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Neuroprotective effect of FK506, an immunosuppressant, on transient global ischemia in gerbil

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Abstract

Delayed neuronal death (DND) in CA1 region after transient global ischemia is a well-known phenomenon, but its mechanism has not been clarified. In order to examine the involvement of nitric oxide (NO) in DND, 7-nitro indazole (7NI), a selective neuronal NO synthase (nNOS) inhibitor, and FK506, an immunosuppressant which also inhibits nNOS, were administered intraperitoneally during and after transient global ischemia in gerbil. FK506 moderately ameliorated DND in a dose-dependent manner. However, 7NI showed only minor neuroprotective effects. These results show that DND is not mainly mediated by NO production via nNOS, and FK506 acts as a neuroprotective agent via unknown pathways other than nNOS inhibition.

Keywords: Delayed neuronal death; Global ischemia; Nitric oxide; FK506; 7-Nitro indazole; Gerbil

FK506 was developed as a new immunosuppressant in 1987 [8]. It affects calcium-dependent steps of the transcription of the genes for interleukin-2 and its receptor in T-cells [15]. FK506 binds to a small soluble protein, named FK506 binding protein (FKBP12) [3,11]. FK506-FKBP12 complex inhibits a calcium and calmodulin-dependent protein phosphatase, calcineurin [7]. One of the substrates for calcineurin is a nuclear factor which activates T-cells and regulates interleukin 2 gene transcription in the immune system. FK506 therefore acts as a potent immunosuppressant through inhibiting calcineurin activity.

In 1992, Steiner et al. showed that FKBP12 distributed abundantly in the central nervous system (CNS) and colocalized with its substrate calcineurin in neuronal cells [14]. The precise functions of FKBP12 in the CNS are unknown but it is supposed that one of the substrates of calcineurin in the CNS is neuronal nitric oxide synthase (nNOS). In 1994, Dawson et al. showed that FK506 enhanced phosphorylation of nNOS, reduced nitric oxide (NO) production and thus attenuated glutamate neurotoxicity in cultured neurons [1]. Recently, Sharkey and Butcher have shown that FK506 has a neuroprotective effect in the rat focal ischemia model, possibly through inhibiting nNOS [13].

Transient forebrain ischemia causes delayed neuronal death (DND) in CA1 region of hippocampus in gerbil [6]. But the mechanism of DND has not been elucidated. The purpose of this paper is to examine whether FK506 and 7-nitro indazole (7NI), a selective nNOS inhibitor, have a neuroprotective effect in DND in global ischemia, and to clarify the involvement of NO in DND.

Adult male Mongolian gerbils ($n = 51$), weighing 60–70 g, were divided into ten groups as below according to doses of drugs and timing of drug administration: (1) 7NI (25 mg/kg) 1 min after ischemia ($n = 9$); (2) 7NI (25 mg/kg) 1 min and 24 h after ischemia ($n = 3$); (3) FK506 (0.1 mg/kg) 1 min after ischemia ($n = 3$); (4) FK506 (0.5 mg/kg) 1 min after ischemia ($n = 3$); (5) FK506 (1 mg/kg) 1 min after ischemia ($n = 5$); (6) FK506 (1 mg/kg) 1 min and 24 h after ischemia ($n = 6$); (7) FK506 (1 mg/kg) 1 min, 24 and 48 h after ischemia ($n = 3$); (8) vehicle of 7NI (peanut oil) 1 min after ischemia ($n = 7$); (9) vehicle of FK506 (saline) 1 min after ischemia ($n = 9$); (10) sham-operated ($n = 3$). 7NI (Lancaster Co. Ltd., Edinburgh, UK) was suspended and sonicated in peanut oil (Nakarai Tesq., Kyoto, Japan). FK506 (Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan) was diluted in saline. Anesthesia was induced with 4% halothane in a

