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Single-electron reduction of quinone and nitroaromatic xenobiotics by recombinant rat neuronal nitric oxide synthase

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We examined the kinetics of single-electron reduction of a large number of structurally diverse quinones and nitroaromatic compounds, including a number of antitumour and antiparasitic drugs, and nitroaromatic explosives by recombinant rat neuronal nitric oxide synthase (nNOS, EC 1.14.13.39), aiming to characterize the role of nNOS in the oxidative stress-type cytotoxicity of the above compounds. The steady-state second-order rate constants (k_{cat}/K_m) of reduction of the quinones and nitroaromatics varied from 10^2 $M^{-1}s^{-1}$ to 10^6 $M^{-1}s^{-1}$, and increased with an increase in their single-electron reduction potentials (E^1_7). The presence of Ca^{2+}/calmodulin enhanced the reactivity of nNOS. These reactions were consistent with an "outer sphere" electron-transfer mechanism, considering the $FMNH^{\cdot}/FMNH_2$ couple of nNOS as the most reactive reduced enzyme form. An analysis of the reactions of nNOS within the 'outer sphere' electron-transfer mechanism gave the approximate values of the distance of electron transfer, 0.39–0.47 nm, which are consistent with the crystal structure of the reductase domain of nNOS. On the other hand, at low oxygen concentrations ($[O_2]$ = 40–50 μM), nNOS performs a net two-electron reduction of quinones and nitroaromatics. This implies that NOS may in part be responsible for the bioreductive alkylation by two-electron reduced forms of antitumour aziridinyl-substituted quinones under a modest hypoxia.

Key words: Neuronal nitric oxide synthase (nNOS), quinones, nitroaromatic compounds, electron transfer mechanism, antitumour agents, oxidative stress

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INTRODUCTION

Nitric oxide synthases (NOS, EC 1.14.13.39) are dimeric flavohemoproteins that catalyze the conversion of *L*-arginine to citruline and nitric oxide (NO) at the expense of NADPH. Each monomer of NOS consists of a heme domain with tetrahydrobiopterin bound at its N-terminus, and a FAD- and FMN-containing reductase domain at its C-terminus. The reductase and oxygenase domains are linked by a calmodulin (CAM)-binding sequence (Masters *et al.*, 1996; Alderton *et al.*, 2001; Xia *et al.*, 2009, and references therein). The reductase domain of NOS is highly similar to the same as the microsomal NADPH:cytochrome P-450 reductase (P-450R, EC 1.6.2.4) (Zhang *et al.,* 2001). In the catalytic cycle, redox equivalents are transferred in the pathway NADPH → FAD → FMN → heme (tetrahydrobiopterin), involving mainly one- and three-electron reduced states of the reductase domain in the turnover (Matsuda & Iyanagi, 1999; Guan *et al.,* 2003; Wei *et al.,* 2008, and references there). The Ca^{2+}-dependent binding of CAM enhances the rate of electron transfer in the reductase domain and from the reduced FMN to heme, inducing the conformational changes and the displacement the repeatedly bound NADPH at the FAD subdomain (Craig *et al.,* 2002; Garcin *et al.*, 2004, and references therein)

NOS reduces quinones and nitroaromatic compounds in a single-electron way *via* $FMNH_2$ and, possibly, $FADH_2$. without an involvement of the heme moiety (Matsuda & Iyanagi, 1999; Kumagai *et al.*, 1998; Fu *et al.*, 2004; Kumagai *et al.*, 2004; Chandor *et al.*, 2008, and references therein). Under aerobic conditions, the quinone and nitroaromatic radicals are reoxidated by dioxygen, which is accompanied by the formation of superoxide and other reactive oxygen species. Thus, NOSs may contribute to the 'oxidative stress-type' cytotoxicity of quinones and nitroaromatics. In addition, quinones and nitroaromatics inhibit the formation of NO by NOS, either by diverting the electron flux from the flavin reductase domain to the heme (Kumagai *et al.*, 1998, 2004) or by trapping of NO with superoxide resulting in the formation of peroxynitrite (Miller, 2002). However, the quantitative structure-activity relationships in the reduction of quinones and nitroaromatic compounds by NOS have not been studied are so far.

