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原　　著

Effects of 1-[bis(4-fluorophenyl)methyl]-4-(2,3,4-trimethoxybenzyl) piperazine dihydrochloride, a New Synthesized Ca2+ Blocker KB-2796, on Free Fatty Acid Liberation in Ischemic Brain in Rats

Toshiro Kanazawa, Minoru Kidooka, Masayuki Matsuda, and Jyoji Handa

Department of Neurosurgery, Shiga University of Medical Science
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Introduction

Since the reports of Bazán[1] and Bazán et al.[2], brain free fatty acid (FFA) has been measured as one of the biochemical parameters of ischemic brain cell injury[3,7,13]. FFA accumulation in the ischemic brain is believed to be a result of its liberation from membrane phospholipids mediated by phospholipases[9]. In this respect, an increase in intracellular free calcium ion (Ca2+) has been found to stimulate phospholipase A2 and other phospholipases, and hence to trigger brain cell damage[4,10]. The effects of several Ca2+ entry blockers against FFA accumulation in the ischemic brain[8] would substantiate an important role of Ca2+ influx into the cells played in an increase in intracellular Ca2+.

1-[bis(4-fluorophenyl)methyl]-4-(2,3,4-trimethoxybenzyl) piperazine dihydrochloride (KB-2796) is a recently synthesized Ca2+ entry blocker and reported to increase cerebral blood flow in unanesthetized immobilized cats[6]. We examined the effects of this new Ca 2+ entry blocker on FFA accumulation in the ischemic brain induced by decapitation, and compared its effects with those of pentobarbital (PB) and flunarizine (FNZ). Both PB and FNZ have been reported to possess the protective effects against FFA accumulation in the brain, using various models of experimental cerebral ischemia including decapitation[8,12].

Material and Methods

Male Wistar rats weighing 220 to 300 g were used. After decapitation, the heads were sealed in plastic bags and placed in an incubator at 37°C for 30 minutes. Then the brains were removed quickly from calvaria and immersed into liquid nitrogen. The frozen brains were weighed and

Key words: Brain protection, Calcium entry blocker, Cerebral ischemia, Free fatty acid, KB-2796.
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Present address: Department of Neurosurgery, Shiga University of Medical Science, Seta, Ohtsu, 520-21 Shiga-ken, Japan.

homogenized with an ice-cold teflon homogenizer containing 20 volumes of chloroform and methanol (2 : 1, v/v), in which 500 nanomoles of heptadecanoic acid (C 17 : 0) was added as an internal standard. The filtered lipid extract was washed with 0.1% NaCl solution and then with methanol-saline solution (1 : 1, v/v). The samples were evaporated dry under a stream of nitrogen in 40°C water bath, and redissolved in 500 μl of chloroform.

The FFA's were separated using thin-layer chromatography according to the method described by Snyder and Blank [11] and extracted with chloroform-acetic acid (99 : 1, v/v). After three times of centrifugation to remove silica gel, the extract obtained was washed with 2 ml of distilled water and evaporated. The dried extracts were methylesterified with diazomethane, and whole brain palmitic (C 16 : 0), stearic (C 18 : 0), oleic (C 18 : 1) and arachidonic (C 20 : 4) acids were determined from peak area measurements for C 16 : 0, C 18 : 0 and C 18 : 1, and peak height for C 20 : 4, using gas liquid chromatograph (Hewlett Packard model 5830A). The total FFA level was calculated as a sum of the above 4 FFA's.

In the second series of experiments, effect of KB-2796 on FFA accumulation in the brain after various periods of global ischemia (1, 10, 30 and 60 minutes) was examined. After decapitation, the heads of animals were kept in an incubator at 37°C for 10, 30 and 60 minutes, then brains were removed quickly and immersed into liquid nitrogen. The brains of rats in the 1-minute-ischemia group were not placed in an incubator but were immediately removed from calvaria and immesed into liquid nitrogen exactly 1 minute after decapitation. The extraction and quantitation protocols were the same as mentioned above.

