

Biochemical and Histochemical Studies of the Effects of Cerebral Metabolism-Improving Drugs on NADPH Diaphorase Activity in Mouse Brain

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ABSTRACT—The effects of cerebral metabolism-improving drugs on NADPH diaphorase activity in the mouse brain were studied, and we found that diaphorase activity in the post-mitochondrial fraction of brain homogenate was enhanced by idebenone in a concentration-dependent manner. Histochemical studies also indicated that diaphorase staining was intensified by idebenone at the same concentration. These results suggest that idebenone may stimulate the production of nitric oxide, probably through its direct action on nitric oxide synthase, thus producing its protective action on neurological disorders due to cerebral hypoxia or ischemia as a consequence of dilating the cerebral blood vessels.

Keywords: NADPH diaphorase, Idebenone, Brain (mouse)

Nitric oxide has recently been proposed to play a physiologically important role as an endogenous factor regulating vascular tone in cerebral blood vessels (1–5). In addition to its role as a vascular relaxing factor derived from endothelium, nitric oxide has also been proposed to play a role as a putative neurotransmitter in the non-adrenergic and non-cholinergic neurons, based on the recent immunohistochemical studies indicating that nitric oxide synthase is localized in the central and peripheral nervous systems (6–8). In view of these findings, it seems reasonable to consider that nitric oxide may be able to influence the cerebral metabolism, presumably through the modulation of cerebral blood flow that results from its dilatory action on cerebral blood vessels. On the other hand, several drugs affecting the cerebral metabolism and/or blood flow have been developed in expectation of their clinical effectiveness for treating neurological disorders due to cerebral hypoxia or ischemia. The effects of these drugs on the metabolism of neurotransmitters have been studied to elucidate the mechanism of their pharmacological actions. However, the mechanism of their improving actions on cerebral metabolism still remains to be elucidated. As a possible mechanism, it is conceivable that the improvement of cerebral blood flow induced by these drugs may be closely related to their stimulatory actions on the production of nitric oxide in the brain. In the recent stud-

ies, NADPH diaphorase has already been reported to be a nitric oxide synthase (9), and the nerve fibers containing NADPH diaphorase have been shown to innervate the cerebral blood vessels (10). In the present study, the activity of NADPH diaphorase was therefore determined as an index for the activity of nitric oxide synthase, and the effects of cerebral metabolism-improving drugs on the production of nitric oxide were examined by measuring this enzyme activity using biochemical and histochemical techniques.

Adult male ddY mice (25–30 g) were killed by decapitation, and whole brains were quickly removed and homogenized in 10 volumes of 10 mM Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at $20,000 \times g$ for 20 min, and the resulting supernatant fraction was used for the determination of NADPH diaphorase activity as a crude enzyme. The enzyme activity was assayed by measuring the reduction of nitro blue tetrazolium (NBT) to its formazan product during the incubation period. The mixture containing 50 mM Tris-HCl (pH 8.0), 0.5 mM NBT, 1 mM NADPH, and the crude enzyme (240 μ g protein) in a volume of 0.5 ml was incubated at 37°C for 20 min, and the reaction was terminated by adding 0.5 ml of 0.1 M sulfuric acid. The incubation mixture was centrifuged at $1,500 \times g$ for 10 min to clarify the solution, and the absorbance of formazan product was determined

at 585 nm.

Mice were transcardially perfused in deep anesthesia with 0.1 M phosphate buffer (pH 7.4) containing 10 units/ml of heparin sulfate followed by 0.1 M phosphate buffer containing 4% paraformaldehyde and 0.2% picric acid. Brains were removed and postfixed for 2 hr in the same fixative, and then placed in 10%, 15% and 20% sucrose in 10 mM phosphate-buffered saline for cryoprotection. The tissues were sectioned serially with a cryostat at 10- μ m thickness for the histochemical study of NADPH diaphorase activity. The sections were incubated at 37°C for 10–40 min in 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM NADPH, 0.2 mM NBT and 0.2% Triton X-100, and the reaction was terminated by washing them with 50 mM Tris-HCl buffer (pH 8.0). The specimens were examined and photographed by a light microscope.

