

## Effects of Flavonoid Compounds on the Activity of NADPH Diaphorase Prepared from the Mouse Brain

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**ABSTRACT**—The effects of flavonoids on NADPH diaphorase activity were studied *in vitro*, and we found that the enzyme activity was markedly inhibited by quercetin. This inhibitory action was shown to be accompanied by an increase in the apparent  $K_m$  value of the enzyme for the cofactor NADPH, with a decrease in the  $V_{max}$ , and an increase in the apparent  $K_m$  for the substrate nitro blue tetrazolium, without any significant change in the  $V_{max}$ . These results indicate that quercetin may directly inhibit NADPH diaphorase, thus suggesting the possibility that this compound may be able to inhibit the production of nitric oxide in the brain.

**Keywords:** NADPH diaphorase, Flavonoid, Quercetin

Flavonoids have generally been appreciated as a factor effective against the hemorrhagic symptoms of scurvy (1), but little is known about the detailed mechanism underlying the antiscorbutic actions of these compounds. On the other hand, flavonoids have been reported to inhibit various enzymes involved in the physiologically important metabolic pathways. Particularly, various protein kinases have been shown to be inhibited by flavonoids (2–4), and these findings seem to indicate that the pharmacological actions of flavonoid compounds may be attributed to their actions on the phosphorylation of functional proteins in various cells and tissues. In addition to these inhibitory actions, quercetin has been shown to inhibit catecholamine secretion from cultured bovine adrenal chromaffin cells, presumably through its inhibitory action on protein kinase C (5). Furthermore, this compound has also been shown to inhibit catecholamine biosynthesis as a consequence of direct inhibition of tyrosine hydroxylase in the adrenal chromaffin cell (6). Thus, the possibility that flavonoid compounds may be able to cause a decline in the sympathoadrenergic function has been proposed. However, although considerable studies on the biochemical and pharmacological actions of flavonoids have already been carried out, the question of whether these biochemical and pharmacological actions are related to their antiscorbutic action still remains to be elucidated.

Nitric oxide has already been proposed to play a physiologically important role as a factor regulating vascular

tone in the cerebral blood vessel (7–10). In addition to its role as an endogenous vascular relaxing factor derived from endothelium, nitric oxide has also been proposed to play a role as a putative neurotransmitter in non-adrenergic and non-cholinergic neurons, based on recent immunohistochemical studies indicating that nitric oxide synthase, an enzyme catalyzing the production of nitric oxide, is localized in the central and peripheral nervous systems (11–13). It therefore seems reasonable to consider that nitric oxide may be able to change the cerebral blood flow, presumably through its dilatory action on the cerebral blood vessel. However, although the effect of nitric oxide on the vascular tone has already been proposed, it is still not known if this vasoactive factor can affect the other properties of the cerebral blood vessel, such as the permeability of cerebral microvessels. In contrast, the effects of flavonoid compounds on the permeability of capillary vessels have already been established, but it may be fair to say that the effects of these compounds on the cerebral vascular tone have hardly been investigated before. In view of these earlier studies, it would be interesting to investigate whether flavonoid compounds have any significant influence on the production of the endogenous vascular relaxing factor nitric oxide in cerebral blood vessels. In the present study, because brain NADPH diaphorase has previously been reported to be a nitric oxide synthase (14) and the nerve fibers containing this enzyme have recently been shown to innervate the cerebral blood vessels (15), the effects of

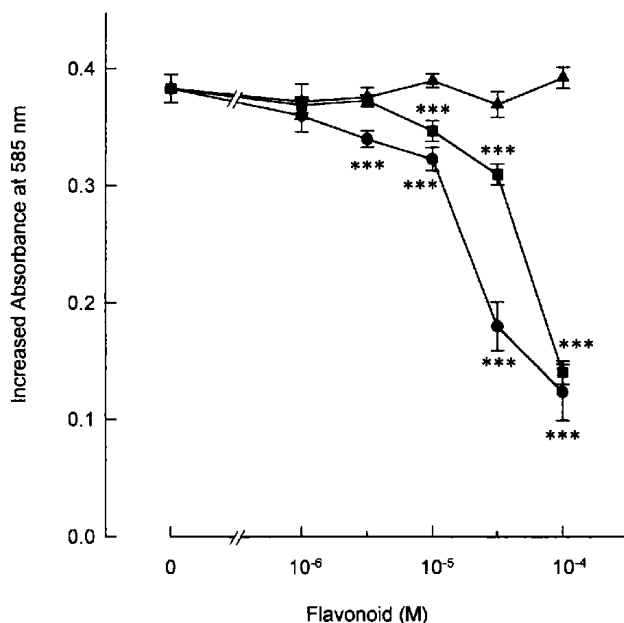
flavonoid compounds on the ability of nitric oxide production in the mouse brain were studied by measuring the activity of NADPH diaphorase as an index for the activity of nitric oxide synthase.

Adult male ddY mice (25–30 g) were killed by decapitation, and the 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 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 reaction mixture containing 50 mM Tris-HCl (pH 8.0), 0.5 mM NBT, 1 mM NADPH and the crude enzyme (550  $\mu$ g protein) in a volume of 0.5 ml was incubated at 37°C for 30 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 the formazan product was determined at 585 nm.