KB-2796 (10 mg/kg), PB (60 mg/kg) and distilled water (5 ml/kg) were administered intraperitoneally 15 minutes before, and FNZ (10 mg/kg) was administered 30 minutes before decapitation. Values were expressed as mean $\pm$S.E. of the mean. Statistical analysis was performed using unpaired t-test and p values less than 0.05 were considered to be significant.

Results

Experiment 1: *Effects of KB-2796, flunarizine and pentobarbital on brain free fatty acid accumulation in 30-minute-ischemia model*

Palmitic, stearic, oleic and arachidonic acid levels (nmol/g) in the control group treated with distilled water only were 468$\pm$8, 567$\pm$1, 355$\pm$7 and 787$\pm$21, respectively. KB-2796 tended to decrease palmitic, stearic and arachidonic acids, and significantly reduced oleic acid and total FFA levels. Although FNZ slightly reduced arachidonic acid and total FFA levels, changes were not significant. Liberation of palmitic acid was significantly decreased by FNZ. PB significantly lowered the levels of all FFA's except for stearic acid (Fig. 1, Table 1).

Experiment 2: *Duration of ischemia and the effects of KB-2796 on brain free fatty acid accumulation*

In the control group, the levels (nmol/g) of palmitic, stearic, oleic and arachidonic acids 1 minute after decapitation were 91$\pm$15, 207$\pm$11, 113$\pm$11 and 338$\pm$24, respectively. They rapidly increased at 10 minutes of ischemia, and more or less gradually thereafter.

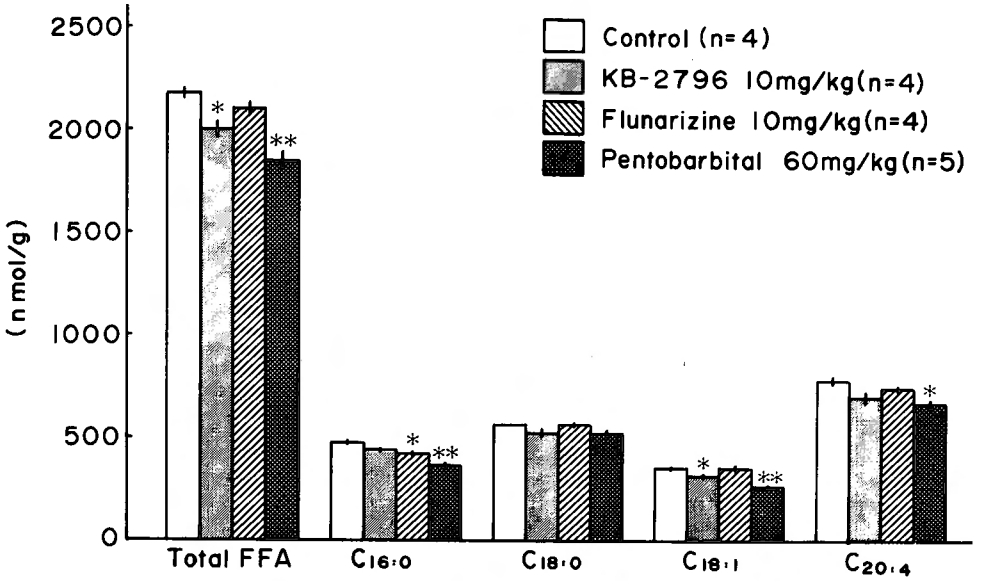

Fig 1. Effects of KB-2796, flunarizine, and pentobarbital on rat brain free fatty acids at 30 minutes post-decapitation. Vertical bars represent the mean±S.E.M.. C16 : 0: palmitic acid, C18 : 0: stearic acid, C18 : 1: oleic acid, C20 : 4: arachidonic acid. Total free fatty acid (FFA) was calculated as a sum of palmitic, stearic, oleic and arachidonic acids. *: $p<0.05$, **: $p<0.01$, compared to the control for each.

Table 1. Free fatty acid levels in the rat brain at 30 minutes post-decapitation.

