Evaluation of the Neuroprotective Action of WEB 1881 FU on Hypoglycemia/Hypoxia-Induced Neuronal Damage Using Rat Striatal Slices

Shuichi KOIZUMI, Yasufumi KATAOKA, Kazuto SHIGEMATSU, Masami NIWA and Showa UEKI

Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka 812, Japan
Department of Pharmacology and Pathology, Nagasaki University School of Medicine, Nagasaki 852, Japan

Accepted February 15, 1990

Abstract—Effect of WEB 1881 FU on hypoglycemia/hypoxia-induced brain damage in rats was evaluated and compared to findings obtained with idebenone. We used an in vitro model that facilitated the direct monitoring of dopamine release from striatal slices. The response to high K+ stimulation under perfusion of the slices with D-glucose-free Ringer solution (hypoglycemia) decreased at 40 min, and then practically disappeared. WEB 1881 FU at 10^-6 M or idebenone at 10^-6 M significantly protected against impairment of the striatal responses under the conditions of hypoglycemia. Hypoglycemic injury, evidenced by a remarkable neuron loss, necrosis and spongiosis was also ameliorated by these drugs. WEB 1881 FU at 10^-6 M had a protective action against the impairment of striatal responses evoked by NaCN (electron transport inhibitor at site 3) and oligomycin (inhibitor of mitochondrial ATP synthesis), but idebenone at 10^-6 M did not. In light of these observations, the possibility that WEB 1881 FU and idebenone exert neuroprotective actions against hypoglycemic/hypoxic brain injury by activating energy metabolism with different mechanisms from each other has to be considered.

High energy demands of the brain are met exclusively by the oxidation of D-glucose. Since neither oxygen nor D-glucose is stored in the brain, this organ is vulnerable to any deficiency in these elements. As in the case of hypoglycemia, cerebral ischemia leads to extensive energy deprivation and an increase in extracellular levels of excitatory amino acids (1, 2). Since N-methyl-D-aspartate antagonists ameliorate acute morphological changes in the hippocampus and the striatum, as induced by ischemia and hypoglycemia, respectively, similar pathogenic mechanisms may prevail in the two disorders (3, 4). Thus, an in vitro model of hypoglycemia-induced brain damage would provide an experimental system with which one could examine mechanisms involved in ischemic neuronal damage, without systemic vascular and other multiple variables which accompany ischemia, in vivo. In addition, a hypoxic condition was also set up for inhibition of oxidative phosphorylation, using NaCN (cytochrome c oxidase inhibitor) and oligomycin (mitochondrial ATP synthetase inhibitor).

Direct monitoring using cell bed perfusion coupled to an amperometric detector enabled the detection of the real-time dynamics of the secretory responses of cultured chromaffin cells (5). The striatum was used because this part of the brain has a high level of dopamine (DA) (6). Additional factors favoring the use of striatal slices are the existence of corticostriatal fibers which contain glutamate (7) and high vulnerability to ischemia (8, 9) and hypoglycemia (10). Therefore, we used a system to directly monitor the real-time dynamics of DA released from striatal slices,
as an index of functional alterations in the process of the hypoglycemic/hypoxic neuronal injury, in vitro.

WEB 1881 FU, 4-aminomethyl-1-benzylpyrrolidin-2-one-fumarate, has been developed as a potential nootropic drug (11). The present study was designed to evaluate the effect of WEB 1881 FU on hypoglycemic/hypoxic brain injury, as compared to findings with idebenone, a cerebral metabolic enhancer (12, 13), using an in vitro model that we have designed (14).

Materials and Methods

Animals: Male Wistar rats (Kyushu University Institute of Laboratory Animals) aged 7–9 weeks and weighing 200–250 g were housed in an air-conditioned room at 23±2°C with free access to food and water and maintained on a 12 hr light-dark schedule (lights on 7:00 a.m.).