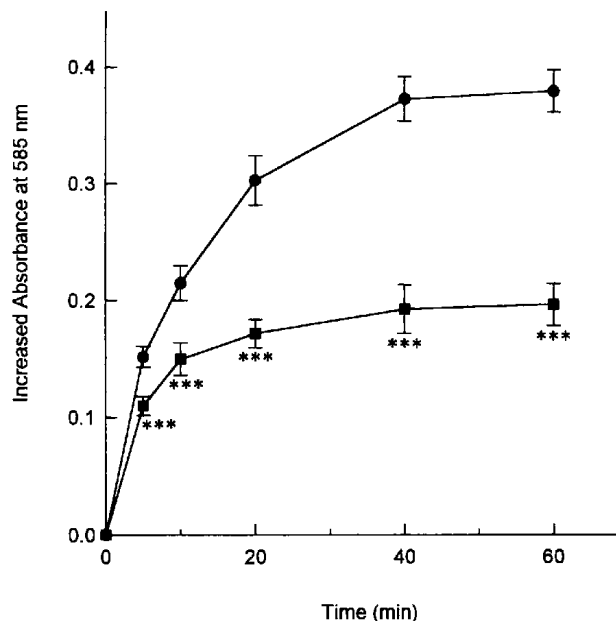
Flavonoid compounds (quercetin, apigenin, flavone) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals used were commercially available reagent grades.

To elucidate the effects of various flavonoids on the

production of nitric oxide in the brain, the direct actions of these compounds on the activity of NADPH diaphorase in the mouse brain were studied using the post-mitochondrial fraction of brain homogenate. As shown in Fig. 1, NADPH diaphorase was inhibited by quercetin and apigenin in a concentration-dependent manner. In contrast, flavone failed to cause any significant alteration in the enzyme activity under the same experimental conditions. Approximately 50% of the enzyme activity was inhibited by  $3 \times 10^{-5}$  M quercetin, and only approximately 20% of the activity was reduced by the same concentration of apigenin. However, the inhibitory actions of these two compounds obtained at  $1 \times 10^{-4}$  M were almost similar. This enzyme is therefore considered to be a little more sensitive to the inhibitory action of quercetin in comparison with that of apigenin. Furthermore, because the inhibitory action of quercetin on NADPH diaphorase was already observed at a 5-min incubation (Fig. 2), the rapid inhibition of the enzyme observed here is presumed to reflect its direct action on the enzyme. Furthermore, the effect of quercetin on kinetic properties of NADPH diaphorase was examined to characterize the inhibitory action of this compound on the enzyme. Quercetin was shown to cause an increase in the apparent  $K_m$  of the enzyme and a decrease in the  $V_{max}$  for NADPH, and also shown to cause an increase in the apparent  $K_m$  of the en-



**Fig. 1.** Effects of flavonoids on the activity of NADPH diaphorase prepared from mouse brain. Enzyme was incubated at 37°C for 30 min in the mixture containing various concentrations of quercetin (●), apigenin (■) and flavone (▲), and the absorbance was determined as described in the text. Values are the mean  $\pm$  S.D. ( $n=6$ ). \*\*\* $P < 0.001$ , significantly different from the basal value.



**Fig. 2.** Time course of the inhibitory action of quercetin on NADPH diaphorase prepared from mouse brain. Enzyme was incubated at 37°C for different time periods in the presence (■) and absence (●) of  $3 \times 10^{-5}$  M quercetin, and the absorbance was determined as described in the text. Values are the mean  $\pm$  S.D. ( $n=6$ ). \*\*\* $P < 0.001$ , significantly different from the basal value.

**Table 1.** Effect of quercetin on kinetic properties of mouse brain NADPH diaphorase

	NADPH		NBT	
	$K_m$ ( $\mu$ M)	$V_{max}$	$K_m$ ( $\mu$ M)	$V_{max}$
Control	74.20	0.381	15.85	0.373
Quercetin	191.88	0.248	32.04	0.313

Enzyme was incubated with or without quercetin ( $3 \times 10^{-5}$  M) at 37°C for 30 min in the mixture containing 0.5 mM NBT with four different concentrations of NADPH (0.1–0.6 mM) or in the mixture containing 1 mM NADPH with four different concentrations of NBT (20–50  $\mu$ M), and the kinetic parameters were calculated from the mean values of three experiments using linear-regression analysis.  $V_{max}$  values were expressed as the 585-nm absorbance of the formazan product formed during the incubation period.

zyme without any significant alteration in the  $V_{max}$  for NBT (Table 1). Thus, quercetin was clearly shown to be a mixed-type inhibitor vs. NADPH (higher  $K_m$ , lower  $V_{max}$ ) and a competitive inhibitor vs. NBT (higher  $K_m$ , similar  $V_{max}$ ). The results obtained from these kinetic studies indicate that the inhibition of NADPH diaphorase caused by quercetin is accompanied by an alteration in kinetic properties of the enzyme, thus suggesting the possibility that quercetin may cause the inhibition of NADPH diaphorase as a result of the direct interaction between this inhibitor and the enzyme.

The present study indicates that both quercetin and apigenin can inhibit NADPH diaphorase in mouse brain, thus proposing the possibility that these flavonoid compounds may be able to inhibit the production of nitric oxide as a consequence of inhibiting nitric oxide synthase in the cerebral vascular system. It therefore seems likely that flavonoids may be able to modulate the vascular tone as well as the permeability of cerebral microvessels. However, because the effects of flavonoid compounds on both the vascular tone and the nitric oxide production in the cerebral microvessels have not been investigated yet, the relevance of this speculation still remains to be testified.

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