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Aniracetam, a Pyrrolidinone-type Cognition Enhancer, Attenuates the Hydroxyl Free Radical Formation in the Brain of Mice with Brain Ischaemia

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Abstract

We demonstrate here that aniracetam has the ability to block the formation of cytotoxic hydroxyl radicals (·OH) during ischaemia-reperfusion of mouse brain.

The fact that brain ischaemia for 40 min followed by reperfusion increased ·OH was evidenced by detection of a peaked increase at 20 min after an ischaemic insult in the formation of 2,3-dihydroxybenzoate (DHBA) from salicylate in cerebroventricular perfusate, a means of monitoring ·OH formation. A clearcut increase in dopamine was also observed during and after brain ischaemia. The ischaemia-reperfusion mice given aniracetam at an intraperitoneal dose of 30 or 100 mg kg^{-1} showed a smaller increase in the formation of DHBA than those given the vehicle only.

Aniracetam at 100 mg kg^{-1} significantly suppressed the formation of DHBA by approximately 80%, becoming evident at 20 min after reperfusion and thereafter. Protection against death in mice insulted with a 40-min brain ischaemia (3/13 vs 13/25) was observed following 100 mg kg^{-1} aniracetam. The increase in the dopamine levels was substantially reduced following aniracetam treatment and the reduction became significant at 20 min after reperfusion and thereafter in parallel with attenuation by aniracetam of DHBA formation.

This finding suggests that the inhibitory activity of aniracetam in attenuating the hydroxyl free-radical formation in ischaemic mice is probably due, at least in part, to its palliative action on the dopaminergic neurons.

Recent electrophysiological and biochemical studies have been reported describing the modulatory properties of the pyrrolidinone-type cognition enhancer aniracetam in terms of allosteric potentiation of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)-specific glutamate receptors (Ito et al 1990), a reduction of glutamate receptor desensitization (Isaacson & Nicoll 1991), and potentiation of metabotropic glutamate-receptor activity (Pizzi et al 1993). Cholinergic neuronal modulation by aniracetam is also presumed, according to a variety of experimental data encompassing down-regulation of muscarinic acetylcholine receptors (Nakajima et al 1986), increased acetylcholine content in the hippocampus (Toide 1989), reversal of increased choline uptake by scopolamine into the dentate gyrus (Yoshizaki & Okada 1986), and facilitated acetylcholine release (Giovannini et al 1993). These modulatory properties may make aniracetam very efficient at amplifying synaptic efficacy and at generating long-term potentiation (Satoh et al 1986; Okuyama & Aihara 1988).

Aniracetam may also prove to be advantageous for use in symptomatic therapy (Otomo et al 1987, 1991; Himori 1993; Himori & Mishima 1994). Aniracetam has been clearly demonstrated to improve impaired cognitive function and disrupted behaviour in a battery of tests with laboratory animals (Cumin et al 1982; Gamzu 1985; Kubota et al 1986; Martin et al 1992; Martin & Haefely 1993; Himori & Mishima 1994). Two double-blind clinical trials have shown the benefit of aniracetam, especially for treating psychiatric symptoms, such as nocturnal wandering, delirium, and emotional disturbance observed following such cerebrovascular disorders as infarction and bleeding (Otomo et al 1987, 1991). Since cerebrovascular circulation, brain energy metabolism, and neuronal function are usually regarded as being closely linked, it seems likely that the demonstrated efficacy of aniracetam in ameliorating the condition of laboratory animals and patients with cerebral blood flow interruption (Kubota et al 1986; Otomo et al 1987, 1991; Himori & Mishima 1994) is not solely due to its neuronal modulatory properties. It is widely believed that ischaemic conditions bring about a diversity of responses that lead to the production of the reactive, toxic hydroxyl radicals (DeLeo et al 1986; Cao et al 1988; Phebus & Clemens 1989; Damsma et al 1990; Liu et al 1991). In the central nervous system, where there is an abundance of membrane lipid, free radicals react with membrane polyunsaturated fatty acids, resulting in a sequence of lipid peroxidation, membrane damage, and cell death.

The present studies using the previously described salicylate trapping method for detecting the levels of hydroxyl radicals (Floyd et al 1984) were undertaken in a first attempt to determine the effect of aniracetam on the early generation of this key radical species in mice following an inflicted transient cerebral ischaemic attack. The results suggest that aniracetam, in addition to its neuromodulatory properties, is also a neuroprotectant.