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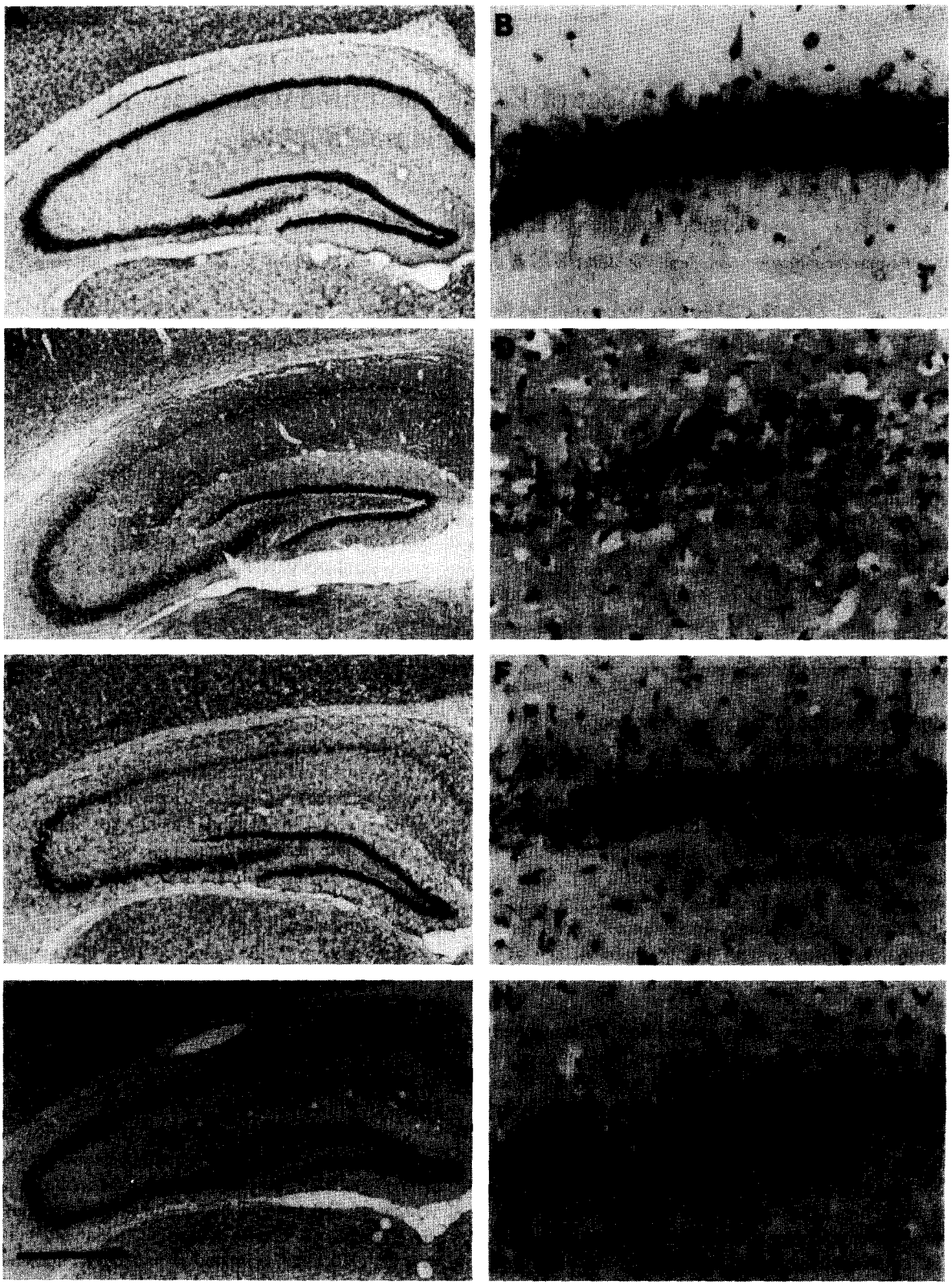

Fig. 1. Representative microphotographs of dorsal hippocampus (A,C,E,G) and CA1 pyramidal cells layer (B,D,F,H) in animals which suffered from sham operation and survived for 7 days (A,B), suffered from BCCA occlusion and were treated with the vehicle of FK506 (C,D), 25 mg/kg of 7NI (E,F), or 1.0 mg/kg of FK506 (G,H). Typical loss of pyramidal neurons in CA1 region is observed following ischemic insults (C,D). While FK506 shows moderate neuroprotective effects (G,H), 7NI shows minimal protective effects (E,F). Bar = 1 mm (A,C,E,G), 0.1 mm (B,D,F,H).

