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# Influence of the Antioxidant Quercetin *In Vivo* on the Level of Nitric Oxide Determined by Electron Paramagnetic Resonance in Rat Brain during Global Ischemia and Reperfusion

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**ABSTRACT.** We characterized the changes in nitric oxide (NO) levels in the brain during global forebrain ischemia and reperfusion and tested the ability of the natural flavonoid, quercetin, and a synthetic flavonoid, FB277, to increase the amount of available NO by elimination of the superoxide radicals produced during reperfusion. In Sprague–Dawley rats, we used a four-vessel occlusion model of forebrain ischemia (15 min) and reperfusion (30 min). Brain NO was measured on samples of cerebral cortex and cerebellum *ex vivo* by electron paramagnetic resonance (EPR) spectroscopy. The spin trap used was diethyldithiocarbamate sodium salt (DETC) associated with ferrous citrate. The complex $Fe(DETC)_2NO$ was detected at 77 K as a triplet signal at $g = 2.035$. Groups of animals were treated with quercetin or FB277 (3-morpholinomethyl-3′,4′,5,7-tetramethoxyflavone) or polyethylene glycol-conjugated superoxide dismutase (PEG-SOD). In control (intact anesthetized animals), the signal was about 3 times greater in the cortex than in the cerebellum. During ischemia, the signal rose to 110% in cortex (NS) and 283% in cerebellum ($P < 0.05$). In reperfusion, it fell again to 91% of control in cerebellum (NS) and 35% in cortex ($P < 0.05$). Treatment by quercetin (5 mg/kg i.v.) of intact and ischemia-reperfusion groups did not significantly change the signal amplitude in the cerebellum, but did double it in the cortex (to 76% of control) for the ischemia-reperfusion group ($P < 0.05$). In contrast, FB277 (3.75 mg/kg i.v.) did not increase the signal in the cortex during ischemia-reperfusion, but did do so in the cerebellum (to 152% of control, $P < 0.05$). The results obtained for PEG-SOD (10,000 U/kg i.v.) were similar to those for FB277. In separate *in vitro* measurements, we found that quercetin but not FB277 efficiently scavenged superoxide. We hypothesize that quercetin but not FB277 scavenged superoxide anions released in the cortex during reperfusion, thus diminishing the amount of NO removed by the formation of peroxynitrite. The lack of effect of PEG-SOD may be related to the need for chronic treatment to obtain protection. BIOCHEM PHARMACOL **57**;2:199–208, 1999. © 1998 Elsevier Science Inc.

**KEY WORDS.** nitric oxide measurement; electron paramagnetic resonance; superoxide scavenging; flavonoids; global brain ischemia and reperfusion; antioxidants

The deleterious influence of ROS‖ in the pathogenesis of neurodegenerative disorders has been widely discussed in recent years [1–4]. It has been established that excessive release of the excitatory neurotransmitter glutamate and the associated overactivation of glutamate receptors is an important source of free radicals in neurons and that, in turn, free radicals may promote an increase in the extracellular concentration of glutamate [4–6].

The mammalian brain is exceptionally vulnerable to the cytotoxic effects of ROS [1]. The main energy supply of this highly oxygenated organ is the oxidative metabolism of the mitochondrial respiratory chain. In oxidative stress, the superoxide anion and hydrogen peroxide formed cannot be readily neutralized because of the low catalase, SOD, and glutathione peroxidase activities present in the brain. Moreover, brain membrane lipids are very rich in polyunsaturated fatty acids which are especially sensitive to free radical-induced lipid peroxidation [7]. Little is known about the mechanism(s) and the sequence of events by which free radicals interfere with cellular functions, but lipid peroxidation is likely to be one of the most important events. Individually, the production of superoxide and nitric oxide (NO) has been associated with the development of several diseases, but only recently has it been

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‖ *Abbreviations:* CCA, common carotid arteries; DETC, diethyldithiocarbamate sodium salt; EPR, electron paramagnetic resonance; NBT, nitroblue tetrazolium; NO, nitric oxide; PEG-SOD, polyethylene glycol-conjugated SOD; ROS, reactive oxygen species; SOD, superoxide dismutase; and XOD, xanthine oxidase.

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realized that their interactions may also be important in disease pathology including atherosclerosis, neurodegenerative diseases, ischemia-reperfusion injury, and cancer [8]. NO toxicity may arise through inhibition of mitochondrial respiration [9]. However, there is no doubt at present that the toxic reactions of NO result in part from its rapid reaction with superoxide, leading to peroxynitrite and peroxynitrous acid formation [10–13], which can initiate lipid peroxidation [14], oxidize protein and nonprotein sulfhydryls [15, 16], and hydroxylate and nitrate aromatic compounds [17, 18].