In this study, we examine the steady-state reduction kinetics of a large number of structurally diverse quinones and nitroaromatic compounds by recombinant rat neuronal NOS (nNOS). Apart from the elucidation of the reactivity and structural features of nNOS relevant to the electron transfer, our data demonstrate that nNOS may be responsible for a net two-electron reduction of several antitumour low-potential aziridinyl-substituted quinones under a modest hypoxia. Our data show that single-electron transferring flavoenzymes may be part-

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Abbreviations: AZQ, 2,5-diaziridinyl-3,6-*bis*(ethoxycarbonylamino)-1,4-benzoquinone; BZQ, 2,5-*bis*(2′-hydroxyethylamino)-3,6-diaziridinyl-1,4-benzoquinone; CAM, calmodulin; CB-1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide; DZQ, 2,5-diaziridinyl-1,4-benzoquinone; E^1_7, single-electron reduction potential at pH 7.0; k_{cat}, catalytic constant; k_{cat}/K_m, apparent second-order rate constant; k_{11}, k_{22}; electron self-exchange rate constant; MeDZQ, 2,5-diaziridinyl-3,6-dimethyl-1,4-benzoquinone; nNOS, neuronal nitric oxide synthase; $nNOS_{red}$, reductase domain of neuronal nitric oxide synthase; P-450R, cytochrome P450 reductase; RH1, 2,5-diaziridinyl-3-hydroxymethyl-6-methyl-1.4-benzoquinone; tetryl, 2,4,6-trinitrophenyl-*N*-methylnitramine; TNT, 2,4,6-trinitrotoluene.

ly responsible for the specific mode of cytotoxicity of aziridinyl-substituted quinones, their bioreduction to DNA-alkylating species.

MATERIALS AND METHODS

Materials. Recombinant rat neuronal nitric oxide synthase (nNOS) was purified as described (Chandor *et al.*, 2008), and its concentration was determined from the absorbance of its ferrous heme-CO complex using an extinction coefficient of $\varepsilon_{450} = 76$ mM^{-1} cm^{-1} (Stuehr & Ikeda-Saito, 1992). The activity of nNOS determined according to the assay of [^{3}H]-L-citrulline formation (Chandor *et al.*, 2008), was equal to 280 ± 50 nmol × min^{-1} × mg protein^{-1}. Aziridinyl-substituted quinones DZQ (2,5-diaziridinyl-1,4-benzoquinone), AZQ (2,5- diaziridinyl-3,6-*bis*(ethoxycarbonylamino)-1,4-benzoquinone), RH1 (2,5-diaziridinyl-3-hydroxymethyl-6-methyl-1.4-benzoquinone), MeDZQ (2,5-diaziridinyl-3,6-dimethyl-1,4-benzoquinone), and BZQ (2,5-*bis*(2'-hydroxyethylamino)-3,6-diaziridinyl-1,4-benzoquinone) (Fig. 1) were synthesized as described previously (Nemeikaitė-Čėnienė *et al.,* 2003). 2,4,6-Trinitrotoluene (TNT) and 2,4,6-trinitrophenyl-*N*-methylnitramine (tetryl) were synthesized as previously (Šarlauskas *et al.*, 2006). 5-(Aziridin-1-yl)-2,4-dinitrobenzamide (CB-1954), synthesized as previously (Miškinienė *et al.*, 1999), was a generous gift of Dr. Vanda Miškinienė (Institute of Biochemistry of Vilnius University, Vilnius, Lithuania). All the synthesized compounds were characterized by their melting points, ^{1}H-NMR and IR spectra. Nifuroxim, nitrofurantoin, other nitroaromatic compounds and quinones, NADPH, cytochrome *c*, CAM, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and other chemicals were obtained from Merck, Fluka, or Sigma. The formulae of nontrivial quinone and nitroaromatic compounds are given in Fig. 1.