Preparation of striatal slices: The rats were decapitated and the brain removed immediately and placed in ice-cold Krebs-Ringer bicarbonate solution, pH 7.4, of the following composition: 118.0 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgCl₂, 1.0 mM NaH₂PO₄, 25.0 mM NaHCO₃ and 11.0 mM D-glucose. The brain was sliced at a thickness of 450 μm using a McIlwain tissue chopper, and striatal slices were obtained by free hand dissection, in ice-cold Krebs-Ringer buffer.

Monitoring system: The monitoring system is shown in Fig. 1. This system is fundamentally composed of a tissue perfusion component and an electrochemical detector (ECD) monitoring component, as described by Kumakura et al. (15). In brief, two striatal slices sandwiched between two membrane filters ( pore size, 3 μm; Nuclepore, CA, U.S.A.) were placed in the chamber (250 μl inner volume) and perfused with oxygenated (95% O₂/5% CO₂) Krebs-Ringer solution, as described above, at a flow rate of 1 ml/min at 37°C. These slices were subjected to pulsatile stimulation by injecting 50 mM KCl into the flow stream in a volume of 500 μl through a loop injector (Rheodyne, CA, U.S.A.). The duration of the stimulation given by a single injection was 45 sec. However, the tissue was stimulated only for 15 sec, with the same concentration as that of the injected solution since dilution occurred when the pulse was introduced into the tissue chamber.

The perfusate from the tissue chamber was directly introduced into the ECD monitoring component (LC-4B and TL-5A, Bioanalytical System, West Lafayette, U.S.A.), setting the electrode potential at +0.45 V versus Ag/AgCl reference electrode to detect oxidizable substances released from the striatal slices. There was a high correlation between

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**Fig. 1.** Diagram of the real-time monitoring system consisting of a perfusion component for the striatal slices enclosed in the chamber and an electrochemical detector (ECD) component. Stimulus (Δ), released dopamine (DA) (●), DA oxidized at the surface of the electrode (○).
the magnitude of the output current from the ECD and the amount of released DA in each response to the stimuli under normal and hypoglycemic conditions (Fig. 2), although 3,4-dihydroxyphenylacetic acid (DOPAC) also contributed to the output current. In some

Fig. 2. Representative current-time curves (1, 2, 3) and chromatographs (DA: 1', 2'; DOPAC: 1'', 2'', 3'') obtained by real-time monitoring and high performance liquid chromatography (HPLC), respectively. Each chromatograph (HPLC) corresponds to the current-time curves at the bottom (real-time monitoring). Inset indicates correlations between the peak height of the current-time curve (nA) and the amount of DA (ng) released in the striatal response to high K+ under conditions of perfusion with normal Ringer (○, r²=0.993) and D-glucose free Ringer (△, r²=0.987).
cases, the concentrations of catecholamines in each individual response were determined by collecting the corresponding aliquot of the perfusate, using high performance liquid chromatography (HPLC) with an ECD, as described by Tani et al. (16).

**Hypoglycemia-induced brain damage:** Krebs-Ringer solution containing D-glucose as described above (normal Ringer) and that containing sucrose as the substitution for D-glucose (D-G free Ringer) were prepared.

Two separate monitoring systems were run in parallel, so that the responses in perfusion with normal Ringer and D-G free Ringer were monitored, respectively, under exactly the same conditions. Striatal slices were first perfused with the normal Ringer for 60 min, as a pre-incubation period, and then stimulated at 20-min intervals by injecting normal or D-G free Ringer containing 50 mM KCl (high K+) through a loop injector. Perfusion with D-G free Ringer was initiated in one system just after monitoring the response to the 3rd preliminary high K+-stimulation, which served as the control, while for the other system, continuous perfusion with normal Ringer was carried out. Test stimulation was performed 9 times (S1–S9) to detect the influence of D-G free Ringer (hypoglycemia) on the striatal responses.

DA release was expressed as the relative response, that is the ratio of the peak height of the current-time curve in the high K+-evoked response to that in the control response to the 3rd prestimulation.

**NaCN- and oligomycin-induced brain damage:** The procedure is the same as that used for the hypoglycemic brain damage except for the following: the striatal slices were exposed to Krebs-Ringer solution containing NaCN (10^{-4} M for 5 min) or oligomycin (5\times10^{-8} M for 180 min) after a pre-incubation period.