In a number of investigations, it has been shown that SOD [19–21], free radical scavengers [22], and agents forming stable adducts with free radicals [23] are able to alleviate the neuronal damage caused by ischemia. Certain inhibitors of XOD, a source of superoxide radicals, are also able to attenuate ischemic-induced brain injury [24]. It seems, therefore, that reduction of superoxide levels, which are particularly increased during reperfusion, may protect the ischemic brain, especially by limiting the amount of peroxynitrite formed through reaction with NO. The results of Kumura *et al.* [25] support this hypothesis. They found that the NO levels measured in jugular blood during reperfusion after 2 hr middle cerebral artery occlusion were significantly increased by treatment with SOD.

It seems likely, therefore, that any factor which alters the balance between NO and superoxide will change the balance between useful and toxic ROS. In a previous study, we showed that superoxide scavenging by a synthetic flavonoid could favor NO-induced vascular relaxation *in vitro* [26]. Our present objective was thus to examine the possible use of flavonoids to control the balance of NO and superoxide in ischemia-reperfusion. Flavonoids are a group of naturally occurring benzo-γ-pyrone derivatives, many of which have been found to be strong free radical scavengers and antioxidants [27–30]. In spite of the rather well established effects *in vitro* of the natural flavonoid, quercetin [27], and its ability to scavenge superoxide, directly inhibit the activity of XOD, and limit the level of superoxide produced [31], little or nothing is known about its influence on the concentration of free radicals *in vivo*. The present study of NO detection carried out by EPR spectroscopy was therefore conducted to characterize the changes in the NO level during brain global ischemia and reperfusion in the rat and the comparative ability of quercetin and a synthetic flavonoid to increase the amount of available NO by the elimination of superoxide radicals, thus decreasing the formation of peroxynitrite. The effect of the flavonoids was compared with the effect of SOD administered in the same manner as the flavonoids. *In vitro*, the superoxide scavenging activity of flavonoids in enzymatic and nonenzymatic systems was compared.

## MATERIALS AND METHODS

### *Chemicals*

DETC, ferrous sulfate, sodium citrate, HEPES, quercetin, SOD, NBT, xanthine, EDTA, BSA, DMSO, XOD, copper sulfate, NADH, phenazine methosulfate, and PEG-SOD were purchased from Sigma Chemical Co. The flavonoid FB277, 3-morpholinomethyl-3′,4′,5,7-tetramethoxyflavone (Fig. 1), was synthesized at the Institut de Chimie des Substances Naturelles, Gif sur Yvette, France.

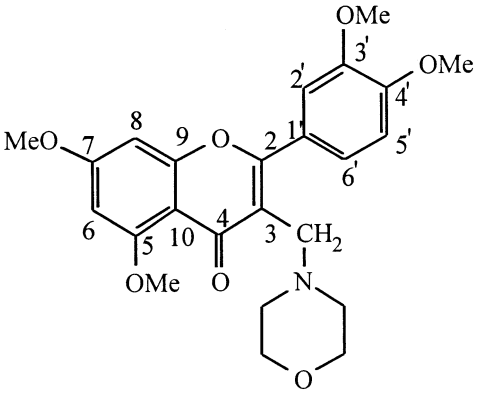


**FIG. 1. Molecular structure of FB277.**

### *Animals*

Adult male Sprague–Dawley rats weighing 220–300 *g* were obtained from Iffa Credo. The animals were fed a standard laboratory diet and water *ad lib.* and maintained at 22° under a constant 12-hour light-dark cycle.

### *Animal Preparation*

Rats were subjected to the four-vessel occlusion method of Pulsinelli and Brierley [32], carried out in two stages. On the first day, rats were anesthetized with pentobarbital, 40 mg/kg, i.p., and electrocoagulation of both vertebral arteries was performed. Twenty-four hours later, anesthesia was induced with 4% halothane mixed with oxygen via a facial mask and CCA clamping was performed under maintained 0.75–1.5% halothane anesthesia. The occurrence of global ischemia was confirmed by a flat electroencephalogram recording.

The duration of ischemia was 15 min. The rats were either killed or reperfused by unclamping of CCA for 30 min and then decapitated. Sham-operated animals were subjected only to the first step of surgery, i.e. vertebral artery electrocoagulation, and killed the following day under halothane anesthesia 30 min after spin trap injection. Control and sham animals were subjected to the same anesthesia as operated/treated animals.

### *Administration of Spin Trap Agents*

The spin trap DETC was injected (400 mg/kg, i.p.) simultaneously with ferrous citrate (40 mg/kg of $FeSO_4$ + 200 mg/kg of sodium citrate, s.c.) 30 min before sacrifice (control, sham, and ischemia-reperfusion groups) or 30 min before CCA clamping (ischemia group) [33, 34].