Kinetic studies. The steady-state kinetic measurements were carried out spectrophotometrically in 0.1 M Tris/HCl (pH 7.0) containing 1 mM EDTA at 25°C, using Hitachi-557 or Perkin-Elmer Lambda 25 UV-VIS spectrophotometers. The concentrations of 10-100 nM nNOS were used in the experiments. The initial rates of reduction of quinones and nitroaromatics were monitored according to the oxidation rates of 50 μM NADPH ($\Delta\varepsilon_{340} = 6.2$ mM^{-1} cm^{-1}). The rates obtained were corrected for the intrinsic NADPH oxidase activity of nNOS. The rates of reduction of cytochrome *c* and ferricyanide were determined using $\Delta\varepsilon_{550} = 20$ mM^{-1} cm^{-1} and $\Delta\varepsilon_{420} = 1.0$ mM^{-1} cm^{-1}, respectively. For nNOS-catalyzed reduction of high-potential 1,4-benzoquinone compounds ($E'_7 > 0.0$ V) and ferricyanide, the initial rates were corrected for the nonenzymatic oxidation of NADPH. k_{cat} corresponded to the number of moles of NADPH oxidized per mole of enzyme per second. In some experiments, CAM (10 μg/ml) and 1 mM $CaCl_2$ were added to the reaction mixture. The reduction of azidinyl-substituted 1.4-benzoquinones and *p*-dinitrobenzene (50 μM) by nNOS in the absence of external oxygen supply was monitored using an NADPH-regeneration system (15 μM NADPH, 10 mM glucose-6-phosphate, and 50 U/ml glucose-6-phosphate dehydrogenase) at the respective specific absorbance maximum wavelengths of the compounds. In those experiments, a sealed 3.5-ml spectrophotometer cell was completely filled with the buffer solution containing the reaction system. The reaction was initiated by the injection of enzyme. In parallel experiments, the time course of the oxygen uptake was monitored using Clark electrode (Digital Model 10, Rank Brothers) at 25°C.

Kinetic parameters were determined at a fixed concentration of NADPH (50 μM) and varied concentrations of oxidants. Typically, 6–8 concentrations of the compounds were used. The catalytic (k_{cat}) and apparent second-order rate (k_{cat}/K_m) constants of reduction of the compounds were obtained in a nonlinear way by a standard hyperbolic expression using SigmaPlot 2000 (version 6.1) or Statistica (version 4.30, Statsoft, 1993).

RESULTS

Quantitative structure-activity relationships in nNOS-catalyzed reduction of quinones and nitroaromatics

Because the K_m for NADPH in NOS-catalyzed reactions is in the micromolar range (Guan *et al.,* 2003), the measurements were performed at a single saturating concentration of NADPH, 50 μM. We examined the steady-state reactions of nNOS with quinones and nitroaromatic compounds with a wide range of single-electron reduction potentials, E'_7 = from –0.06 V to –0.485 V, which reflect the energetics of their single-electron reduction. The corresponding kinetic parameters (k_{cat} and k_{cat}/K_m) for the examined compounds are given in Table 1, together with the data of several single-electron inorganic oxidants for comparison. One may note that the k_{cat} and k_{cat}/K_m for reduction of 2-methyl-1,4-naphthoquinone (menadione), ferricyanide, and cytochrome *c* are close to those previously reported for their reduction by the flavin reductase domain of nNOS (Matsuda & Iyanagi, 1999; Matsuda *et al.,* 2000). The addition of Ca^{2+}/CAM increased the reactivity of some aziridinyl-substituted 1,4-benzoquinones and ferricyanide by 2–5-fold (Table 1), which is in line with the previously observed enhancement of $FMNH_2$-dependent reactions by CAM (Matsuda & Iyanagi, 1999; Matsuda *et al.,* 2000; Chandor *et al.*, 2008). It is important to note that in the reactions of nNOS, the reactivity of a large number of quinones and nitroaromatic compounds ($n = 34$) was characterized by two separate parabolic dependences of their log k_{cat}/K_m on E'_7 (Fig. 2A, B). We failed