Free fatty acid	Control (n=4)	KB-2796 10 mg/kg (n=4)	Flunarizine 10 mg/kg (n=4)	Pentobarbital 60 mg/kg (n=5)
Palmitic acid	468± 8	450±13	435± 5*	370±11**
Stearic acid	567± 1	528±18	569± 6	533±20
Oleic acid	355± 7	323± 8*	358± 6	265±14**
Arachidonic acid	787±21	707±30	750±17	681±22**
Total	2177±23	2008±45*	2112±28	1849±51**

Each value represents the mean±S.E.M. (nmol/g). n: Number of animals.
*: $p<0.05$, **: $p<0.01$.

KB-2796 attenuated the accumulation of FFA at all periods of ischemia except for 10 minutes (Fig. 2, Table 2). This effect was remarkable at 1 and 60 minutes of ischemia. All FFA's and thereby total FFA in 1-minute-ischemia model showed significantly lower levels than those in the control group. With 30 minutes of ischemia where the effects of KB-2796 were obvious in experiment 1, however, significant effect was not provided in this experiment 2 except for stearic acid, which was significantly lower than in the control group. With 10 minutes of ischemia, oleic and arachidonic acid levels in the KB-2796-treated group were higher than those in the control group although these differences were minor and not significant.

Proportion of palmitic acid in 1-minute-ischemia model was 5.7% of total FFA's in the KB-2796-treated group, and this value was significantly lower ($p<0.01$) than 12.1% in the

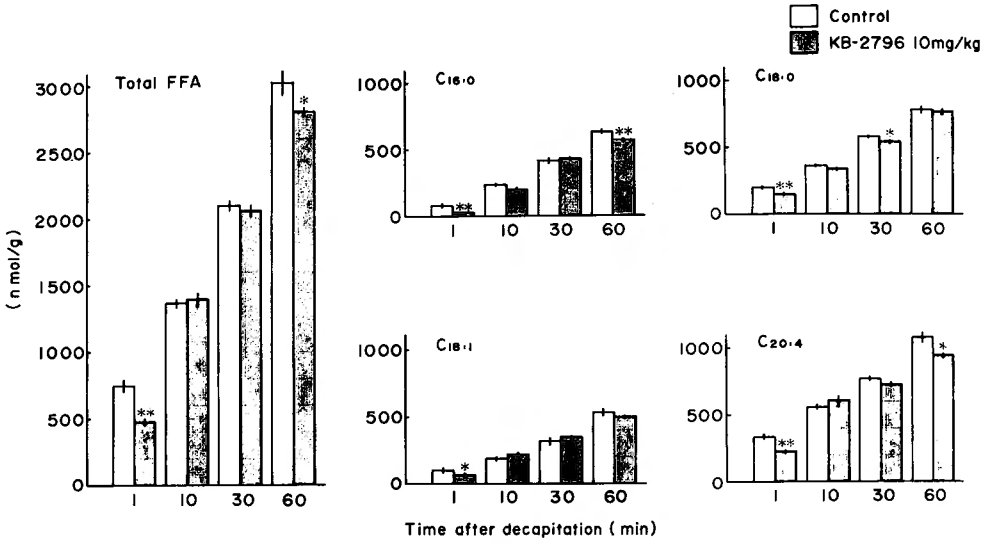

Fig 2. Effects of KB-2796 on rat brain free fatty acids at 1, 10, 30 and 60 minutes post-decapitation. Vertical bars represent the mean±S.E.M. of 8 to 9 rats. C16 : 0: palmitic acid, C18 : 0: stearic acid, C18 : 1: oleic acid, C20 : 4: arachidonic acid. *: $p<0.05$, **: $p<0.01$, compared to the control for each.

Table 2. Changes in free fatty acid levels in rat brain after various periods of ischemia.

