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SHORT COMMUNICATION

Protective Action of Cardiac DT-diaphorase against Menadione Toxicity in Guinea Pig Isolated Atria

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ABSTRACT. In myocardial preparations isolated from guinea pigs, 2-methyl-1,4-naphthoquinone (menadione) causes an increase in contractility that is strictly related to the generation of reactive oxygen species (ROS) as a consequence of quinone metabolism. In heart, menadione undergoes one-electron reduction to semiquinone, a reaction mainly catalysed by mitochondrial NADH: ubiquinone oxidoreductase. It is also converted to hydroquinone by the soluble two-electron reductase, DT-diaphorase, and is conjugated with GSH by glutathione *S*-transferase. In order to assess the role of DT-diaphorase in cardiac responses to menadione, we examined the effects of both a specific inhibitor (dicoumarol) and an inducer (β-naphthoflavone) of the enzyme on the inotropic action of the quinone. In electrically driven left atria of guinea pig, 4 μM dicoumarol significantly enhanced the positive inotropic effect of menadione, especially at the lower concentrations of the quinone. In myocardial preparations isolated from guinea pigs treated with β-naphthoflavone (80 mg/kg i.p.for 2 days), DT-diaphorase activity was enhanced (+36% with respect to control animals, $P < 0.01$), whereas the activities of the other enzymes involved in menadione metabolism were not modified. In these preparations, menadione caused a significantly lower increase in the force of contraction than in atria from untreated animals; moreover, pretreatment with β-naphthoflavone caused a significant decrease in the menadione-induced oxidative stress, as evaluated from the GSH redox index. Taken together, these results demonstrate that cardiac DT-diaphorase does not contribute to ROS generation, but represents a detoxification system. BIOCHEM PHARMACOL **60**;4: 601–605, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. DT-diaphorase; menadione; reactive oxygen species; dicoumarol; β-naphthoflavone; guinea pig atria

In guinea pig isolated myocardial preparations, menadione produces an increase in the force of contraction, which is mainly due to catecholamine release from adrenergic stores [1, 2]. This inotropic effect is followed by a marked increase in resting tension, leading to systolic arrest. The catecholamine-mediated positive inotropic effect is strictly related to the amount of ROS† produced by the reduction of the quinone [3]. In heart, menadione is converted to a semiquinone free radical by the mitochondrial NADH: ubiquinone oxidoreductase and, to a much more limited extent, by the microsomal NADPH-cytochrome P450- and NADH-cytochrome b_5 reductases [3–5]. The semiquinone enters a redox cycle, with generation of the parent compound and ROS [6, 7]. It has also been shown that menadione can be transformed in liver [8] and heart [4] to hydroquinone via a two-electron reduction catalysed by the soluble NAD(P)H: (quinone acceptor) oxidoreductase, also known as DT-diaphorase. It has been proposed that in liver DT-diaphorase serves as a cellular control device against quinone toxicity, since inhibition of the enzyme by its specific inhibitor dicoumarol potentiates the toxicity of the quinone [9], whereas its induction by 3-methylcholanthrene protects rat hepatocytes against menadione toxicity [8]. However, it was not verified whether 3-methylcholanthrene also induced GST, an enzyme known to protect cells from free radical damage [10].

The role of cardiac DT-diaphorase in the heart responses to menadione has not been assessed. The uncertainty regarding the role of this enzyme derives from the *in vitro* observation that the hydroquinone of menadione can also generate ROS, following autoxidation to the semiquinone [3, 4, 11]. Indeed, the reaction catalysed by DT-diaphorase contributes to the cytotoxic activity of certain quinones, such as diaziquone [see 12 and references therein].

The aim of the present study was to ascertain whether cardiac DT-diaphorase contributes to the ROS-mediated positive inotropic effect of menadione or represents a detoxification system. For this purpose, we tested the effect of menadione on isolated atria either in the presence of dicoumarol or following treatment of guinea pigs with BNF, a synthetic flavonoid with induction properties quite similar to those of 3-methylcholanthrene [13].

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† *Abbreviations:* BNF, β-naphthoflavone; ROS, reactive oxygen species; SOD, superoxide dismutase; 7-EROD, 7-ethoxyresorufin *O*-deethylase; and GST, glutathione *S*-transferase.