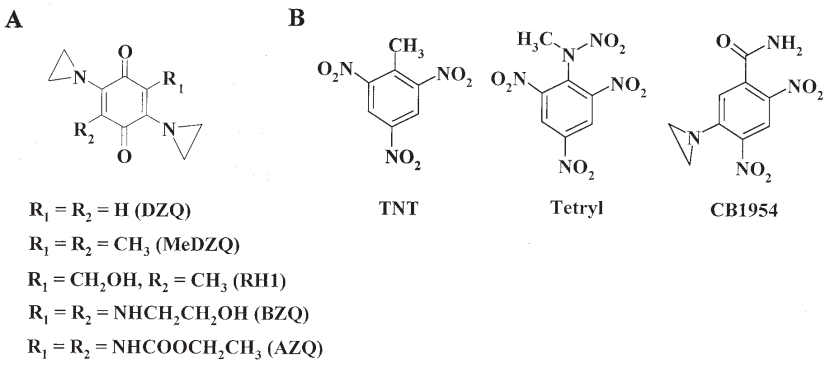

Figure 1. Formulae of aziridinyl-substituted 1,4-benzoquinones (A) and nontrivial nitroaromatic compounds (B) used in this study

Table 1. Kinetic parameters of quinone- and nitroaromatics reduction by nNOS.
Single-electron reduction potentials (E^1_7) of quinones, nitroaromatics and single-electron acceptors (Čėnas *et al.,* 2004; Šarlauskas *et al.,* 2006; Wardman, 1989), catalytic (k_{cat}) and apparent second-order rate (k_{cat}/K_m) constants of their reduction by nNOS in the absence of Ca^{2+}/CAM (50 μM NADPH, 0.1 M Tris/HCl, pH 7.0, 25°C). In some experiments, the kinetic parameters of reduction of aziridinyl-substituted quinones and ferricyanide by nNOS were determined in the presence of 1.0 mM Ca^{2+} and 10 μg/ml CAM (data in parentheses).

