Protection against reactive oxygen species by NAD(P)H:quinone reductase induced by the dietary antioxidant butylated hydroxyanisole (BHA)

Decreased hepatic low-level chemiluminescence during quinone redox cycling

Heribert Wefers, Toru Komai, Paul Talalay[+] and Helmut Sies*

Institut für Physiologische Chemie I, Universität Düsseldorf, D-4000 Düsseldorf, FRG and [+]*Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA*

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Menadione elicits low-level chemiluminescence ($\lambda > 620$ nm) associated with redox cycling of the quinone in mouse hepatic postmitochondrial fractions. This photoemission is suppressed when the animals are fed a diet containing the anticarcinogenic antioxidant, 2[3]-(*tert*-butyl)-4-hydroxyanisole (BHA), which leads to a 13-fold increase in NAD(P)H:quinone reductase (EC 1.6.99.2). Inhibition of the enzyme by dicoumarol completely abolishes the protective effect of BHA treatment and leads to higher chemiluminescence, reaching similar photoemission for BHA-treated and control animals. These findings indicate that the two-electron reduction promoted by quinone reductase prevents redox cycling and that BHA protects against reactive oxygen species by elevating the activity of this enzyme.

Reactive oxygen species *Dietary antioxidant* *DT-diaphorase* *Low-level chemiluminescence* *Dicoumarol* *Quinone redox cycling*

1. INTRODUCTION

Cytotoxic effects of quinones, including certain chemotherapeutic agents, have been related to the enzymatic or nonenzymatic formation of the corresponding semiquinones in the cell and their subsequent reaction with molecular oxygen yielding superoxide anion radicals ($O_2^{\cdot -}$) and regenerating the quinone, thus bringing about a redox cycling at the expense of cellular reducing equivalents (review [1]).

For isolated perfused rat liver it has been shown that redox cycling of menadione is accompanied by increased oxygen uptake, loss of cellular GSH and increases in $NADP^+$ and GSSG as well as increased low-level chemiluminescence in the red spectral region [2]. Similar results were reported for isolated hepatocytes with respect to O_2 uptake [3], GSH level [3] and chemiluminescence [2].

The enzyme NAD(P)H:quinone reductase (EC 1.6.99.2; also designated DT-diaphorase) is believed to exert a protective function against such semiquinone redox cycling since it mediates obligatory two-electron reductions [4] to hydroquinones which can be detoxified by conjugation to yield the glucuronide and sulfate in preparation for elimination from the cell ([5,6]; see also [2,3]). Inhibition of this enzyme by dicoumarol produced marked morphological effects in the form of blebbing of isolated hepatocytes supplemented with menadione [3] and was accompanied by an increase of

* To whom correspondence should be addressed

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menadione-induced chemiluminescence in perfused rat liver and isolated hepatocytes [2].

A dramatic increase of the NAD(P)H:quinone reductase specific activity in mouse liver cytosols occurs upon treatment of the animals with the anticarcinogenic antioxidant, 2[3]-(*tert*-butyl)-4-hydroxyanisole (butylated hydroxyanisole; BHA) [7,8]. Thus, the question arises whether quinone toxicity can be lowered by BHA treatment. We present results of chemiluminescence measurements on mouse hepatic post-mitochondrial supernatant fractions supplemented with menadione, providing direct evidence for the protective effect of increased NAD(P)H:quinone reductase activity in BHA-treated mice.

2. MATERIALS AND METHODS

2.1. *Animal treatment*

Female NMRI mice, 5 weeks old, were fed a standard diet (Altromin) containing 7.5 g/kg BHA [7], and experiments were carried out at 8 weeks of age.

2.2. *Preparation of the post-mitochondrial supernatant fraction*

After anaesthetizing the mice the livers were excised, washed and homogenized with 4 vols ice-cold 0.25 M sucrose. Centrifugation of the homogenate for 20 min at 9000 × *g* provided the supernatant fractions (S9) which were used for enzyme assays and chemiluminescence measurements.