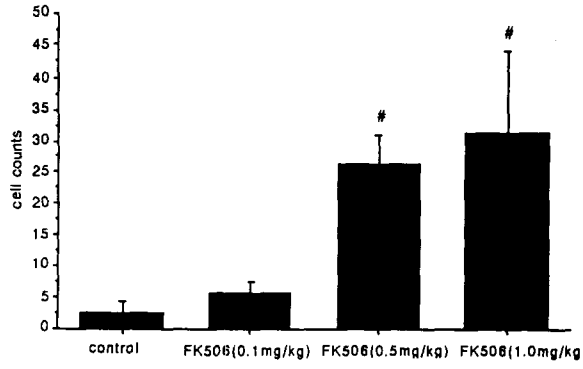

Fig. 2. Numbers of survival neurons in CA1 region of the hippocampus after FK506 treatment at various doses. FK506 shows neuroprotective effects in a dose-dependent manner. Neuroprotective effects with 0.5 mg/kg and 1 mg/kg of FK506 are significant compared with the vehicle (#$P < 0.01$).

gas mixture of 30% oxygen/70% nitrous gas and maintained at 2% halothane in the same gas mixture. Bilateral common carotid arteries (BCCA) were dissected and occluded with microaneurysmal clips for 5 min, and then halothane-reduced to 1%. One minute after BCCA occlusion the drugs or the vehicles were injected intraperitoneally. During the operation, and until they were awake, rectal temperature was monitored and kept at 37–38°C by means of a heating pad. Some gerbils were given additional drugs without anesthesia 24 h or 24 and 48 h after ischemia.

Seven days after the operation, the animals were perfused transcardially with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). The brains were removed, cut coronally in 20 μm thickness, and then coronal sections were stained with cresyl violet.

The severity of neuronal damage in CA1 region was evaluated by the number of surviving neurons. The mean number of surviving neurons per 450 μm length was calculated in CA1 region for each group. Cell counting was performed in four serial sections, using the light microscope equipped with a 10× objective. The data was statistically analyzed by unpaired Student t-test. Data are presented as mean ± SD, and when $P < 0.05$, differences were considered significant.

The rectal temperature was kept at 37.5 ± 0.5°C and did not change significantly during the operative procedure and postoperative period (about 2 h). In sham-operated group, no significant neuronal damage was detected (Fig. 1A,B). The mean number of surviving neurons in CA1 region was 132 ± 5. In control groups (groups 9 and 10), almost all neurons in CA1 region were lost, and only less than 2% of neurons were observed compared with sham-operated group (Fig. 1C,D). No significant deference was seen between the two control groups.

Single intraperitoneal administration of FK506 (0.1–1.0 mg/kg) during ischemia showed a moderate neuroprotective effect in a dose-dependent manner (Fig 2). The maximum effect was observed at a dose of 1.0 mg/kg, and 25% of neurons survived (Fig. 1G,H). Post-ischemic additional administration of FK506 (1.0 mg/kg at 24 h, or 24 and 48 h after BCCA occlusion) did not produce further neuroprotective effects when compared with single administration (Fig. 3).

7NI showed only a minor neuroprotective effect, and 8 and 13% of neurons in CA1 region survived in groups 2 and 3, respectively (Fig. 1E,F and Fig. 3).