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MATERIALS AND METHODS

Animal Treatment

Male albino guinea pigs (300–350 g body weight), obtained from Rodentia, were treated i.p. with 80 mg/kg of BNF (dissolved in corn oil) daily for 2 days [14]. An equal amount (1 mL) of the vehicle was injected into the control animals for 2 days. Twenty-four hours after the last injection, all animals were killed by a blow to the head, followed by exsanguination, and the organs (heart and liver) were removed immediately.

Isolated Myocardial Preparations

Left atria isolated from control and BNF-treated guinea pigs were suspended vertically in a 16-mL organ bath containing a solution constantly gassed with 95% O_2 and 5% CO_2, at 29°. The bathing solution contained (in mM): NaCl 120, KCl 2.7, $CaCl_2$ 1.36, $MgCl_2$ 0.09, NaH_2PO_4 0.4, $NaHCO_3$ 12, glucose 5 (pH 7.4). The atria were electrically driven at 1 Hz by square-wave pulses of 0.6–0.9 msec duration (S44 stimulator, Grass Instruments). The force of contraction was recorded on an isometric force transducer (7003 Basile, Comerio) connected to a rectilinear recorder (KV 135 Battaglia Rangoni, Casalecchio di Reno). In each atrial preparation, we determined the response to 0.2 μM isoprenaline before the addition of menadione. This response was considered the maximum positive inotropic effect (E_{max}) obtainable in such preparations. Menadione was added cumulatively, and the inotropic effect caused by each quinone concentration was recorded before a higher concentration was tested. The effect of menadione was defined as the difference between the force of contraction before and after its addition to the bathing fluid, and was expressed as a percentage of the response (E_{max}) induced by isoprenaline (0.2 μM) in the same preparation.

Preparation of Subcellular Fractions from Heart and Liver

The cardiac tissue remaining after isolation of the left atrium and the liver of each animal were homogenised in 3 volumes (w/v) of 0.25 M sucrose–100 mM Tris (pH 7.4). From this homogenate, liver and heart microsomal and soluble (105,000 *g* supernatant) fractions were prepared by conventional techniques [15]. Protein content was determined by the method of Lowry *et al.* [16].

Enzymatic Assays

In the liver microsomal fraction, 7-EROD activity was measured by the fluorometric method of Burke *et al.* [17] using 5 μM 7-ethoxyresorufin as substrate. Fluorescence emission at 585 nm was monitored, for 2 min at 37°, in a Perkin-Elmer LS3-50 spectrofluorometer after excitation at 530 nm. The amount of resorufin formed was calculated from a calibration curve obtained with known amounts (from 50 to 600 pmol) of authentic resorufin. In the cardiac-soluble fraction, the activity of DT-diaphorase was evaluated by determining the rate of the two-electron reduction of 1 μM menadione according to the method of Lind *et al.* [8], modified as previously described [4]. Briefly, the rate of menadione reduction was determined by following, for 3 min at 30°, the disappearance of NADPH at 340 nm, and DT-diaphorase activity was calculated as that portion of the total activity that was inhibited by dicoumarol (30 μM). GST activity was determined using 0.25 mM 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, according to the method of Habig *et al.* [18]. The reaction was followed (for 2 min at 30°) at 340 nm, and GST activity was calculated using a $\Delta\epsilon$ of 9.6 mM^{-1} cm^{-1} [18].

GSH and GSSG Content

Left atria isolated from normal and BNF-treated guinea pig were rapidly frozen in liquid nitrogen. They were then pulverised in a cooled ceramic percussion mortar after the addition of 2 mL of 6% metaphosphoric acid. The tissue suspension was briefly homogenised, centrifuged at 27,000 *g* for 20 min, and the pellet then discarded. On the clear supernatant, GSH and GSSG contents were immediately determined by an enzymatic procedure, as previously described [19].

Statistical Analysis

Results were expressed as arithmetic means ± SE. Comparisons were made by one-way analysis of variance (ANOVA). In the case of significant differences (α: 0.05), the ANOVA was followed by the Student–Newman–Keuls test for a pairwise comparisons of the means. A P value of less than 0.05 was considered statistically significant.

Chemicals

Menadione, BNF, 7-ethoxyresorufin, resorufin, dicoumarol, NADPH, CDNB, GSH, GSSG, isoprenaline, albumin, SOD from bovine erythrocytes, and DMSO were from Sigma Chemical Co. All other reagents were of analytical grade and were used as received.