2.3. *Enzyme assay*

Determination of NAD(P)H:quinone reductase activity was performed as in [9]. The reaction mixture contained 0.2 mM NADH, 0.7 mg/ml bovine serum albumin and an appropriate amount of S9 in 25 mM potassium phosphate buffer (pH 7.6) at 25°C. The reaction was started by addition of menadione (final concentration, 60 μM) as electron acceptor and was monitored at 340–400 nm using a dual-wavelength spectrophotometer (Model ZWS 11, Biochem. Co., München). Enzyme specific activities presented in table 1 are considerably lower than those reported for CD-1 mice [7,8] although a comparable degree of induction by BHA was observed. These discrepancies are probably attributable to differences in the assay procedure and possibly the strain of mice.

2.4. *Chemiluminescence measurements*

Low-level chemiluminescence measurements were performed as in [9] using a photon-counter equipped with a red sensitive EMI 9658 photomultiplier, maintained at −25°C by a thermoelectric cooler. Photoemission is expressed in counts·s^{-1}. As the involvement of singlet molecular oxygen (dimol emission at 634 and 703 nm) in chemiluminescence during quinone redox cycling has been suggested [2], a 620 nm cut-off gelatin filter (Kodak) was placed between the cuvette and the light guide. Thus, light emission in the blue and green regions of the spectrum is not recorded in our experiments. Chemiluminescence was measured from S9 samples (1.5 mg protein per ml) in 0.1 M potassium phosphate buffer (pH 7.4) in a final volume of 6.5 ml. Samples were gassed with oxygen. NADPH (0.4 mM) and menadione (100 μM) were added through polyethylene tubing.

2.5. *Chemicals*

BHA and dicoumarol were purchased from Sigma (München) and menadione from Fluka (Buchs). Other chemicals or biochemicals were from E. Merck (Darmstadt) or Boehringer-Mannheim (Mannheim).

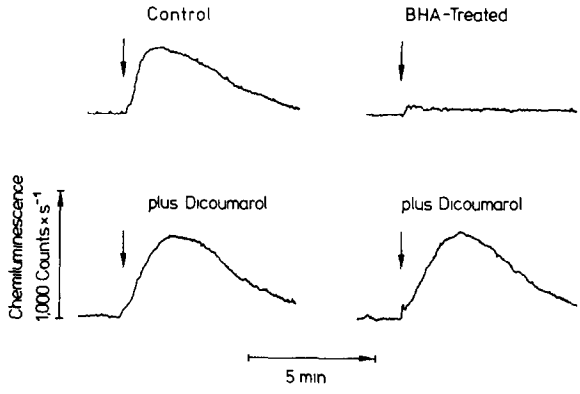

Fig.1. Menadione-induced low-level chemiluminescence (>620 nm) of post-mitochondrial supernatant fractions of mouse liver of controls and animals treated with BHA. Menadione (100 μM) was added at the times indicated by the arrows. NADPH (0.4 mM) had been added 1 min prior to menadione. Protein content was 1.5 mg/ml, dicoumarol 30 μM.

Table 1

Effects of dietary BHA on menadione-induced low-level chemiluminescence and NAD(P)H:quinone reductase activities of mouse hepatic supernatant fractions

Prior treatment of mice	Addition of 30 μM dicoumarol	Activities in post-mitochondrial supernatant fractions		
		NADH oxidation (nmol·min^{-1}·mg^{-1} protein)	NAD(P)H:quinone reductase (nmol·min^{-1}·mg^{-1} protein)	Low-level chemiluminescence (counts·s^{-1})
Control diet	–	10.2 ± 0.4	8.4 ± 0.7	482 ± 47
Control diet	+	1.8 ± 0.8		648 ± 72
BHA diet	–	113.8 ± 6.4	109.6 ± 5.6	68 ± 26
BHA diet	+	4.2 ± 0.8		597 ± 69

The NAD(P)H:quinone reductase activity is defined as the dicoumarol-sensitive part of the total NADH oxidation rate. Chemiluminescence (>620 nm) was detected after addition of menadione (100 μM) under conditions described in fig.1. Data are given as means ± SE of 3–4 experiments

3. RESULTS

Fig.1 shows chemiluminescence traces of mouse post-mitochondrial supernatant (S9) fractions after addition of menadione (100 μM). NADPH was added 1 min prior to menadione and did not produce any light emission. NADPH was added to provide reducing equivalents for both the NADPH-cytochrome P-450 reductase (present as microsomal enzyme in the supernatant) and NAD(P)H:quinone reductase which is located mainly in the cytosol [5]. In controls, 1 min after starting the reaction with menadione, light emission reached a maximum, followed by a slow decrease. In contrast, supernatant fractions from BHA-treated mice yielded a very small light signal upon addition of menadione. After inhibition of the NAD(P)H:quinone reductase by dicoumarol (fig.1, lower) both the post-mitochondrial supernatant fractions of treated animals and controls exhibited increased chemiluminescence, reaching similar levels (fig.1, table 1).

