (dotted) or 5 min (gray) after the addition of the enzyme(s). Scan speed was 480 nm/min. A typical example is shown. The reduction of the absorbance at 340 nm (panel A and C), reflecting NADH consumption, demonstrates that QQ and menadione are good substrates for DT-diaphorase. Ascorbate prevented NADH consumption in the incubation mixture where QQ was generated (panel D) but not in the incubation containing menadione (panel B).