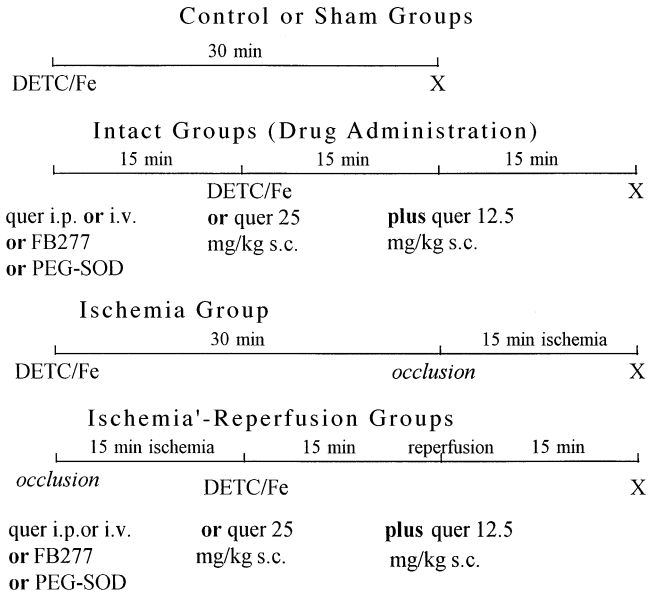


**FIG. 2. Diagram of protocols for injections of spin trap and drugs. Drugs administered were either quercetin (quer) [20 mg/kg i.p. *or* 5 mg i.v. *or* 25 and 12.5 mg/kg s.c. (2 injections)] *or* FB277 3.75 mg/kg i.v. *or* PEG-SOD 10,000 U/kg i.v. X denotes sacrifice.**

### *Flavonoid Administration*

The natural flavonoid quercetin was administered at 20 mg/kg i.p. or 5 mg/kg i.v. 45 min before sacrifice. In the case of s.c. administration, quercetin was injected twice: 25 mg/kg and 12.5 mg/kg 15 min later for intact animals, and 25 mg/kg at the beginning of reperfusion and 12.5 mg/kg 15 min later for the rats subjected to ischemia-reperfusion. Rats were thus killed 30 min after the first s.c. administration of the flavonoid. The synthetic flavonoid FB277 was administered i.v. at the dose of 3.75 mg/kg (equimolar with quercetin), also 45 min before sacrifice. Figure 2 summarizes the injection protocols. For i.p. and s.c. administrations, quercetin was suspended in peanut oil; for i.v. administrations, quercetin and FB277 were suspended in 10% BSA solution.

### *SOD Administration*

10,000 U/kg of PEG-SOD were injected i.v. 45 min before killing the animals (Fig. 2).

### *Preparation of Tissue for EPR Spectrometry*

The cerebral cortex and the cerebellum were rapidly removed and homogenized in an equal volume (w/v) of 0.2 M of HEPES buffer (pH 7.4). The homogenate was extruded into a quartz EPR tube (inner diameter 3 mm) in a volume sufficient to exceed the EPR cavity height, immediately frozen, and kept at liquid nitrogen temperature until EPR spectra recording.

### *EPR Spectroscopy*

EPR spectra of paramagnetic mononitrosyl–iron DETC complex were recorded at 77 K using a Varian E109 spectrometer. The experimental conditions were as follows: klystron frequency 9.32 GHz, microwave power 20 mW, field modulation amplitude 0.5 mT at 100 kHz frequency, time constant 0.5 sec and $2.5 \times 10^4$ gain, field range 40 mT. The magnetic field was calibrated with the stable radical 1,1-diphenyl-2-picrylhydrazyl ($g = 2.0036$). The EPR signals of different brain preparations were compared, based upon the peak-to-peak amplitude of the triplet signal at $g = 2.035$, between the peak at 2.047 and the trough at 2.025, produced from identical volumes of tissue homogenate.

### *Measurement of the Inhibitory Effect of the Flavonoids on In Vitro Superoxide Anion Generation*

INHIBITION OF XANTHINE OXIDASE. A mixture of 2.4 mL of 50 mM sodium carbonate buffer (pH 10.2), 0.1 mL of 0.75 mM NBT solution, 0.1 mL of a 3 mM xanthine solution, and 0.1 mL of a 0.15% BSA solution was preincubated at 25° for 20 min. It was then added to 20 μL of DMSO containing the flavonoids at the final concentrations of 0.67, 1.00, and $1.33 \times 10^{-6}$ M. After the addition of XOD (0.14 U per tube) and further incubation for 20 min at room temperature, 0.1 mL of a 6 mM $CuCl_2$ solution was added to the reaction mixture, and the absorbance at 560 nm was measured against the blank samples containing no xanthine. In the measurements with quercetin, each concentration had its own control, containing the same amount of quercetin but added after the reaction was finished. The results were expressed in percent of control absorbance [35].

SCAVENGING OF SUPEROXIDE ANION GENERATED NONENZYMATICALLY. Superoxide anions were generated in samples which contained 10 μM phenazine methosulfate, 78 μM NADH and 25 μM NBT in 0.1 M phosphate buffer, pH 7.4, and the flavonoids dissolved in 0.7 mL of DMSO at various concentrations. The total volume of the incubation mixture was 2 mL. The absorbance at 560 nm reached the maximum and became stable at 7 min: it was measured against the blank containing no phenazine methosulphate. $IC_{50}$ values (concentrations that cause 50% inhibition) were calculated for the inhibition by the flavonoids of the generation of superoxide anions by calculation of the linear regression of absorbance plotted against log concentration [27, 29]. The experiments *in vitro* were repeated twice in three replicates.