Free fatty acid	Duration of Ischemia							
	1 minute		10 minutes		30 minutes		60 minutes	
	Control (n=8)	KB-2796 (n=8)	Control (n=8)	KB-2796 (n=8)	Control (n=8)	KB-2796 (n=8)	Control (n=9)	KB-2796 (n=8)
Palmitic acid	91±15	27± 3**	235± 7	216± 9	424±17	431±11	627±12	572± 6**
Stearic acid	207±11	151± 9**	360± 9	346± 9	582± 8	547±13*	780±22	773±12
Oleic acid	113±11	72±11*	195±10	223±11	315±20	348±11	536±30	499± 9
Arachidonic acid	338±24	229± 9**	579±29	617±40	770±12	737±19	1076±45	953± 9*
Total	749±52	479±28**	1369±43	1401±51	2092±38	2063±35	3018±92	2797±29**

Values represent the mean±S.E.M. (nmol/g). n: Number of animals in each group. KB-2796 was administered intraperitoneally 15 minutes before decapitation. *: $p<0.05$, **: $p<0.01$.

control group. In contrast, proportion of stearic acid was significantly higher ($p<0.01$) in the KB-2796-treated group than that in the control group, that is, 31.5±0.45% in the KB-2796-treated group against 28.0±0.93% in the control group. Except for this difference of proportions of palmitic and stearic acids in 1-minute-ischemia model, no obvious differences of the compositions of brain FFA's were observed between the control and the KB-2796-treated groups in any other durations of ischemia (Fig. 3).

Discussion

Present results have shown that FFA's accumulate in the brain following global ischemia induced by decapitation, and that KB-2796, a Ca2+ entry blocker, attenuates this FFA accumulation effectively, as does PB.

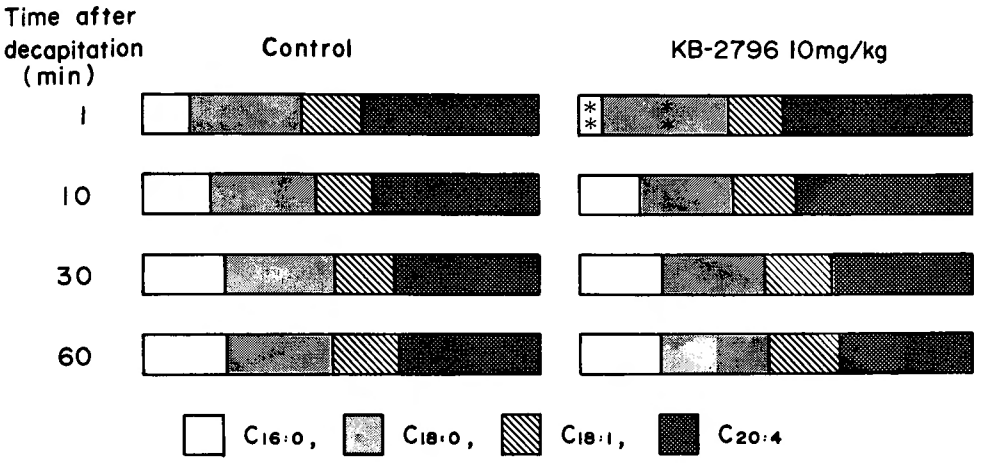

Fig 3. Changes in the proportion of brain free fatty acids at 1, 10, 30 and 60 minutes post-decapitation. Each column represents the proportion against total free fatty acid. C16 : 0: palmitic acid, C18 : 0: stearic acid, C18 : 1: oleic acid, C 20 : 4: arachidonic acid. *: $p<0.05$, **: $p<0.01$, compared to the control for each.

The reported values of FFA contents in the brain vary among investigators, probably due to the differences in experimental conditions employed. Although we did not measure the FFA levels in the non-ischemic rat brain, our values obtained at 1, 10, 30 and 60 minutes post-decapitation were similar to those reported by Shiu and Nemoto[7] who also used decapitation-induced ischemia model, except that arachidonic acid levels were 1.5- to 2-fold higher in our study.

PB has been known to reduce the accumulation of FFA's in the ischemic brain[7,14] and to proect the brain from hypoxic injury[5]. In consonant with these previous results, a significant decrease was found in the liberation of each FFA in the PB-treated rats with 30-minute-ischemia in the present study. The presnet study has shown that KB-2796 also attenuates the FFA accumulation in the ischemic brain induced by decapitation.