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Materials and Methods

Animals

Male ddY mice, aged 7–9 weeks (35–40 g), were kept under

regular lighting conditions (lights on at 0700 h and off at 1900 h) in an animal room with a controlled temperature of 22 ± 2°C and relative humidity of 55 ± 10%, and had free access to water and food.

Brain ischaemia/reperfusion

Transient two-vessel occlusion to cause forebrain ischaemia was performed according to a procedure similar to that described by Himori et al (1990). Under intraperitoneal hexobarbitone anaesthesia (120 mg kg^{-1}), an occluder and two releasers were loosely placed around both common carotid arteries of mice. The mice were allowed 3–5 days for recovery before the experiments were begun.

Cerebroventricular perfusion

The technique for perfusing the cerebral ventricle under ischaemia-reperfusion has been presented to the Japanese Pharmacological Society (Ueno et al 1994). On the experimental day, the mice that had undergone surgery for brain ischaemia intervention were anaesthetized with urethan (500 mg kg^{-1}) in combination with α-chloralose (50 mg kg^{-1}) and were placed on a thermopad (Model PHT-1302, Toshiba, Tokyo, Japan) kept at 37°C during the entire experiment. The brain ventricle was perfused with artificial cerebrospinal fluid (ACSF) containing salicylic acid (1 mM) through two small implanted cannulae (27 gauge) by means of a Harvard Digital Infusion Pump (Model 22, South Natik, MO, USA) at a rate of 5 μL min^{-1}; approximately 3 h before the experiments, the two cannulae were respectively inserted, by using a stereotaxic instrument, into the right and left lateral brain ventricles at sites 1·6 mm lateral (right and left) from a midsagittal line, 0·5 mm posterior to the bregma and 2·2 mm below the dura. The trachea of each test animal was intubated by a small tracheal tube to allow smooth breathing. Two hours after the initiation of cerebroventricular perfusion, the occluder was tightened for 40 min to occlude the bilateral common carotid arteries, causing brain ischaemia and then loosened again by pulling the releasers to allow for reperfusion. The experiments lasted for 2 h after reperfusion. Aniracetam (Ro 13-5057, 1-*p*-anisoyl-2-pyrrolidinone, F. Hoffmann-La Roche, Basle, Switzerland) or its vehicle (0·25% carmellose) was given intraperitoneally (10 mL kg^{-1}) 45 min before the brain ischaemia.

Quantitation of hydroxyl radical generation in the brain perfusate

We utilized salicylic acid, a highly effective ·OH trapper, which upon scavenging ·OH forms the stable product 2,3-dihydroxybenzoate (DHBA) by a hydroxylation reaction. The DHBA formed was quantified by high performance liquid chromatography (HPLC) separation coupled with electrochemical detection (ECD), using a slight modification of the method of Floyd et al (1984). Brain perfusate (ACSF sample) was collected every 20 min before, during, and after brain ischaemia, and stored at −80°C until the time of analysis. Ten microlitres of 1 M perchloric acid was added to each ACSF sample (60 μL). After centrifugation at 7000 *g* for 10 min at 4°C, 10 μL of each sample was applied to HPLC-ECD (Eicom, Kyoto, Japan) under the following HPLC conditions: an analytical column (LiChrosphere 100 RP-18 (e), particle size 5 μm, 25 cm × 4 mm i.d.) was guarded by a LiChrosorb column (RP-18, particle size 30 μm, 4 mm × 4 mm i.d.), the mobile phase contained 30 mM citrate/30 mM acetate buffer (pH 3·6) consisting of 0·2 g L^{-1} sodium azide and 20% methanol, and the applied voltage was +0·65 V vs a Ag/AgCl reference electrode. The flow rate of the mobile phase was 0·7 mL min^{-1}.

Quantitation of dopamine in the brain perfusate

Ten microlitres of the perchloric acid-treated ACSF samples was applied to another HPLC-ECD system (Eicom) under the following conditions (Murai et al 1988). An Eicompak MA-50DS column (particle size 5 μm, 15 cm × 4·6 mm i.d.) was used for the analysis. The mobile phase consisted of 20 mM sodium acetate/12·5 mM sodium citrate buffer (pH 3·92) containing 13% methanol, 0·033% heptanesulphonic acid, and 0·1 mM EDTA, and the applied voltage was +0·65 V vs a Ag/AgCl reference electrode. The mobile phase was delivered by a pump at 1·2 mL min^{-1}. A precolumn (LiChrosorb, RP-18) was placed between the pump and analytical column.