### *Statistics*

All results are expressed as means ± SD. Intergroup comparisons were made by *t*-test for 2 groups or ANOVA for 3 or more groups; when the ANOVA was significant, a

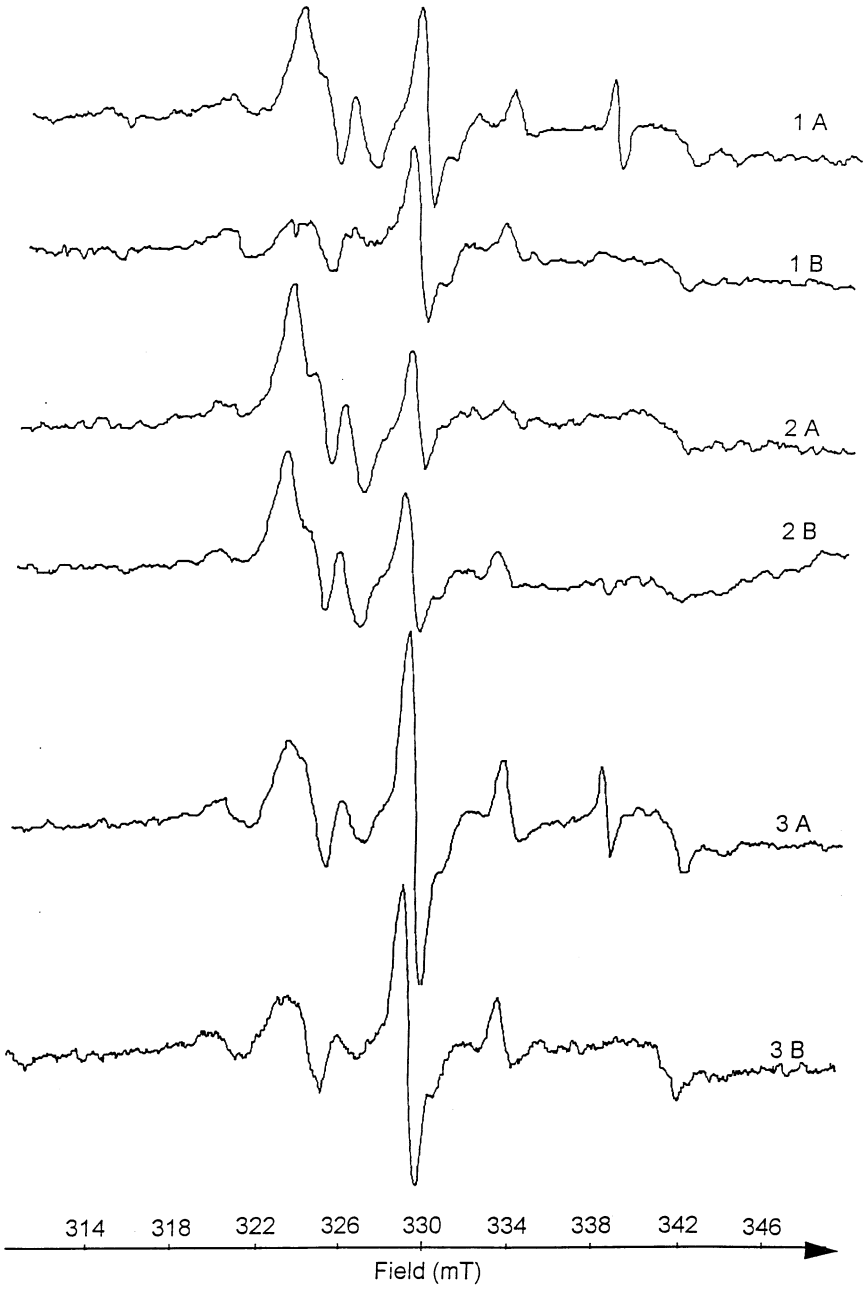


**FIG. 3. EPR spectra at 77 K of $Fe(DETC)_2NO$ and Cu–DETC complexes in rat brain homogenates: 1, control animals; 2, 15-min global ischemia; 3, 15-min global ischemia followed by 30-min reperfusion. A: cerebral cortex; B: cerebellum. EPR conditions are given in Materials and Methods.**

Tukey or Dunnett test was applied as appropriate to determine which pairs of groups differed. When normality tests were failed or variances were significantly different, nonparametric tests were used: Mann-Whitney for 2 groups and Kruskall-Wallis (plus Dunn's test) for 3 or more groups. $P < 0.05$ was considered a significant probability.