**Drugs:** WEB 1881 FU (Nippon Boehringer Ingelheim) and idebenone (6-(10-hydroxydecyl)-2,3-dimethoxy-5-methyl-1,4-benzoquinone, Takeda Chemical Industries) were dissolved at a concentration of 10^{-3} M in distilled water and ethanol, respectively; and then they were diluted with Krebs Ringer buffer to concentrations of 10^{-6}, 10^{-7} and 10^{-8} M. Drugs were applied from the initiation of the pre-incubation period to the end of the experiment.

**Histological examination:** Striatal slices were collected from the chamber after monitoring the response to the 9th test stimulation and were then placed in 15% formaldehyde solution. Formalin fixed and paraffin embedded tissues were sectioned at a 5 \mu m thickness. Deparaffinized sections were stained with hematoxylin-eosin. The percent of normal neurons in complete neurons was calculated by counting the number of intact and degenerative neurons in 6 separate areas (1 mm^{2}), selected at random for each section. These microscopic observations were performed on 3–5 sections of each striatal slice obtained from 4–6 separate experiments.

**Statistical analysis:** Data were expressed as the mean±S.E. Significance of the difference was calculated by analysis of variance followed by the Duncan test for individual comparisons.

**Results**

Figure 3 shows the process of impairment of the striatal responses to high K+ following hypoglycemia. Under perfusion with D-G-free Ringer, the response to the first high K+ stimulation was significantly large, and the shape of the current-time curve differed from that seen in the case of normal Ringer. Striatal responses suddenly decreased 40 min after and then practically disappeared. Striatal responses under conditions of perfusion with normal Ringer were not influenced either by WEB 1881 FU or by idebenone (Fig. 4, A and C). WEB 1881 FU and idebenone, at concentrations of 10^{-6} M, significantly protected the tissue from this damage, although the response was not completely restored to that obtained in the presence of normal Ringer (Fig. 4, B and D). Both drugs at concentrations of 10^{-7} and 10^{-8} M showed no protective action.

Histological examination demonstrated a remarkable neuron loss, neuron necrosis and spongiosis, and normal neurons were significantly decreased to 10%, in the presence of hypoglycemia (180 min) (Table 1 and Fig. 5). WEB 1881 FU at 10^{-6} M or idebenone at 10^{-6} M clearly diminished these losses (Table 1 and Fig. 5).
Exposure to $10^{-4}$ M cyanide for 5 min elicited a severe impairment even when the striatal slices had been perfused with normal Ringer (Fig. 6). When the tissue was exposed to cyanide in the presence of WEB 1881 FU at $10^{-6}$ M, the response gradually recovered.

Fig. 3. Time course of striatal response to high $K^+$ under conditions of perfusion with normal Ringer and D-glucose-free Ringer (D-G-free Ringer) (A) and typical records of ECD-monitored current-time curves of striatal responses to high $K^+$, under conditions of perfusion with normal Ringer and D-G-free Ringer (B). The relative response was expressed as a ratio of the peak current in the test stimulation-evoked response to that in the 3rd pre-stimulation-evoked response. Values represent the means±S.E. of 18 experiments. pre: the 3rd pre-stimulation, S1–S9: test stimulation.
and then reached the normal level at the 5th stimulation (Fig. 6A). In this case, the abnormal response to the first test-stimulation, similar to that under hypoglycemia, was also diminished by WEB 1881 FU at $10^{-6}$ M. Idebenone at $10^{-6}$ M had no significant action on cyanide-induced damage (Fig. 6B). Functional impairment gradually appeared and the evoked responses disappeared at the 7th stimulation of the tissue perfused with normal Ringer containing $5 \times 10^{-8}$ M oligomycin. WEB 1881 FU at $10^{-6}$ M but not idebenone at $10^{-6}$ M had a significant protective action (Fig. 6, C and D).