Data analysis

Results are expressed throughout the text as either the absolute number of mice showing respective variables or the percent change of hydroxyl radical formation expressed as means ± s.e.m., and n is the sample size or number of animals used. The following statistical analyses were made to assess the differences in values between groups: Mann-Whitney U-test for the studies relating to hydroxyl radical formation, Mann-Whitney U-test and Williams multiple comparison test for changes in dopamine levels, and Fisher's exact probability test for the incidence of death or the number of animals showing outlying DHBA values outside the range of those from the corresponding sham-operated animals. $P < 0.05$ was considered to be statistically significant.

Results

Time-course change in the generation of hydroxyl radicals during ischaemia-reperfusion

Fig. 1 shows the time course for changes in DHBA levels in mice subjected to a 40-min period of brain ischaemia. The DHBA levels increased to a peak at 20 min after reperfusion (approx. 60%) ($P < 0.01$) and then fell. For the sham-operated control group, the change in the DHBA levels was insignificant (within ± 20%).

Time-course change in the incidence of death during ischaemia-reperfusion

Table 1 shows the number of mice that died during ischaemia after reperfusion, and the cumulative incidence during the whole experimental period of 160 min. Brain ischaemia caused by occlusion of the bilateral common carotid arteries resulted in approximately 50% incidence of death: 13 out of 25 animals died within the observation period. Deaths occurred mainly during the brain ischaemia

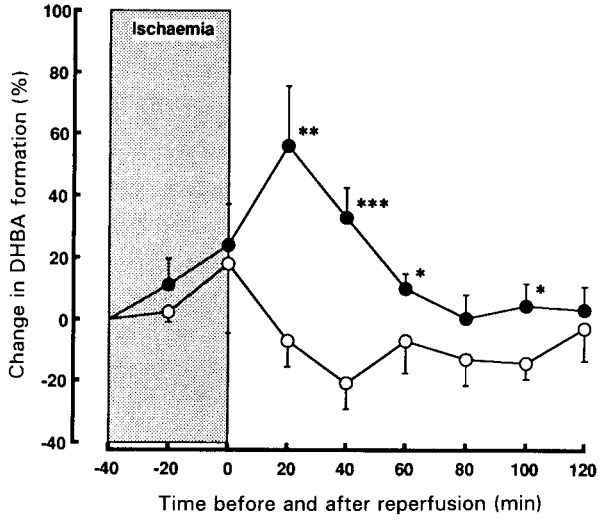

FIG. 1. Changes in 2,3-dihydroxybenzoate (DHBA) levels of brain perfusate during ischaemia-reperfusion in mice (○ sham operated; ● ischaemia reperfusion). The perfusate (ACSF sample) was collected every 20 min during and after a 40-min period of brain ischaemia. Respective data points are shown as the means ± s.e. obtained from 15 animals. Absolute DHBA levels for the sham-operated and ischaemia groups were 0·060 ± 0·006 and 0·061 ± 0·007 pmol min^{-1}, respectively. $*P < 0·05$, $**P < 0·01$, $***P < 0·001$ vs the corresponding samples of the control sham-operated group (Mann-Whitney U-test).

(40 min) and early reperfusion periods (0–80 min). No deaths occurred in the sham-operated group.

Attenuation by aniracetam of the hydroxyl radical generation resulting from an ischaemic insult

Intraperitoneal administration of aniracetam before the ischaemia/reperfusion insult inhibited the induced increase of perfusate DHBA levels. The inhibitory effects of aniracetam on the ischaemia-elicited DHBA generation were dose-dependent; the increase in the DHBA levels in ischaemic mice treated with aniracetam at the doses of 30 and 100 mg kg^{-1} did not reach statistical significance (Fig. 2). The number of mice with outlying DHBA values—those exceeding the value (216·2) of the mean + 1·96 s.d. of the sham-operated group—was much less for the mice given aniracetam, being respectively 6·8 and 1·3% for 30 and 100 mg kg^{-1}, compared with 10·4% for the vehicle-treated ischaemia group (Table 2). One out of 11 animals in the sham-operated group unexpectedly provided an abnormal value of 479·4% only at the 60-min sampling time after reperfusion, hence none of the data derived from this animal were included in the present study.