## RESULTS

### *EPR Spectra Analysis*

Figure 3 shows the 77 K EPR spectra of brain homogenates taken from (1) control, (2) ischemic, and (3) ischemic-reperfused animals. A triplet EPR signal with a peak at $g = 2.047$ and a shallow trough at $g = 2.025$ [33, 34, 36–39] were observed in cerebral cortex homogenate when DETC and Fe were administered to the intact rats (spectrum 1A). The cerebellum homogenate of the same animals presented only the background EPR spectrum of the endogenous Cu–DETC complex (spectrum 1B). The peak-to-peak amplitude is a measure of the relative concentration of the $Fe(DETC)_2NO$ signal. Spectra 2A and 2B are taken from an ischemic brain. Typically, well-resolved triplet signals are observed in this case. Spectra 3A and 3B are taken from brain tissue of a rat subjected to ischemia and reperfusion.

### *NO Levels during Ischemia and Reperfusion*

Figure 4 shows the relative amplitudes of the $Fe(DETC)_2NO$ spectra of the untreated control (intact),

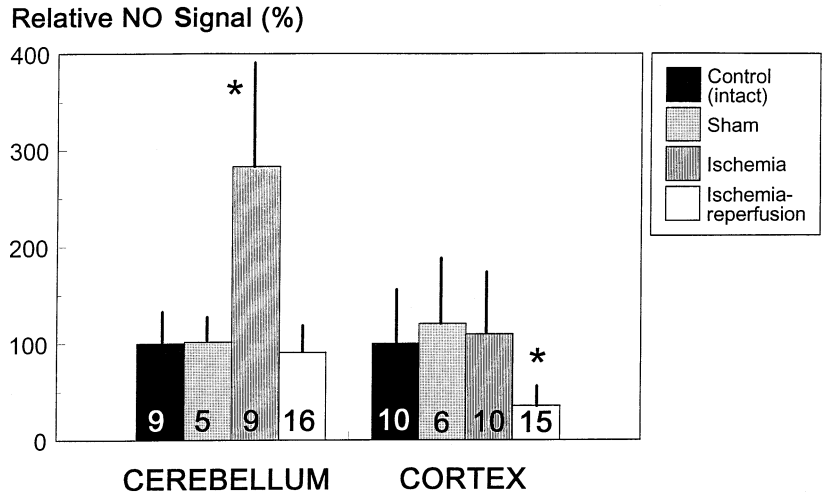


**FIG. 4. Relative amplitude (means ± SD, as % of value in intact animals) of the $Fe(DETC)_2NO$ signal in cerebellum and cerebral cortex of untreated rats. The animals were killed under halothane anesthesia either intact, sham-operated, after ischemia, or after ischemia and reperfusion. The heights of the triplet EPR signals for cerebellum and cortex in intact animals were 2.40 ± 0.72 and 6.93 ± 2.95 arbitrary units, respectively (significantly different, $P < 0.05$, *t*-test). *Significantly different from the other three columns of the same structure, $P < 0.05$.**

sham-operated, ischemia, and ischemia-reperfusion groups of animals expressed relative to the intact group. There were no significant differences (*t*-test) between the control and the sham groups for either the cortex ($P = 0.394$) or the cerebellum ($P = 0.917$). In the cortex, analysis of variance showed no significant changes compared to control except in the ischemia-reperfusion group, where the NO level was reduced to 35% of control. In the cerebellum, a huge increase was observed during ischemia (to 283% of control), whereas in the ischemia-reperfusion group the amplitude of the signal was only slightly below that of the control (91%, NS). The intact control NO level was significantly higher in the cortex than in the cerebellum (6.93 ± 2.95 vs 2.40 ± 0.72 arbitrary units, $P < 0.05$, *t*-test).

### *Effect of Quercetin on NO Levels*

I.p., i.v. or s.c. administration of the natural flavonoid quercetin did not change significantly the level of NO in either brain region of intact animals (Fig. 5).

In the ischemia-reperfusion group, the increases in the NO signal induced by quercetin were significant in the cortex of the rats given 5 mg/kg i.v. but nonsignificant in those given 20 mg/kg i.p. (Fig. 5). For the i.v. group, the NO signal was then more than 2-fold higher than in the untreated animals and also significantly higher than after FB277. Concerning the cerebellum, no significant differences were observed between the signals in the ischemia-reperfusion groups of treated and untreated animals (Fig. 5).

### *Effect of FB277 on NO Levels*

I.v. administration of FB277 provoked a significant increase in the NO signal found in both the cerebellum (to 141%) and cerebral cortex (to 167%) of intact animals (Fig. 5). In the ischemia-reperfusion group, an increase in the NO level compared with the nontreated ischemia-reperfusion group was found in the cerebellum (to 152%), but not in the cortex (Fig. 5).

### *Effect of PEG-SOD on NO Levels*

As in the case of FB277, i.v. administration of PEG-SOD to intact animals induced a significant increase in the NO signal in both brain regions compared with the controls, to 147% in the cerebellum and 182% in the cerebral cortex (Fig. 5). In the ischemia-reperfusion group, a significant increase (to 149%) was found only in the cerebellum (Fig. 5).

### *Effect of Flavonoids on the Activity of XOD In Vitro*

At all the concentrations studied (0.67, 1.00, and 1.33 μM), quercetin exerted a significant inhibitory effect on the activity of XOD. The degree of inhibition was concentration-dependent. The highest level of inhibition of the enzyme activity, observed for the concentration of 1.33 μM, was 18.4% ($P < 0.001$). On the contrary, FB277 was active only at the highest concentration, at which it induced an elevation of the enzymatically produced superoxide by 24.7% ($P < 0.05$).