![Graphs A, B, C, D](image)

**Fig. 4.** Effects of WEB 1881 FU (A, B) and idebenone (C, D) on high K+-evoked responses under the perfusion with normal Ringer and D-G-free Ringer. Values represent the means±S.E. of 7-12 experiments. pre: the 3rd pre-stimulation, S1-S9: test stimulation.

**Table 1.** Effects of WEB 1881 FU and idebenone on histological damage of striatal slices induced by hypoglycemia

| Index               | Intact   | Normal Ringer | D-glucose-free (hypoglycemia) |
|---------------------|----------|---------------|-----------------------------|
|                     |          |               | WEB 1881 FU | idebenone |
| Neuron loss         | −        | +             | + + + + + + + + | + + + + + + |
| Neuron necrosis     | −        | +             | + + + + + + + + | + + + + + + |
| Spongiosis          | − − +    | +             | + + + + + + + + | + + + + + + |
| % of normal neuron  | 80.2±5.4 | 64.5±1.7      | 11.4±8.0     | 48.5±6.1   | 39.8±7.2 |

−: no, +: slight, ++: moderate, +++: marked incidence. The microscopic observations were performed on 3-5 sections of each striatal slice obtained from 4-6 separate experiments. Values represent the means±S.E.
Fig. 5. Typical micrographs of rat striatal slices exposed to normal Ringer (A), D-G-free Ringer (B) and D-G-free Ringer containing WEB 1881 FU (C) for 180 min.

Fig. 6. Effects of WEB 1881 FU (A, C) and idebenone (B, D) on the impairment of the striatal response induced by 10^{-4} M NaCN and 5 \times 10^{-8} M oligomycin. Values represent the means±S.E. of 7–12 experiments. pre: the 3rd pre-stimulation, S1–S9: test stimulation.
**Discussion**

In the present study, glucose as the main source of metabolic energy was excluded from the perfusate (hypoglycemia) and oxidative phosphorylation was inhibited by cyanide (cytochrome c oxidase inhibitor) and oligomycin (ATP synthetase inhibitor); hence, these conditions simulated an hypoxic state.

An in vitro model of brain damage composed of a tissue perfusion component and an ECD monitoring component was found to be a useful technique for identifying neuroprotective properties of drugs, and the real-time monitoring system was simple and efficient. Delicate functional alterations in striatal slices in the process of hypoglycemic/hypoxic injury can also be detected, as the current-time curve of the high K+-evoked response.

WEB 1881 FU and idebenone significantly improved diminution in the high K+-evoked responses and morphological damage of striatal slices, as induced by hypoglycemia, thereby suggesting that these drugs have neuroprotective action. Fatty acids, glycerol and amino acids other than glucose are also converted into the acetyl unit of acetyl coenzyme A, which generates metabolic energy by joining with the citric acid cycle and oxidative phosphorylation. Brain tissue under hypoglycemic conditions can acquire energy by oxidizing lactate and other metabolites and/or by inserting amino acids into the citric acid cycle (17). The increase in the energy pool produced by pathways other than glycolysis can prolong the survival time of the neuron exposed to hypoglycemia. Therefore, we assume that WEB 1881 FU and idebenone protect neurons from hypoglycemic damage by stimulating energy metabolism, other than glycolysis, although this production is not sufficient to replenish the loss of metabolic energy induced by hypoglycemia.

The present findings suggest that WEB 1881 FU and idebenone may mediate the activation of energy metabolism at the stage of oxidative phosphorylation through different mechanisms. WEB 1881 FU but not idebenone protected the striatal tissue from hypoglycemic damage by stimulating energy metabolism, other than glycolysis, although this production is not sufficient to replenish the loss of metabolic energy induced by hypoglycemia.

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**Acknowledgments:** We thank Dr. K. Kumakura (Sophia University) and Dr. Y. Kohno (Nippon Boehringer Ingelheim) for pertinent comments and Ms. T. Usui for secretarial services. WEB 1881 FU and idebenone were generously supplied by Nippon Boehringer Ingelheim and Takeda Chemical Industries, respectively.

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