RESULTS AND DISCUSSION

We have previously demonstrated that a direct relationship exists between the positive inotropic effect caused by menadione in isolated myocardial preparations and the amount of ROS generated through its metabolism [3]. Here, we further confirm the involvement of ROS in the cardiac effect of menadione by showing that the addition of SOD decreases, in a dose-dependent manner, the inotropic action of the quinone (Fig. 1).

As mentioned above, ROS arise from the reductive metabolism of menadione by the one-electron reductases [5–7, 9]. However, *in vitro* experiments [3, 4, 11] indicated

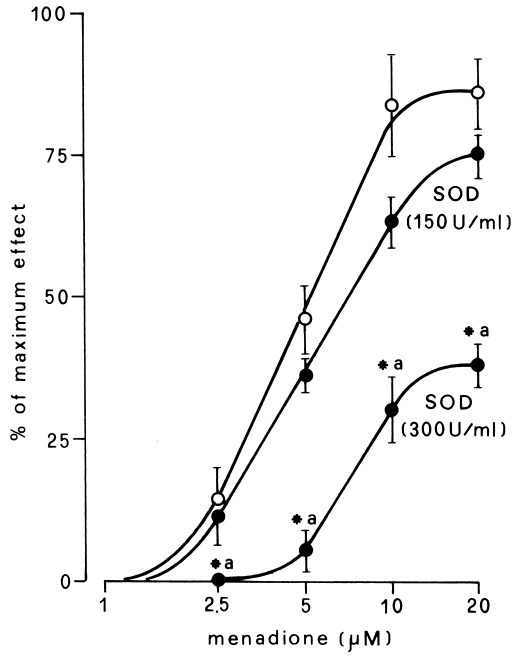

FIG. 1. Cumulative concentration–response curves for the positive inotropic effect of menadione in electrically driven (1 Hz) left atria in the presence of 150 and 300 U/mL of SOD. The enzyme, added 15 min before menadione, did not modify the force of contraction of atria. The effects of menadione were calculated as the differences in the force of contraction before and after menadione addition, and expressed as percentages of the maximum increase in the force of contraction induced by 0.2 μM isoprenaline in the same preparation (+167 ± 7% compared to basal force of contraction). Data are means ± SE of experiments carried out in four different myocardial preparations for each protocol group. The open circles represent the results obtained in left atria in the absence of SOD; the closed circles represent those obtained in the presence of the increasing concentrations (150 or 300 U/mL) of SOD. *P < 0.05 vs control. [a]P < 0.05 vs 150 U/mL of SOD.

that ROS generation may also result from the autoxidation of the hydroquinone formed by the soluble DT-diaphorase [8], the enzyme endowed with the highest affinity for menadione [9]. In order to assess the role of DT-diaphorase in the cardiac metabolism of this quinone and, consequently, in its cardiac effect, two types of experiments were designed. First, dicoumarol, a specific inhibitor of DT-diaphorase, was added to the bathing fluid of left atria before the addition of increasing concentrations of menadione. For these experiments, we used a dicoumarol concentration (4 μM) which has been shown to produce complete inhibition of DT-diaphorase [20] without affecting GSH-related enzymes [21]. As reported in Table 1, dicoumarol produced an increase in the positive inotropic effect of menadione, which was more evident at the lowest concentration of the quinone. Since the positive inotropic effect of menadione is strictly related to ROS generation [3], this result suggests that cardiac DT-diaphorase does not produce ROS but, on the contrary, competes with the one-electron reductases for the biotransformation of menadione. The observation that the effect of dicoumarol

TABLE 1. Effect of dicoumarol on the positive inotropic action caused by increasing concentrations of menadione in guinea pig left atria

	% of E_{max}		
Addition	2.5 μM menadione	5 μM menadione	10 μM menadione
None	14.5 ± 5.5	47.0 ± 7.7	85 ± 15
Dicoumarol	40.6 ± 3.0*	59.8 ± 4.5	82 ± 5

Dicoumarol (4 μM), added in DMSO 10 min before the addition of increasing concentrations of menadione, did not modify the force of contraction of atria. An equal amount of DMSO (10 μL) was added to control atria. The effects of menadione were calculated as reported in the legend to Fig. 1. Data are means ± SE of four experiments carried out on four different myocardial preparations.