The cerebral metabolism-improving drugs were kindly donated. Idebenone was from Takeda Chemical Industries, Ltd. (Osaka); calcium hopantenate was from Tanabe Seiyaku Co., Ltd. (Osaka); propentofylline was from Hoechst Japan, Ltd. (Tokyo); and brovincamine was from Sandoz Pharmaceuticals (Tokyo). Other chemicals were of commercially available reagent grades.

To elucidate the effects of cerebral metabolism-improving

ing drugs on the production of nitric oxide in the brain, the direct actions of these drugs on NADPH diaphorase in the mouse brain were examined using the post-mitochondrial fraction of brain homogenate. As shown in Fig. 1, NADPH diaphorase was markedly activated by the addition of idebenone (10^{-5} M). In contrast, other drugs, such as propentofylline, hopantenate and brovincamine, failed to cause any notable increase in the activity of this enzyme at the same concentration. Because the drug concentrations used here are considered to be sufficient to produce their actions, based on the blood concentrations at which their therapeutic effects are exhibited, it therefore seems reasonable to conclude that NADPH diaphorase is activated only by idebenone under the experimental conditions used here. Furthermore, the stimulatory action of idebenone on the diaphorase activity was also shown to be dependent on its concentration, and a slight increase in the enzyme activity was already observed at 3×10^{-6} M idebenone (Fig. 2). The results obtained in these *in vitro* studies clearly indicate that idebenone can directly activate NADPH diaphorase, thus suggesting the possibility that this drug may stimulate the production of nitric oxide presumably through its stimula-

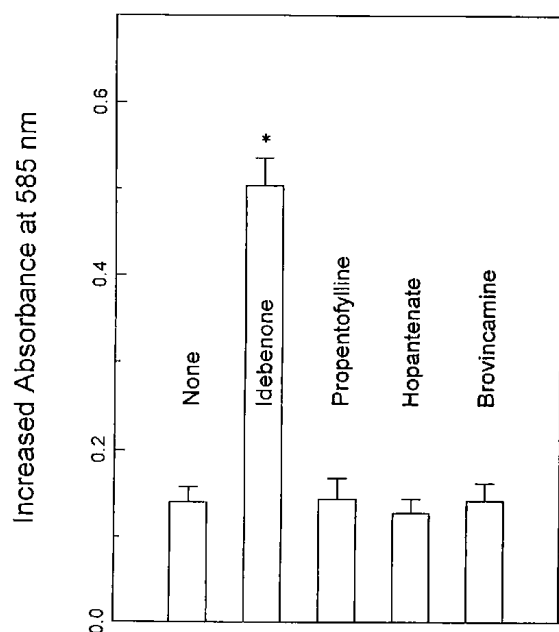


Fig. 1. Effects of various cerebral metabolism-improving drugs on NADPH diaphorase activity in the post-mitochondrial fraction of mouse brain homogenate. The mixture containing 10^{-5} M of each cerebral metabolism-improving drugs was incubated at 37°C for 20 min, and the absorbance was then determined as described in the text. Values are presented as the mean of three experiments with triplicate assays \pm S.E.M. (vertical lines). * indicates a significant increase of absorbance compared to the control value ($P < 0.05$).

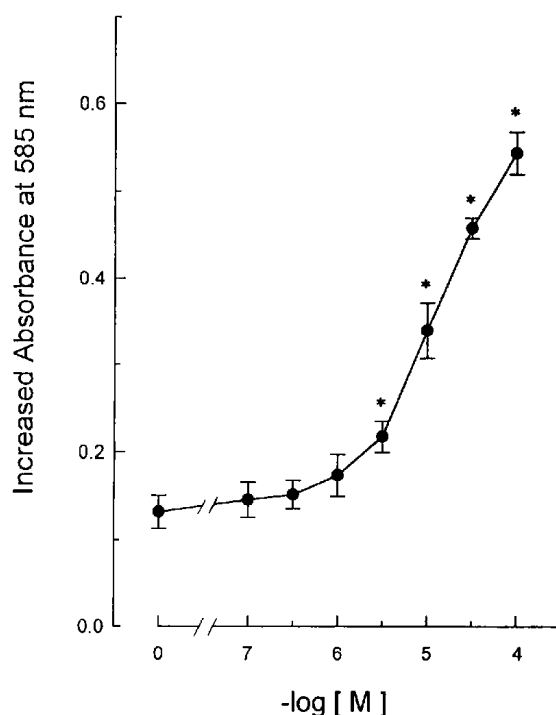


Fig. 2. Stimulatory action of idebenone on NADPH diaphorase as a function of its concentration. The mixture containing different concentrations of idebenone was incubated at 37°C for 20 min, and the absorbance was then determined as described in the text. Values are presented as the mean of three experiments with triplicate assays \pm S.E.M. (vertical lines). * indicates a significant increase of absorbance compared to the control value ($P < 0.05$).