With the dosage used in this study (10 mg/kg), however, KB-2796 (10 mg/kg) was less effective than PB in 30-minute-ischemia model. In the pilot study not reported here, an increase in the dosage of KB-2796 to 30 mg/kg failed to further potentiate the effects of KB-2796 on attenuation of FFA liberation. At the time of decapitation, rats were under the anesthetized condition in the PB-treated group, whereas they were awake when treated with KB-2796. Such a marked difference in the neuronal activities of the brain between the two groups would substantiate the presence of different modes of action between KB-2796 and PB against FFA accumulation.

As summarized by Siesjö[9], it has been considered that in the ischemic brain, an increase in intracellular Ca2+ concentration results in activation of cellular phospholipases and thus the liberation of FFA's, and that both extracellular Ca2+ and intracellularly stored Ca2+ are the source of increased intracellular Ca2+.

In experiment 2, attenuation of FFA accumulation by KB-2796 was more marked with the

shortest duration (1 minute) as well as the longest duration (60 minutes) of ischemia. No significant effects on FFA liberation were observed in 10-minute-ischemia rats, and KB-2796 was less efective in 30-minute-ischemia rats than in 10- and 60-minute-ischemia rats. The present results suggest that an influx of Ca2+ into the cell plays an important role in accumulation of FFA's especially in the early phase of ischemia and it does not play a major role in the intermediate duration of ischemia. Similar results were obtained by Shiu et al[8]. They state that the accumulation of brain FFA's following decapitation consists of two phases and that Ca2+ entry blockers such as FNZ and D-600 affect the early phase of FFA liberation. The present results are consonant with their findings concerning 1- and 30-minute-ischemia models, however, the effects of KB-2796 in 60-minute-ischemia model may well suggest the involvement of more than 2 mechanisms of FFA accumulation during global ischemia of the brain.

In 1-minute-ischemia rats, KB-2796 attenuated the FFA accumulation with reduction in proportion of palmitic acid, whereas it caused no obvious changes in the composition of FFA's in 60-minute-ischemia rats. This fact may also suggest the differences in the action of KB-2796 between early and late periods of ischemia.

Although KB-2796 significantly decreased the total FFA level in 30-minute-ischemia model in experiment 1, it failed to significantly decrease total FFA's in rats with the same period of ischemia in experiment 2. It seems that the ischemia of 10 to 30 minutes' duration is not adequate to reveal the effectiveness of KB-2796, and probably of FNZ also but not of PB.

In conclusion, KB-2796 attenuates brain FFA accumulation following decapitation, and this effect is most evident in 1-minute- and 60-minute-ischemia models. This effects of KB-2796 in attenuating FFA accumulation during ischemia seem to substantiate its clinical usefulness in cerebrovascular diseases.

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和文抄録

新合成カルシウム拮抗剤 1-[bis(4-fluprophenyl)methyl]-4-(2,3,4-trimethoxybenzyl) piperazine dihydrochloride (KB-2796) のラット虚血脳内遊離脂肪酸増加に対する効果

滋賀医科大学脳神経外科
金澤　稔郎，木戸岡　実，松田　昌之，半田　譲二

Bazán and Bazán らの報告以来，脳内遊離脂肪酸は，虚血脳での細胞障害の指標の一つとして測定されている．また，カルシウム拮抗剤による虚血時の脳内遊離脂肪酸増加の抑制が報告されている．

1-[bis(4-fluorophenyl) methyl]-4-(2,3,4-trimethoxybenzyl) piperazine dihydrochloride(KB-2796) は，最近新に合成されたカルシウム拮抗剤であり，先に無麻酔非動化ネコを用い，脳血流増加作用を有することを報告した．

今回ラットを用い，断頭虚血モデルでの脳内遊離脂肪酸増加に対する効果を，すでに脳保護剤と考えられているペントバルビタールとフルナリジンと比較した．

KB-2796 は，断頭後の脳内遊離脂肪酸増加をペントバルビタールと同様，フルナリジンよりはやや強く抑制し，この効果は，虚血1分と60分で顕著であった．

今回の結果は，KB-2796 の脳血管障害改善薬としての有用性を示唆するものと思われた．