No. Compound	E^1_7 (V)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
Quinones			
1. 1,4-Benzoquinone	0.09	11.7±3.9	$3.0\pm0.8\times10^5$
2. 2-Methyl-1,4-benzoquinone	0.01	5.5±1.1	$4.9\pm1.3\times10^5$
3. 2,3-Dichloro-1,4-naphthoquinone	–0.035	11.9±1.3	$1.2\pm0.3\times10^6$
4. DZQ	–0.054	11.9±0.9	$4.8\pm0.4\times10^5$
		(42.7±14.1)	$(1.6\pm0.4\times10^6)$
5. AZQ	–0.07	6.2±0.8	$4.3\pm0.4\times10^5$
		(26.7±4.3)	$(2.4\pm0.2\times10^6)$
6. 2,6-Dimethyl-1,4-benzoquinone	–0.08	10.0±1.5	$1.7\pm0.1\times10^5$
7. 5-Hydroxy-1,4-naphthoquinone	–0.09	12.8±1.2	$1.1\pm0.3\times10^6$
8. 5,8-Dihydroxy-1,4-naphthoquinone	–0.11	16.0±1.3	$1.0\pm0.4\times10^6$
9. 9,10-Phenanthrene quinone	–0.12	8.5±0.5	$8.4\pm1.0\times10^5$
10. 1,4-Naphthoquinone	–0.15	11.8±1.1	$3.3\pm0.4\times10^5$
11. 2-Methyl-5-hydroxy-1,4-naphthoquinone	–0.16	14.6±1.3	$3.2\pm0.4\times10^5$
12. 2-Methyl-1,4-naphthoquinone	–0.20	4.2±0.1	$3.7\pm0.2\times10^5$
13. RH1	–0.23	7.4±0.7	$2.8\pm0.3\times10^5$
		(16.6±1.7)	$(1.1\pm0.3\times10^6)$
14. MeDZQ	–0.23	3.1±0.5	$1.5\pm0.4\times10^5$
15. Tetramethyl-1,4-benzoquinone	–0.26	3.9±0.6	$9.9\pm0.2\times10^4$
16. Riboflavin	–0.32	5.2±0.7	$3.3\pm0.2\times10^4$
17. 1,8-Dihydroxy-9,10-anthraquinone	–0.33	1.0±0.1	$8.0\pm0.7\times10^3$
18. Daunorubicin	–0.34	3.4±0.6	$1.7\pm0.4\times10^5$
19. BZQ	–0.38	0.3±0.2	$6.9\pm1.7\times10^3$
		(1.8±0.2)	$(3.1\pm0.4\times10^4)$
20. 1-Hydroxy-9,10-anthraquinone	–0.39	0.4±0.1	$1.1\pm0.2\times10^4$
21. 2-Hydroxy-1,4-naphthoquinone	–0.41	1.1±0.2	$3.7\pm0.8\times10^4$
22. 2-Methyl-3-hydroxy-1,4-naphthoquinone	–0.46	0.4±0.1	$2.2\pm0.4\times10^3$
Nitroaromatics			
23. 2,4,6-Trinitrophenyl-*N*-methylnitramine	–0.156	5.3±0.5	$2.8\pm0.3\times10^5$
24. 2,4,6-Trinitrotoluene	–0.253	2.0±0.2	$1.8\pm0.2\times10^4$
25. Nifuroxime	–0.255	0.8±0.1	$2.3\pm0.2\times10^4$
26. Nitrofurantoin	–0.255	1.5±0.2	$6.0\pm0.5\times10^4$
27. *p*-Dinitrobenzene	–0.257	4.2±0.3	$1.2\pm0.1\times10^5$
28. *o*-Dinitrobenzene	–0.287	4.4±0.4	$1.5\pm0.2\times10^4$
29. *m*-Dinitrobenzene	–0.348	1.6±0.2	$4.0\pm0.3\times10^3$
30. 3,5-Dinitrobenzoic acid	–0.350	1.6±0.3	$3.2\pm0.3\times10^2$
31. *p*-Nitroacetophenone	–0.355	2.5±0.3	$1.3\pm0.1\times10^3$
32. CB-1954	–0.385	≥0.1	$9.8\pm1.0\times10^2$
33. *p*-Nitrobenzoic acid	–0.425	0.3±0.1	$7.5\pm0.8\times10^1$
34. Nitrobenzene	–0.485	≥0.1	$1.2\pm0.2\times10^2$
Single-electron acceptors			
35. Ferricyanide	0.41	39.8±0.3	$1.5\pm0.2\times10^5$
		(51.7±5.1)	$(2.5\pm0.3\times10^5)$
36. Cytochrome *c*	0.26	3.5±0.2	$8.2\pm1.2\times10^5$
37. $Fe(EDTA)^-$	0.12	4.2±0.6	$.6\pm0.1\times10^3$