Protection by aniracetam against death in mice subjected to an ischaemic insult

Aniracetam was administered to 28 and 13 mice at the respective intraperitoneal doses of 30 and 100 mg kg^{-1}. With the lower dose, the incidence of death was not significantly different from that of the vehicle-treated ischaemia group. The administration of the higher dose, however, produced a significant decrease in the number of deaths (mortality, approx. 20%), compared with that of the vehicle-treated ischaemia group (mortality, approx. 50%) (Table 1).

Inhibitory action of aniracetam on the increase in dopamine levels during ischaemia-reperfusion

The dopamine levels in the brain perfusate significantly increased to a peak at 20 min after brain ischaemia and then waned rapidly. Aniracetam inhibited the increase of perfusate dopamine levels at a dose of 100 mg kg^{-1}, and the dose-dependent attenuation by aniracetam of the dopamine release was significant 40–120 min after reperfusion (Fig. 3).

Discussion

The present study consistently demonstrated the occurrence and time course of increased ·OH formation in an early stage of brain ischaemia-reperfusion; that is, the observed increase of DHBA in these ischaemia-reperfusion experiments was significant after cessation of an ischaemic insult, while only a small increase in DHBA level was observed during the brain ischaemic incident, probably due to leakage of DHBA formed in the ischaemic area from its satellite zone where the vertebral artery irrigates (Figs 1, 2). To explain this post-reperfusion increase in the DHBA levels, there are a number of possible scenarios for potential sources of oxygen radicals that are operative after brain ischaemia-reperfusion, including mitochondrial leak, a chemical modification of the enzyme xanthine oxidase that has been shown to be responsible for producing excessive levels of superoxide radicals upon reperfusion, arachidonic acid oxidation by either prostaglandin synthetase or

Table 1. Effects of aniracetam on the incidence of death of mice during brain ischaemia-reperfusion.

Treatment	Intraperitoneal dose (mg kg^{-1})	No. of animals	Incidence of death		Total no. of deaths	Mortality (%)
			During ischaemia (min)	After reperfusion (min)		
			−40–0	0–40–80–120		
Sham + vehicle	–	10	0	0 0 0	0	0
Ischaemia + vehicle	–	25	3	6 4 0	13*	52·0
Ischaemia + aniracetam	30	28	4	8 4 1	17*	60·7
	100	13	0	2 1 0	3	23·1

The incidence of death was observed during and after an ischaemic insult at 40-min intervals for 160 min. The animals were given aniracetam intraperitoneally 45 min before a 40-min period of brain ischaemia. Vehicle (0·25% carmellose) was given at 10 mL kg^{-1}. $*P > 0·01$ vs control sham-operated group (Fisher's exact probability test).