Fig.2 indicates that, in the absence of dicoumarol, in hepatic supernatants of BHA-treated and control animals, the concentration of menadione has to exceed a certain level to yield a marked chemiluminescence increase, whereas in the controls chemiluminescence is already observed at concentrations somewhat higher than 30 μM. Thus, BHA treatment leads to a shift of sensitivity towards menadione by about one order of magnitude. Interestingly, this corresponds to the increase in enzyme activity by a factor of 13 (table 1). Thus within a certain concentration

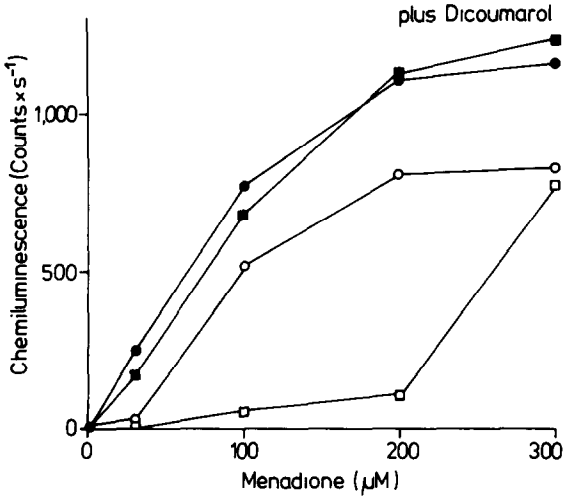

Fig.2. Protective effect of NAD(P)H:quinone reductase on the formation of light-emitting species during menadione redox cycling. Menadione at varying concentrations was added to hepatic post-mitochondrial supernatant fractions of BHA-treated animals (□, ■) and controls (○, ●) in the absence (□, ○) and presence (■, ●) of dicoumarol. Conditions as described in fig.1.

range (roughly between 50 and 250 μM) a clear protective effect by BHA treatment is observed whereas at 300 μM menadione this protection appears to be overwhelmed. At all concentrations examined, the presence of dicoumarol leads to higher chemiluminescence, and there is no significant difference in the levels reached in BHA-treated or untreated animals.

4. DISCUSSION

Low-level chemiluminescence reflects the formation of excited species such as singlet molecular oxygen [8]. Our results provide further evidence for the assumption that these excited species are generated during quinone metabolism when one-electron reduction of the quinone is involved, but not during two-electron reduction. One-electron reduction results from microsomal NADPH-cytochrome P-450 reductase [4] and yields $O_2^{\cdot -}$ via reaction of molecular oxygen with the semiquinone. Two-electron reduction yields the hydroquinone which subsequently is a substrate for conjugating enzymes such as glucuronyltransferases and sulfotransferases. Low-level chemiluminescence as described here is thus interpreted as a consequence of NADPH-consuming redox cycling, promoted by the NADPH-cytochrome P-450 reductase, yielding $O_2^{\cdot -}$ and excited species such as singlet oxygen. Consequently, an increased NAD(P)H:quinone reductase activity with concomitant decrease of semiquinone and $O_2^{\cdot -}$ formation produced markedly lowered photoemission.

Prior treatment with an antioxidant such as BHA has been shown to increase not only the NAD(P)H:quinone reductase but also GSH transferase and epoxide hydrolase activities [9] and these enzymes may also be important in quinone metabolism. However, the effect of dicoumarol as a specific inhibitor of the NAD(P)H:quinone reductase shows that the observed differences in chemiluminescence between post-mitochondrial supernatants of BHA-treated mice and controls are due to the NAD(P)H:quinone reductase, as they disappear in the presence of the inhibitor (fig.1, table 1). The cytotoxicity of quinones, including therapeutic agents such as certain anticancer drugs, is believed to be related to oxidative stress due to $O_2^{\cdot -}$ formation and subsequent reactions of this species. The results presented here support the protective action of BHA treatment, and furthermore, indicate the practicability of chemiluminescence measurements in monitoring changes in quinone metabolism.

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