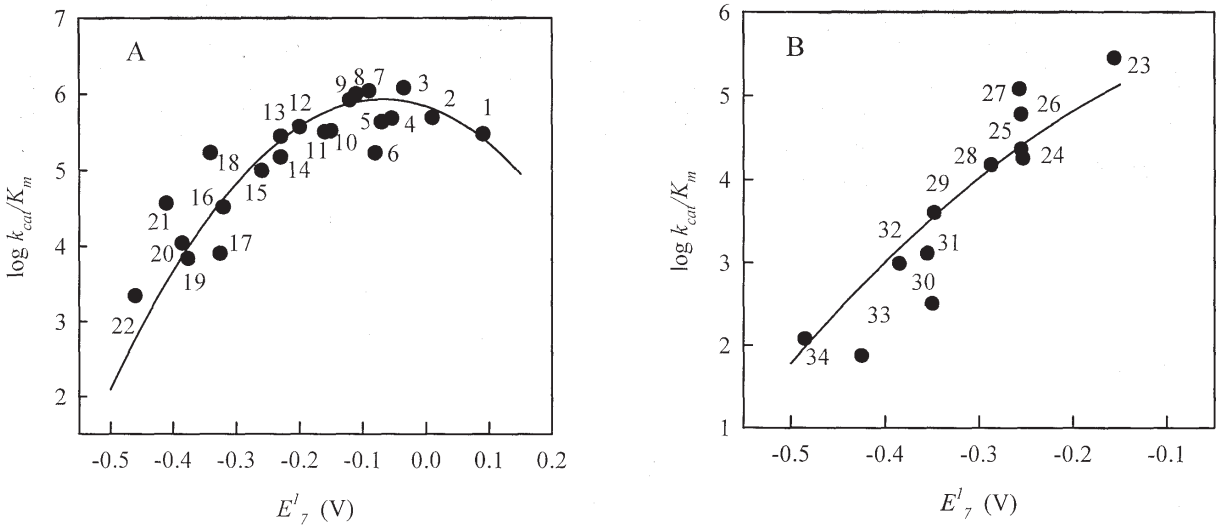

Figure 2. Structure-activity relationships in nNOS-catalyzed single-electron reduction of quinones and nitroaromatic compounds
Dependence of log k_{cat}/K_m of nNOS-catalyzed reduction of quinones (**A**) and nitroaromatics (**B**) on their single-electron reduction potentials (E^1_7). Numbering of compounds is as in Table 1.

to find any pronounced role of the structure of the quinones, *e.g.,* the presence of one, two or three aromatic rings, or the size of their substituents, on their reactivity. This is in line with a single-electron reduction of quinones and nitroaromatic compounds by dehydrogenases-electrontransferases such as P-450R, where the linear or parabolic (square) dependences of log (rate constant) on E^1_7 of oxidants are consistent with an "outer-sphere" electron transfer model, *i.e.* weak electronic coupling between the reactants, and an insignificant role of their structural peculiarities (Marcus & Sutin, 1985; Čėnas *et al.,* 1994, 2004). In accordance with this model, the bimolecular rate constant of single electron transfer between the reagents (k_{12}) depends on the electron self-exchange constants of the reactants (k_{11}, k_{22}), and the equilibrium constant of reaction (K) (log $K = \Delta E^1$ (V)/0.059, where ΔE^1 is the difference between the standard single-electron transfer potentials of the reactants):

$$k_{12} = (k_{11} \times k_{22} \times K \times f)^{1/2} \quad (1),$$

and f is expressed as:

$$\log f = (\log K)^2 / 4 \log (k_{11} \times k_{22} / Z^2), \quad (2),$$

where Z is a frequency factor (10^{11} $M^{-1}s^{-1}$). In accordance with this model, the data of Fig. 2A, B demonstrate a lower reactivity of nitroaromatic compounds towards nNOS as compared with quinones with similar values of E^1_7. This is because the k_{11} values for nitroaromatic compounds, 10^5 $M^{-1}s^{-1}$ (Moetner & Neta, 1986), are lower than those for quinones, 10^8 $M^{-1}s^{-1}$ (Grampp & Jaenicke, 1987). In this aspect, estimation of the electron self-exchange rate constant of nNOS (k_{22}) may be of considerable interest. At $\Delta E^1 = 0$, Eqn. (1) is reduced to $k_{12} = (k_{11} \times k_{22})^{1/2}$. Because the E^1_7 value for the $FMNH^{\cdot}/FMNH_2$ redox couple of nNOS, which is the main electron donor in the quinone reductase reaction, is –0.274 V (Matsuda *et al.,* 2000, and references there), the approximate values of k_{22} for nNOS may be estimated from the data of Fig. 2A, B at $\Delta E^1_7 \sim 0$ V, *i.e.,* at $E^1_7 \sim -0.274$ V for the electron acceptor. They are equal to ~10^2 $M^{-1}s^{-1}$ (reactions with quinones), or ~10^3 $M^{-1}s^{-1}$ (reactions with nitroaromatic compounds). Consequently, the k_{22} values for redox proteins may be related to the distance of electron transfer in the reactions (R_p) (Mauk *et al.*, 1980):