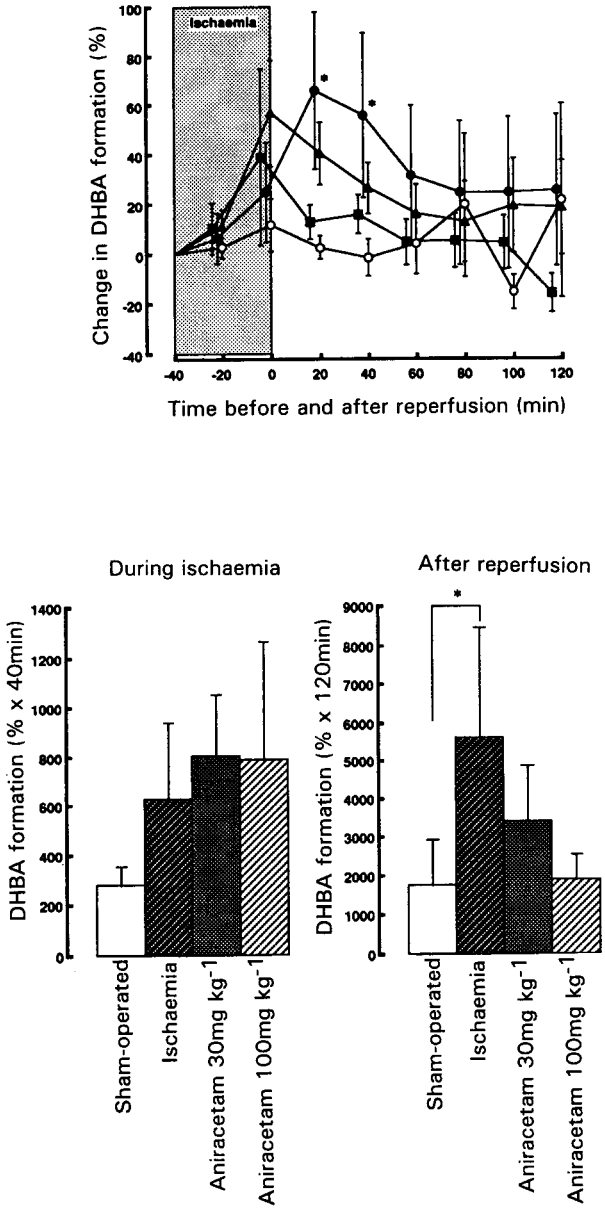

FIG. 2. Attenuation by aniracetam of 2,3-dihydroxybenzoate (DHBA) formation in the brain of mice subjected to a 40-min forebrain ischaemic insult (○ sham operated, ● ischaemia; upper panel) and area under the curve of each group (lower panel). Aniracetam, at a dose of 30 (▲ upper panel) or 100 (■ upper panel) mg kg^{-1}, was given intraperitoneally 45 min before the ischaemic insult. Respective data points are shown as the means ± s.e. obtained from 10–12 animals. Absolute DHBA levels of their respective groups were 0·083 ± 0·010 (sham-operated group), 0·066 ± 0·018 (ischaemia group), 0·092 ± 0·021 (aniracetam, 30 mg kg^{-1}, i.p.), and 0·060 ± 0·010 pmol min^{-1} (aniracetam, 100 mg kg^{-1}, i.p.). *$P < 0·05$ vs the corresponding samples of the control sham-operated group (Mann-Whitney U-test).

lipoxygenase (Kukreja et al 1986), and activated polymorphonuclear leucocytes. An enhanced mobilization of iron from tissue stores may also be likely to cause an excessive generation of hydroxyl radicals converted from hydrogen peroxide via Fenton chemistry (see Braughler & Hall 1989). A possible correlation thus exists between the excessive amount of dopamine and the production of hydroxyl radicals presently observed during the ischaemia-reperfusion episode, since it has been proposed that every monoamine oxidase (MAO)-catalysed deamination event produces one molecule of hydrogen peroxide, a hydroxyl radical precursor (see Halliwell 1987). In the present study, we also recognized that where there was an excessive release or accumulation of dopamine, followed by substantial formation of DHBA during brain ischaemia-reperfusion in normal mice. In contrast, in our preliminary study there was only a slight increase in DHBA generation in dopamine-depleted mice given 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), in keeping with the observation that dopamine depletion protected striatal neurons from cell death caused by global forebrain ischaemia (Clemens & Phebus 1988). The effect of a subsequent increase of dopamine level on brain ischaemia has already been reported (Slivka et al 1988; Obrenovitch et al 1990).

In the present system of an in-situ brain perfusion through the cerebral ventricular space, aniracetam was found, for the first time, to suppress dose-dependently the formation of DHBA in mice subjected to brain ischaemia-reperfusion. This suppression became substantially evident at an intraperitoneal dose of 100 mg kg^{-1} 20 min after reperfusion and thereafter. We are not aware of any earlier work providing possible explanations of why aniracetam has the ability to interfere with certain steps in the biological cascade towards the formation of hydroxyl radicals. We can suggest a number of hypotheses, although a detailed understanding of the mechanism is incomplete. First, a decrease in the level of dopamine is a plausible explanation, as mentioned earlier. Aniracetam affected the dopamine level at the dose that effectively suppressed the formation of DHBA (Figs 2, 3); the reduction of dopamine release in mice dosed with the 100 mg kg^{-1} aniracetam was marked and this change became significant at 20 min after reperfusion in parallel with attenuation of DHBA formation by aniracetam. Based on recent research, together with our experimental data from brains of ischaemic mice, we conclude that the demonstrated inhibitory activity of aniracetam is probably, due at least in part, to its palliative action on the dopaminergic neurons. This hypothesis is supported by our previous experimental findings that aniracetam was efficacious in ameliorating the disrupted choice reaction performance induced by methamphetamine (Himori & Mishima 1994). A second possibility is a MAO inhibitory property of aniracetam described in the study by Stancheva & Alova (1988), where aniracetam was found to inhibit total MAO activity in the rat striatum and hypothalamus in-vitro. Recent reports suggested that treatment with clorgyline and deprenyl at the doses that inhibited both A and B type MAOs in the striatal dialysate completely blocked the generation of DHBA induced by 2′CH_3-MPTP in rats (Chiueh et al 1992). We also observed that clorgyline and lazabemide, the highly selective MAO A and B inhibitors, respectively, inhibited hydroxyl-radical generation in mice with transient forebrain ischaemia (Ueno et al 1994). Third, an improvement in neuronal bioenergy caused by aniracetam might be at least in part responsible for its effect against brain ischaemia-reperfusion. This hypothesis is supported by the finding that production of adenosine triphosphate via the trichloroacetate (TCA) cycle of rat brain mitochondria showed a small but significant increase

Table 2. Effects of aniracetam on the number of ischaemic mice showing an outlying 2,3-dihydroxybenzoate (DHBA) value.