*$P < 0.01$ vs control.

decreases as the concentration of menadione is raised can be explained by two considerations: i) DT-diaphorase possesses a higher affinity for menadione than the one-electron reductases [4, 9]; therefore, at the lower concentrations of menadione, a larger proportion of the quinone is metabolised by DT-diaphorase (see below) and the effect of its inhibition is, consequently, more evident; ii) the increase in contractility caused by dicoumarol may not be evident at the highest concentration of menadione since, at this concentration, the effect of the quinone has virtually reached its maximum (see Fig. 2, below). These results indicate that, in isolated myocardial preparations, DT-diaphorase serves as a detoxification pathway for menadi-

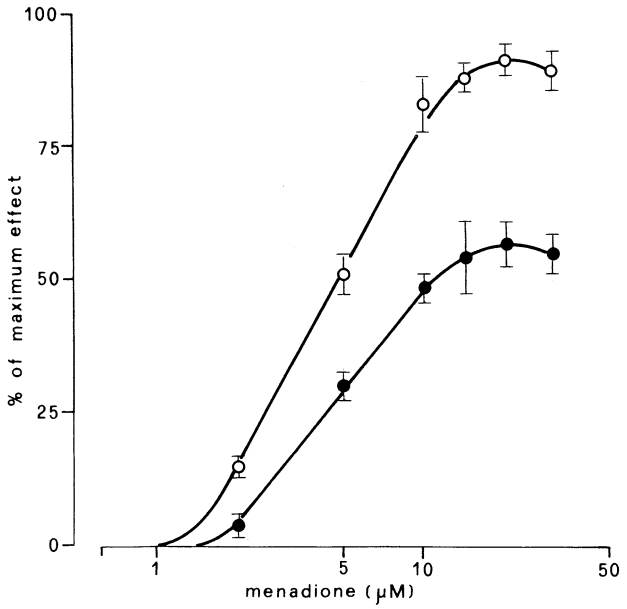

FIG. 2. Cumulative concentration–response curves for the positive inotropic effect of menadione in electrically driven (1 Hz) left atria isolated from control (○) and BNF-treated (●) guinea pigs. Menadione effects were calculated as reported in the legend to Fig. 1. Data are means ± SE of experiments carried out in myocardial preparations from six animals for each protocol group. Differences were statistically significant ($P < 0.05$) at all menadione concentrations.

TABLE 2. Effect of BNF treatment on DT-diaphorase and GST activities of cardiac tissue

Treatment	DT-diaphorase activity (μmol/g tissue/min)	GST activity (μmol/g tissue/min)
None	0.606 ± 0.047	3.03 ± 0.30
BNF	0.825 ± 0.025*	2.96 ± 0.10

DT-diaphorase and GST activities were measured in the soluble fractions isolated from cardiac tissue of four animals for each protocol group (see Materials and Methods). The activities (μmol/g tissue/min) were calculated by referring the activities per milligram of protein to the amount of proteins present in the soluble (105,000 *g* supernatant) fraction recovered from 1 g of cardiac tissue. Amounts of recovered proteins were 29.98 ± 0.58 and 30.05 ± 0.08 mg/g tissue in control and BNF-treated animals, respectively. Results are means ± SE of four determinations carried out in duplicate.

*$P < 0.01$ vs controls.

one, in accordance with previous observations on hepatocytes in which dicoumarol was shown to exacerbate the menadione effects [9].

A second line of experiments was carried out using atria isolated from guinea pigs pretreated with BNF (80 mg/kg i.p. for 2 days) to induce DT-diaphorase. In a set of preliminary experiments designed to assess the efficacy of the BNF protocol, 7-EROD, an activity ascribed to the BNF-inducible CYP1A family of cytochrome P450 [22], was measured in microsomes isolated from livers of control and BNF-treated guinea pigs. Microsomal 7-EROD was induced about 4 times by treatment with BNF, the activity being 0.120 ± 0.017 and 0.422 ± 0.031 nmol/mg protein/min in control and BNF-treated animals, respectively. Having confirmed the effectiveness of the BNF treatment, we measured the metabolism of menadione in subcellular preparations of cardiac tissue. As expected, BNF treatment did not modify the activity of the enzymes responsible for the one-electron reduction of menadione, namely mitochondrial NADH: ubiquinone oxidoreductase, microsomal NADH-cytochrome b_5 reductase and NADPH-cytochrome P450 reductase (data not shown), since these enzymes are not induced by BNF [23, 24]. On the contrary, the activity of soluble DT-diaphorase was significantly affected by BNF treatment (Table 2).