$$R_p \text{ (nm)} = 0.63–0.035 \ln k_{22} \quad (3)$$

In accordance with this, Eqn. (3) gives tentative values of R_p of 0.47 nm (quinones), and 0.39 nm (nitroaromatics) for nNOS-catalyzed reactions. For comparison, the single-eletron quinone reduction by rat NADPH:cytochrome P-450 reductase is characterized by a higher value of k_{22}, ~10^4 $M^{-1}s^{-1}$, and, correspondingly, a the lower value of R_p, ~0.31 nm (Čėnas *et al.*, 1994). It seems that calculation according to this method may give slightly overestimated distances, because the dimethylbenzene part of the isoalloxazine ring of FMN in P-450R is exposed to the solvent (Wang *et al.,* 1997). However, the difference between the calculated values of R_p of P-450R and nNOS in quinone reduction is in agreement with recent crystal structure data of NOS which indicate that FMN may be relatively less accessible to solvent, being buried at the FMN/FAD interface (Xia *et al.*, 2009). This explains the almost tenfold lower reactivity of quinones and nitroaromatic compounds in terms of their k_{cat}/K_m with nNOS in comparison to rat P-450R (Table 1, Čėnas *et al.*, 1994).

nNOS-catalyzed reduction of quinones and nitroaromatics under partial hypoxic condition

We examined another related problem, the possibility of a net two-electron reduction of quinones and nitroaromatic compounds by nNOS. It is generally accepted that under aerobic conditions, these reactions may be performed by obligatory two-electron transferring flavoenzymes, such as NAD(P)H:quinone oxidoreductase (DT-diaphorase, NQO1, EC 1.6.5.2) (Čėnas *et al.,* 2004, and references therein). This reaction initiates alkylation of DNA by reduced forms of anticancer drugs aziridinyl-substituted quinones and nitroaromatics such as CB-1954 (Fig. 1) (Helsby *et al.*, 2003; Nemeikaitė-Čėnienė *et al.*, 2003; Miškinienė *et al.*, 1999, and references there), and contributes to their enhanced cytotoxicity. In contrast, nNOS reduces CB-1954 to its hydroxylamine only under complete anaerobiotic conditions (Chandor *et al.*, 2008). On the other hand, the single-electron transferring P-450R has been found to be involved in the bioreductive activation of antitumour quinone RH1 (Fig. 1) in the cell (Begleiter *et al.*, 2007). We also found that RH1

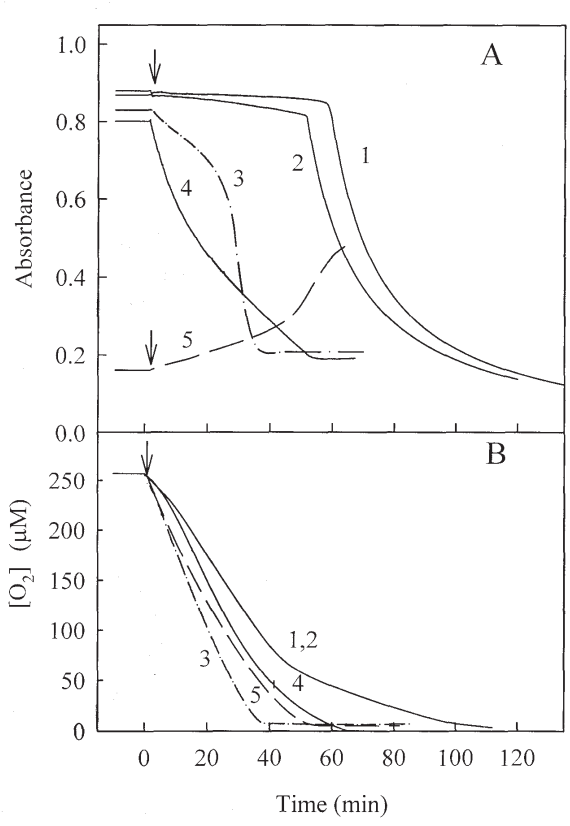

Figure 3. Reduction of quinones and nitroaromatic compounds by nNOS under partially anaerobiotic conditions.
Time course of reduction of quinones and nitroaromatics (50 μM) by 50 nM nNOS in the presence of NADPH-regeneration system in the absence of external oxygen supply (**A**), and parallel oxygen uptake during the reactions (**B**). Changes in absorbance of the examined compounds were followed at the indicated wavelengths: RH1, 325 nm (**1**), MeDZQ, 325 nm (**2**), AZQ, 344 nm (**3**), DZQ, 320 nm (**4**), *p*-dinitrobenzene, 340 nm (**5**). Arrows indicate the time of introduction of nNOS.