### *Effect of Flavonoids on the Nonenzymatic Production of Superoxide Anions*

Quercetin showed a strong scavenging activity of superoxide anions generated nonenzymatically. The $IC_{50}$ (concentration causing 50% inhibition) for quercetin was 94 μM, whereas FB277 was inactive in the range of concentrations studied (100–5000 μM).

## DISCUSSION

### *NO under Control Conditions, in Ischemia, and during Reperfusion*

Using the NO trapping agent DETC, we have demonstrated that NO radicals are produced in the brain of control rats, in agreement with the results of Tominaga *et al.* [39], who carried out validation experiments with this method. The amount of the $Fe(DETC)_2NO$ complex measured, according to our results, was greater in the cerebral

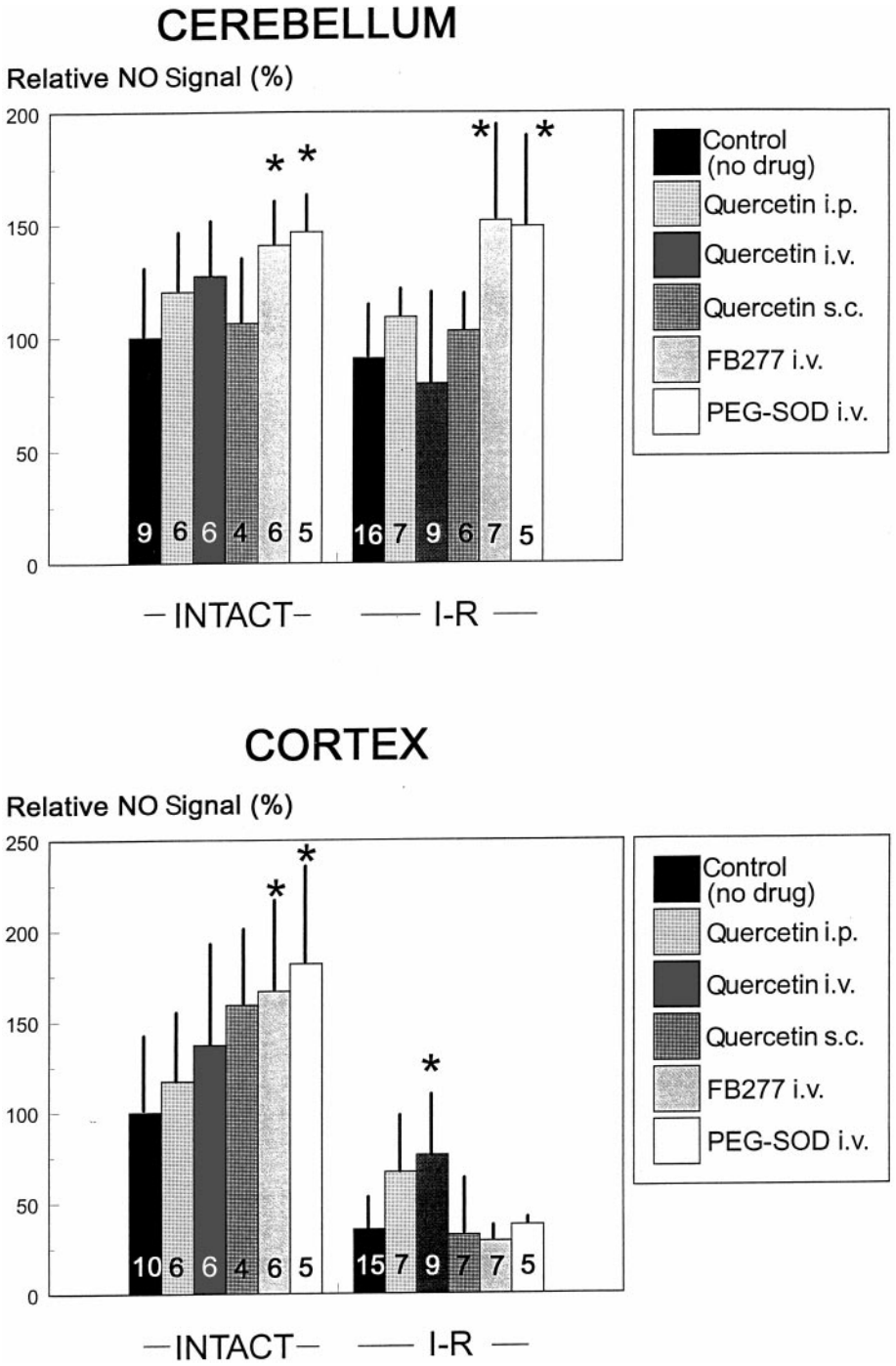


**FIG. 5.** Relative amplitude (means ± SD, as % of value in intact untreated animals) of the $Fe(DETC)_2NO$ signal in cerebellum and cerebral cortex in untreated animals and animals treated with quercetin (20 mg/kg i.p., 5 mg/kg i.v. or 37.5 mg/kg s.c.), FB277 (3.75 mg/kg i.v.) or PEG-SOD (10,000 U/kg i.v.). I-R, ischemia-reperfusion group. *Significantly different from control column of the same series, $P < 0.05$.