Since conjugation with glutathione by GST constitutes a cellular detoxification pathway against electrophilic substances such as menadione [10], and an isoform of GST is induced in mouse by BNF [23, 24], we also measured GST activity in the cardiac tissue from control and BNF-treated guinea pigs. As shown in Table 2, GST activity was not modified by BNF treatment, an observation in accordance with the finding of Falkner *et al.* [25] that BNF does not induce GST in guinea pig liver, kidney, or lung. These results exclude the possibility that the protective effect of BNF treatment against the cardiac action of menadione (Fig. 2) may be mediated by an increased conjugation of the quinone with GSH. The cumulative concentration–response curves shown in Fig. 2 indicate that the positive inotropic effect caused by increasing concentrations of menadione was significantly lower in atria isolated from BNF-treated animals. In contrast, EC_{50} values for the positive inotropic effect remained unchanged (about 5 μM). In atria isolated from BNF-pretreated animals, the response to isoprenaline was not modified (data not shown). This excludes the possibility that the reduced response to menadione following BNF treatment was due to a decreased ability of the heart to respond to catecholamines. Although it may not be readily apparent from Fig. 2, the percent reduction in the positive inotropic effect of menadione in myocardial preparations from BNF-treated animals became progressively lower (from 74 to 39%) upon increasing menadione concentration from 2 to 20 μM. This is consistent with the observation that the contribution of DT-diaphorase to total menadione metabolism (as deduced from measurements of the rate of quinone reduction by the cardiac homogenate in the absence or presence of dicoumarol) decreased from almost 100% to 50% when the menadione concentration was raised from 2 to 20 μM.

The finding that induction of DT-diaphorase, without modification of GST activity, reduces the ROS-mediated inotropic effect of menadione confirms the indication obtained from the experiments with dicoumarol, namely that cardiac DT-diaphorase prevents ROS generation. The results of the measurement of the GSH redox index, a marker of the cell redox state [26], are also consistent with this conclusion. In these experiments, the levels of reduced and oxidised glutathione were determined in the left atria isolated from control and BNF-treated guinea pigs both in the absence and presence of menadione. Table 3 clearly

TABLE 3. Effect of menadione on GSH and GSSG contents of left atria isolated from control and BNF-treated guinea pigs

	Basal		After menadione treatment	
	Controls	BNF-treated	Controls	BNF-treated
GSH (μmol/g tissue)	1.21 ± 0.01	1.25 ± 0.03	0.505 ± 0.06*	0.770 ± 0.039*†
GSSG (μmol/g tissue)	0.045 ± 0.002	0.048 ± 0.002	0.126 ± 0.025‡	0.063 ± 0.004‡§
GSH redox index	0.144	0.140	0.030	0.071

GSH and GSSG contents were measured in atria from control and BNF-treated guinea pigs after incubation in organ bath solution in the absence of menadione (basal values) and in atria isolated from control and BNF-treated animals after the addition of increasing concentrations of menadione for determination of the positive inotropic effect (see Fig. 2). GSH redox index was calculated as ([GSH] + 2[GSSG])/(2[GSSG] × 100), according to Benzi and Moretti [26]. Data are means ± SE of determinations carried out in duplicate using four different myocardial preparations for each protocol group.

§$P < 0.05$ and †$P < 0.01$ vs data obtained after menadione treatment in atria isolated from control animals.

‡$P < 0.05$ and *$P < 0.001$ vs the corresponding data obtained in the absence of menadione.

shows that the basal contents of GSH and GSSG were not modified by BNF treatment. In atria from control guinea pigs, menadione caused a marked reduction in the GSH level with a concomitant increase in GSSG. This resulted in a 5-fold decrease in the GSH redox index, indicating that menadione caused a considerable oxidative stress. By contrast, menadione-induced oxidative stress was much less evident in left atria isolated from BNF-treated animals, where the redox index was only reduced by about 50%.

In conclusion, the results of this study confirm that the cardiac effects of menadione are mediated by ROS generated during reduction of the quinone. They also show that these effects can be enhanced or decreased by inhibition or induction, respectively, of DT-diaphorase. These observations demonstrate that the two-electron reduction of menadione by cardiac DT-diaphorase decreases ROS generation by competition with the one-electron reductases.

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