FK506 is supposed to act as an immunosuppressant by binding to FKBP12 and inhibiting calcineurin activity [7]. In in vitro studies, FK506 has been shown to reduce NO production by enhancing NOS phosphorylation and to be neuroprotective against glutamate-induced excitotoxicity in cultured neurons [1]. Also in in vivo studies, the systemic administration of FK506 has a neuroprotective effect in the rat focal ischemia model. The authors speculated that the inhibition of calcineurin by FK506-FKBP12 complex and nNOS inhibition seemed to be involved in the neuroprotective effect of FK506 [13].

In cultured neurons, NO mediates glutamate neurotoxicity, but in ischemic circumstances, NO plays both beneficial and detrimental roles [5]. Recently it has been shown that a selective nNOS inhibitor, 7NI, is neuroprotective in the rat brain focal ischemia model [18], and that nNOS knock-out mice are resistant to brain focal ischemia [4]. These data suggest that NO produced from neurons is cytotoxic in focal brain ischemia and that FK506 may act as a neuroprotective agent through inhibiting nNOS in focal ischemic circumstances.

On the other hand, the mechanism of DND in CA1 region of the hippocampus is unknown and the involvement of NO in the process of DND has not been clarified yet. Because the hippocampus, the region most vulnerable to transient global ischemia, contains only occasional NOS neurons and sparse nerve fibers in the CA1 through

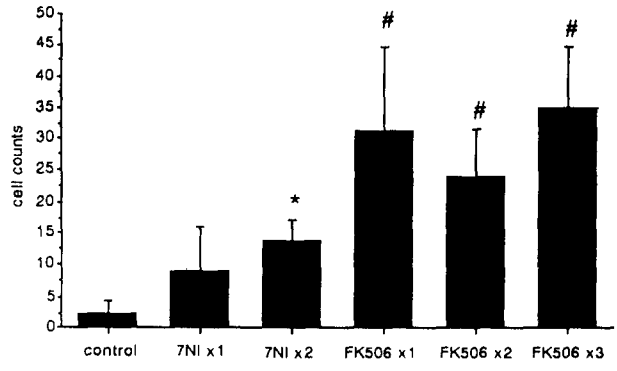

Fig. 3. Numbers of survival neurons in CA1 region of the hippocampus after 7NI or FK506 treatment. Additional postischemic administration of FK506 does not enhance neuroprotective effects. *$P < 0.05$, #$P <$ 0.01 compared with control group. Control includes both groups 9 and 10. 7NIx1, 7NIx2, FK506x1, FK506x2 and FK506x3 indicate groups 1, 2, 5, 6 and 7.

CA3 sectors as well as in the dentate gyrus [16], the involvement of nNOS in the process of DND seems to be less likely. Moreover, previous studies, which examined the effect of non-selective NOS inhibitors, did not offer definite results [10,12,17].

We have shown in the present study that FK506, an immunosuppressant which also inhibits nNOS, has a moderate neuroprotective effect and 7NI, a selective nNOS inhibitor, has only a minor neuroprotective effect. These data imply that DND observed in the CA1 region may not be mainly mediated by NO produced from nNOS, and that most parts of neuroprotective effects by FK506 are derived from other mechanisms than nNOS inhibition by this drug. Steiner et al. showed that FK506 binding activity was abundant in the CNS and that FKBP12 colocalized with calcineurin in neurons in rats [14]. We have also observed that FKBP12 immunoreactivity was present in CA1 region. (data not shown) Therefore, the neuroprotective effect of FK506 for DND in CA1 region may be mediated through the formation of FK506-FKBP12 complex in pyramidal cells. Recent studies have demonstrated that long-term potentiation observed in the CA1 region which is mediated by NO, still exists in mice which are lacking nNOS [9], and that endothelial NOS immunoreactivity and/or NADPH-diaphorase activity are present in CA1 region in the glutaraldehyde fixed section [2]. These data suggest that there may be a substance other than nNOS which can produce NO in the hippocampal pyramidal cells and can be modified by FK506. Further studies are necessary to elucidate the precise intracellular mechanisms of neuroprotective efforts by FK506 in brain ischemia.

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