Treatment	Intraperitoneal dose (mg kg^{-1})	No. of animals	Number of animals with an outlying DHBA value			Incidence (%)
			During ischaemia (min)	After reperfusion (min)	Outliers/ total	
			−40–0	0–40–80–120		
Sham + vehicle	–	10	0	0 1 1	2/80	2·5
Ischaemia + vehicle	–	12	1	5 2 2	10/96*	10·4
Ischaemia + aniracetam	30	11	2	1 1 2	6/88	6·8
	100	10	1	0 0 0	1/80	1·3

Values (216·2) greater than that of the mean +1·96 s.d. (105·5 + 110·7) of the sham-operated group were taken as outliers. The number of animals with outlying DHBA values was observed during and after an ischaemic insult at 20-min intervals for 160 min. Other information is the same as that described in Table 1 or Fig. 2. *$P < 0.05$ vs control sham-operated group, $P < 0.05$ vs ischaemia group (Fisher's exact probability test).

following oral administration of aniracetam (Himori et al 1992). The enhancing effect on brain oxidative metabolism suggests that aniracetam may preserve to some extent mitochondrial function as well as neuronal integrity in animals inflicted with an ischaemic insult, lessening the generation of hydroxyl radicals. Aniracetam has also been known to potentiate metabotropic glutamate receptor-evoked stimulation of phospholipase C, thus counteracting glutamate-elicited neurotoxicity (Pizzi et al 1993). We assume that both the mitochondrial dysfunction and the excessive release of glutamate augment hydroxyl-radical formation. Any of the possible biological pathways described above leading to the formation of hydroxyl radicals might be impeded following aniracetam treatment. However, it seems unlikely that the effects observed with aniracetam involved changes either in arterial CO_2 tension and partial pressure of oxygen or in body temperature, as normal laboratory animals given aniracetam showed no relevant pharmacological effects on bronchopulmonary, cardiovascular or various central nervous system variables (Himori et al 1986).

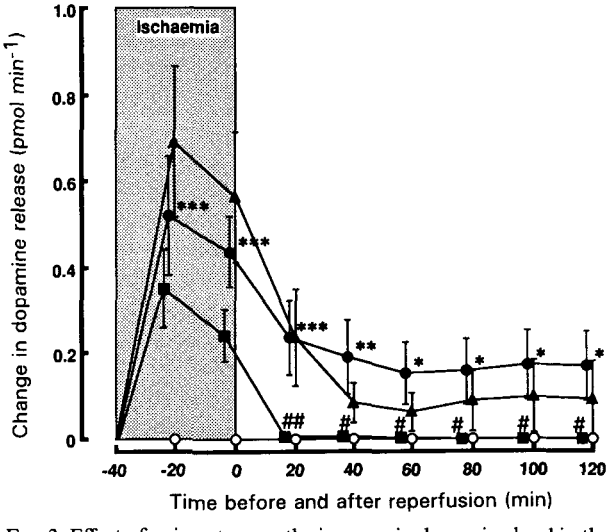

FIG. 3. Effect of aniracetam on the increase in dopamine level in the brain of mice subjected to a 40-min forebrain ischaemic insult (○ sham operated; ● ischaemia). Aniracetam, at a dose of 30 (▲) or 100 mg kg^{-1} (■), was given intraperitoneally 45 min before the ischaemic insult. Respective data points are shown as the means ± s.e. obtained from 10–12 animals. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs the corresponding samples of the control sham-operated group (Mann-Whitney U-test), #$P < 0.05$, ##$P < 0.001$ vs the corresponding samples of the ischaemia group (Williams multiple comparison test).

In summary, these findings are the first direct demonstration of attenuation by aniracetam of hydroxyl-radical formation in mice insulted with brain ischaemia. The neuroprotective property of aniracetam thus appears to offer a supplementary advantage as a means for treating the subsequent neurologic liabilities following cerebrovascular disorders.

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