cortex than in the cerebellum (see legend of Fig. 4), in accordance with the recent results of Olesen *et al.* [40] using essentially the same technique. This appears paradoxical in view of (a) the quantitative estimates of the NOS concentrations made by autoradiography in these two structures [41, 42] indicating an approximately 2-fold higher concentration in the cerebellum and (b) the higher values of cerebellar NOS activity determined *in vitro* [43, 44]. Assuming that there was no significant difference between regions in the distribution of DETC, Fe and/or $Fe(DETC)_3$, the present result suggests that the actual *in vivo* activity of NOS in various regions may not be simply related to the quantity of enzyme present. The prevailing physiological conditions, such as neuronal activity and the availability of substrates and cofactors and other regulators, may significantly affect the amount of NO produced or available. For example, one factor that should be envisaged is the thiol-mediated turnover of NO [45–47]. In particular, GSH under aerobic and anaerobic conditions might stimulate NOS activity [48] and participate in the mobilization and, perhaps, in the catabolism of nitric oxide. Although NO does not require a carrier or transporter to reach its pharmacological target, it has to diffuse through cells in the presence of 5–10 mM of GSH as nitrosylated glutathione

[49], which may represent a means of stocking NO in a readily accessible form. The association with GSH limits the inhibition of respiration by NO and could allow the slow release of NO into the extracellular environment [50]. Thus, the amount of NO trapped in our measurements might be greater in tissue with higher concentrations of such thiols. Despite some disparity between authors, it seems likely that GSH concentration is a little higher in cortex than in cerebellum [e.g. 51].

We did not find any significant differences in the amount of NO trapped in sham-operated animals compared to controls (Fig. 4), showing that the surgical preparation did not significantly affect NO production. This is also compatible with the notion that occlusion of the CCA is a more critical factor in the induction of ischemic conditions likely to modify NO production than is electrocoagulation of the vertebral arteries, since carotid occlusion alone has been shown to cause a rise in NO levels [37].

The role of halothane in modifying NOS activity *in vitro* has been studied in several reports, with varying results; both significant inhibition, about 30% at 0.5 minimal alveolar concentration of halothane [52], and an absence of effect [53] have been reported. The animals in our study were maintained systematically under about 1% halothane, but since none of the compounds tested is expected to interfere directly with NOS activity, we assume that the anesthetic regimen did not significantly influence the comparison of NO levels.

Although it has been shown that 3 days are required after global forebrain ischemia before significant induction of type 2 NOS [54], we found that the cumulated NO radical production determined after 15 min of global ischemia in the cerebellum increased by almost 3-fold with respect to control (Fig. 4), which is probably a physiological response to the decreased blood flow, i.e. the activation of NOS [55]. Under such conditions, this compensatory NO production probably plays a beneficial role by increasing the vasodilator tone [33]. In the cortex, however, the amount of NO trapped was scarcely elevated (Fig. 4). This observation may be related to the fact that the various regions of the brain have differing reactions to global forebrain ischemia, the most marked lesions occurring in the cortex, the hippocampus, and the striatum [56–58]. The cortex probably had a too low partial pressure of oxygen during ischemia to react by a compensatory stimulated NO production [59]. In their experiments on the effects of global ischemia on NO production, Tominaga *et al.* [39] estimated that a large increase in NO production occurred in the cortex. It is not clear why there was such a difference compared to our result, except for the possibility that the method used in inducing global ischemia (carotid ligation and hypotension) might have allowed a higher $PO_2$ to persist in the cortex. In the experiments by Olesen *et al.* [40], the proportionately larger effects of forebrain ischemia in general may be explained by their choice of briefer measurement periods (7 mins) englobing only the most intense increases in NO production. This strong initial stimulation followed by a progressive diminution of the effect has been noted in focal ischemia (middle cerebral artery occlusion) [60–62] and is attenuated by specific inhibitors of NOS.

It should be noted that the measurement period for NO determination in the ischemia group (Fig. 2) was 45 min, from the injection of the spin trap to the end of the ischemia, thus differing from the other groups (the 30 min preceding sacrifice). We considered that, blood flow being virtually zero in the cortex during 15 min CCA occlusion, a 30-min period would not allow complete equilibration of the spin trap in the brain parenchyma. Possibly, this choice led to some degree of underestimation of the NO level in ischemia due to slow dissociation of the complex after the initial formation. However, the measurements of the effects of flavonoids or PEG-SOD were not made in the ischemia group and so are not concerned by this uncertainty.