and MeDZQ (Fig. 1) may be reduced to corresponding hydroquinones by single-electron transferring *Plasmodium falciparum* ferredoxin: $NADP^+$ reductase (FNR, EC 1.18.1.2) under partly anaerobic conditions (Grellier *et al.*, 2010). Our data also show that the reduction of RH1 and MeDZQ (E^1_7 = –0.23 V) by isolated nNOS starts with a significant delay (Fig. 3A) only after oxygen exhaustion, when the residual O_2 concentrations reaches 40–50 μM (Fig. 3B). On the other hand, the reduction of DZQ (E^1_7 = –0.054 V) and AZQ (E^1_7 = –0.07 V) starts immediately, although it is accompanied by oxygen consumption, and the reduction of AZQ accelerates after significant consumption of O_2 (Fig. 3A, B). Interestingly, the reduction of *p*-dinitrobenzene (E^1_7 = –0.257 V) also starts immediately, but accelerates significantly after the exhaustion of O_2 (Fig. 3A, B). Thus, our data show that NOS and presumably other single-electron transferring flavoenzymes may contribute to a net two-electron reduction of aziridinyl-substituted quinones and nitroaromatics, *i.e.,* the generation of their alkylating products, under aerobic or modestly anaerobic conditions. The efficiency of this process, caused by the reduction of free radicals and their parallel dismutation, increases with an increase in E^1_7 values of compounds. This may explain inefficient reduction of low-potential CB-1954 (E^1_7 = –0.385 V) by NOS even under partially anaerobic conditions (Chandor *et al.*, 2008).

DISCUSSION

Several studies have demonstrated the ability of NOSs to catalyze a single-electron reduction of anti-tumor drugs bearing a quinone moiety such as mitomycin and adriamycin (Garner *et al.*, 1999; Matsuda *et al.,* 2000; Fu *et al.*, 2004), or a nitroaromatic moiety, such as nilutamide and CB-1954 (Ask *et al.*, 2003; Chandor *et al.*, 2008). NOSs also catalyze a single-electron reduction of environmental pollutants such as TNT and dinitrobenzenes (Kumagai *et al.,* 2004; Miller, 2002). Our study demonstrates that rat neuronal NOS catalyzes a single-electron reduction of diverse quinones and nitroaromatic compounds following an 'outer-sphere' electron transfer model that is almost independent of the structure of the compound. The lower activity of nNOS in comparison with NADPH:cytochrome P-450 reductase may be explained by the poorer accessibility of reduced FMN as observed by crystallographic study (Zhang *et al.,* 2001; Garcin *et al.*, 2004). Stopped-flow kinetic studies have shown that the rate of electron flux through $nNOS_{red}$ is more than sufficient to support both NO and L-citrulline production as well as simultaneous electron transfer to exogenous electron acceptors (Miller *et al.*, 1999). These reactions may significantly contribute to the oxidative stress-type cytotoxicity of quinones and nitroaromatic compounds in target organs such as the brain, where nNOS contributes to neurodegenerative diseases (Steinert *et al.*, 2010).

On the other hand, under modestly anaerobic conditions, nNOS performs a net two-electron reduction of quinones and nitroaromatics, even those with a relatively low single-electron reduction potential, from –0.23 V to –0.257 V, and thus may contribute to the bioreductive alkylation mechanism of action of antitumour compounds with similar reduction potentials. The relative importance of the contribution of NOSs in the metabolism of quinones and nitroaromatic compounds *in vivo* remains poorly studied. Under aerobic conditions, the contribution of NOSs to metabolic reduction should be weak as O_2 inhibits the NOS-catalyzed reduction. However, the contributions of NOSs could be more important in pathological situations resulting in severe hypoxia, as in tumors. Furthermore, in hypoxic tumoral tissues, inducible NOS is often over-expressed and could play a role in the bioactivation of cytotoxic quinones and generation of reactive oxygen species.

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