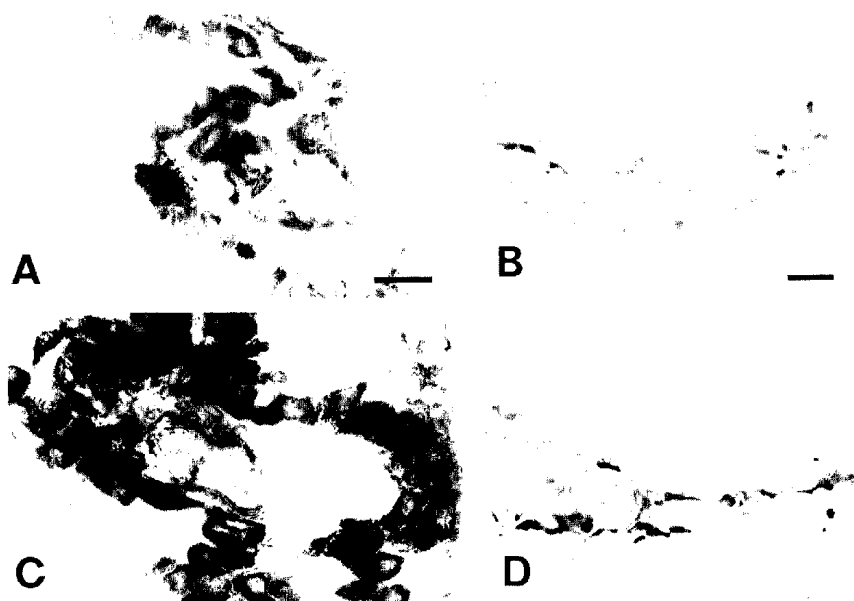


Fig. 3. Effect of idebenone on NADPH diaphorase staining in the choroid plexus (A, C) and blood vessel (B, D) of mouse brain. The adjacent cryostat sections were stained at 37°C for 20 min in the presence (C, D) or absence (A, B) of 10^{-4} M idebenone. Scale bar = 10 μ m (A, C and B, D).

tory action on nitric oxide synthase in the brain.

Histochemical studies of NADPH diaphorase were furthermore carried out to test whether the activity of NADPH diaphorase in a particular region of the mouse brain was specifically altered by idebenone. As shown in Fig. 3, the choroid plexus revealed a highly positive reaction to NADPH diaphorase, and the positive enzyme staining was observed in the epithelial cells and the nerve fibers in the blood vessel (Fig. 3, A and B). On the other hand, the enzyme was positive in the neurons of both the paraventricular nucleus and supraoptic nucleus (pictures not shown). In addition, the staining of NADPH diaphorase in these specific cells and neurons was markedly intensified by the addition of idebenone to the reaction mixture (Fig. 3, C and D as compared with A and B). These results indicate that NADPH diaphorase is activated by idebenone, and this activation is similarly observed in different types of cells in different regions of the mouse brain.

The findings presented here suggest that idebenone may improve the cerebral metabolism and blood flow as a consequence of stimulation of nitric oxide production, thus providing evidence for a possible involvement of nitric oxide in the mechanism underlying the improving action of this drug on neurological disorders. In fact, the previous study has shown that L-arginine, a substrate for nitric oxide production, can reduce the infarct size in a focal ischemia and this effect is likely due to the dilation of cerebral blood vessels (11). However, conclusive evidence

for a possible relation of nitric oxide production to the improvement of neurological disorders has not yet been obtained. Histochemical studies on the localization of NADPH diaphorase and the effect of idebenone on this enzyme in other regions of the brain are furthermore required to obtain a definite answer to this critical question.

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