In reperfusion following ischemia, the amount of free NO measured was drastically reduced to approximately the control level in the cerebellum and down to 35% of control in the cerebral cortex (Fig. 4). Both these decreases are likely to be connected with the large amounts of superoxide formed during the first few minutes of reperfusion [63] and the ability of this radical to rapidly react with NO, producing the highly toxic and potent oxidant peroxynitrite [12, 13]. Other factors, different in the two structures, might also contribute to these large drops in NO levels during reperfusion. In the cerebellum, the return of a normal level of circulation may have removed the signal for NOS stimulation (excess glutamate, intracellular $Ca^{2+}$) which was present during ischemia. On the contrary, in the cortex the reoxygenation after the quasi-anoxic period may have allowed a transient increase in the NOS activity as well as in superoxide production. However, membrane damage (including the blood–brain barrier) in this structure is likely to be more extensive because of the greater initial energy deficit and cell depolarization (during ischemia) and the greater production of peroxynitrite (during reperfusion), leading to greater washout of NO in the blood, either alone or in combination with thiols.

Thus, the acute utilization of superoxide scavengers at an early stage of ischemia and reperfusion should be able, by decreasing the concentration of available superoxide, to reduce the possibility of peroxynitrite formation and increase the levels of NO.

### *Effects of Treatment with Flavonoids or SOD*

QUERCETIN. The elevation in the NO level induced by i.v. quercetin in the cerebral cortex of animals subjected to ischemia-reperfusion may result from its two mechanisms of action, i.e. inhibition of hypoxanthine–XOD activity and scavenging of superoxide (current *in vitro* results and [27, 29, 30]). The results of our investigation thus confirm *in vivo* the properties of quercetin previously demonstrated *in vitro*. The elevation in the NO concentration was statistically significant only after i.v. administration (Fig. 5),

probably due to the higher molecular bioavailability of quercetin thus attained compared to the other modes of administration. Intraperitoneal (but not subcutaneous) administration induced changes in the same direction, but these did not reach statistical significance. It is notable that the efficiency of quercetin in increasing the NO level during reperfusion was limited to the cerebral cortex. In contrast, the synthetic flavonoid did not raise the NO level during reperfusion in either structure. This point is discussed later.

**FB277.** This compound differs importantly from quercetin in that it possesses a carbon chain at position 3 instead of a hydroxyl group, and its phenol groups are all *O*-methylated (Fig. 1). It proved to be incapable of restoring the level of NO in the cerebral cortex of rats subjected to ischemia-reperfusion (Fig. 5), although it did increase the NO measured in the cerebellum and in the intact animal in both structures. The absence *in vitro* of any superoxide scavenging activity or inhibitory effect on XOD (there was rather a stimulation) emphasizes that not all flavonoid compounds possess such activity. As discussed above, in this model the ischemic conditions are certainly much more severe in the cortex than in the cerebellum, with probably a much greater tendency for superoxide scavenging of NO at the beginning of cortical reperfusion. In the cerebellum, the NOS activity was apparently maintained and may have been stimulated by the flavonoid. This hypothesis would also explain the increased levels of NO in intact animals.

An additional possible explanation for the effectiveness of quercetin but not FB277 in restoring the NO level in the cortex is provided by the study of Haenen *et al.* [64]. In an *in vitro* comparison of seven flavonoids, they identified quercetin as the most active scavenger of peroxynitrite, superior to the well-known scavenger ebselen. The substitutions on the flavonoid moiety all being hydroxyl groups in the case of quercetin, and larger groups in the case of FB277, it seems highly probable that the latter molecule is a much weaker scavenger of such ROS. Although certain flavonoids have been shown to scavenge NO [65], this seems unlikely to be significant here since the flavonoids tested tended to increase the NO levels in intact animals.

**PEG-SOD.** Although PEG-SOD administered acutely like the flavonoids raised the level of NO determined in intact animals (in both brain structures), like FB277 it was unable to restore any part of the NO lost in the cortex during reperfusion (Fig. 5). This compound has been demonstrated to exert a protective action on the outcome of ischemia-reperfusion [19], but in a protocol involving chronic pretreatment. A relatively permeable polyamine-modified SOD has also been shown to decrease hippocampal cell death resulting from 12-min global ischemia in rats, following twice daily treatment for three days after ischemia [66]. In the present experiments, the quasi-absence of circulation in the cortex during ischemia probably prevented sufficient access of this large molecule to the parenchyma. This reduced access associated with the briefness of the critical early period of reperfusion would then prevent the administration of PEG-SOD from exerting any beneficial effect (Fig. 5).

In conclusion, the present results are compatible with the hypothesis that superoxide scavenging by quercetin during reperfusion after a period of global forebrain ischemia can restore at least partly the fall in the NO level in the cortex. In the cerebellum, on the other hand, the olighemia (not ischemia) created by this model probably did not induce conditions conducive to large-scale superoxide production, so that no such beneficial effect was seen. The influence of treatment with this natural flavonoid on the balance of nitric oxide and superoxide and, therefore, on peroxynitrite concentrations suggests that such compounds, if active scavengers, might be capable of a protective role in various human disorders such as cerebral ischemia.

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