Protective Effects of a Monosialoganglioside Derivative Following Transitory Forebrain Ischemia in Rats

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We evaluated the effects of treatment with the inner ester derivative of the monosialoganglioside GM1 on cortical electroencephalographic activity and hippocampal CA1 morphology after transitory, near-complete cerebral ischemia in rats. Ischemia was induced by the four-vessel occlusion method, and we studied only the 58 rats that showed flattening of the cortical electroencephalogram for the entire 30 minutes of occlusion. The ganglioside (n = 30) or saline (n = 28) was administered intravenously immediately after release of the carotid clips and then intramuscularly for 21 days of observation. Cortical electroencephalographic activity was monitored throughout the experiment. After 21 days of recirculation we assessed hippocampal CA1 damage by light microscopy. The results indicate that treatment with the ganglioside reduces postischemic secondary damage to the cortical circuitry (as indicated by significantly higher cortical electroencephalographic activity late after reperfusion) and limits neuronal loss in the CA1 region. Our results lend support to the possible therapeutic use of the ganglioside in human pathologic conditions associated with cerebrovascular insufficiencies. (Stroke 1990;21:1607-1612)

Transient global cerebral ischemia induces a relatively selective pattern of neuronal degeneration. Recent investigations into the underlying pathogenesis suggest that excitatory amino acid neurotransmission plays an important role. Neurons selectively vulnerable to an ischemic episode receive prominent excitatory amino acid transmitter inputs, and ablation of these pathways reduces ischemia-induced neuronal loss. In addition, the extracellular concentrations of glutamate and aspartate—potential neurotoxins both in vitro and in vivo—increase during ischemia. A current hypothesis is that excessive accumulation of glutamate or related compounds, via specific postsynaptic receptors, cause neuronal overactivation, triggering a cascade of cellular events that ultimately lead to cell death.1

A corollary to the above hypothesis is that agents capable of antagonizing specific excitatory amino acid receptor–related recognition sites or postreceptor effects may be of potential therapeutic value. In particular, great attention has recently been focused on N-methyl-D-aspartate (NMDA) receptor antagonists. Although these compounds are protective against ischemia-induced neuronal death,1 at effective doses they cause widespread central nervous system (CNS) depression and learning impairment,2 as well as acute pathomorphologic changes in defined populations of CNS neurons.3

Another approach has made use of gangliosides, in particular the monosialogangliosides GM1 and its inner ester derivative.4 This latter ganglioside, like its parent compound, is efficacious in limiting excitatory amino acid–related neurotoxicity in cerebellar granule cell cultures,5,6 an effect not associated with interference with excitatory amino acid recognition sites.5,7,8 The inner ester derivative has also proved useful in ameliorating outcome following various types of CNS insult in adult rodents.9-11 Furthermore, there is evidence that the inner ester derivative of GM1, although transformable to GM1 per se, is more active than GM1 at lower doses, an effect most likely related to its higher bioavailability and incorporation into brain following its systemic injection.

We designed the present investigation to evaluate the capability of the inner ester derivative of GM1 (designated siagoside in our study and GM1-lactone9 or AGF29 by others) to limit neuronal damage over time following transitory near-complete forebrain ischemia in adult rats.

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Materials and Methods

We subjected 92 adult male Wistar rats weighing 200–250 g to four-vessel occlusion according to the methods of Pulsinelli and Brierley. Following induction of anesthesia with 50 mg/kg pentobarbital sodium, the vertebral arteries were permanently occluded and stainless steel screws were mounted over the dura to record cortical electrical activity throughout the experiment. The rats were allowed to recover from anesthesia for 24 hours with an overnight fast. The following day, under anesthesia with 1.5% halothane in 30% O2 and 70% N2O, the tail artery was catheterized for measurements of mean arterial blood pressure, arterial blood gases, and arterial pH.

The carotid arteries were then exposed, occluded for 30 minutes with clips, and released as described by Schmidt-Kastner and Hossmann. At all times, body temperature was kept at 37°C by using a heated operating table and physiologic parameters were within normal ranges. In 62 rats (68%) the cortical electroencephalogram (EEG) became isoelectric 1–2 minutes after carotid occlusion and the spontaneous righting reflex was absent. Since preliminary experiments conducted by implanting an electrode deep within the hippocampus (stereotactic coordinates -3.5 anteroposterior, -2.9 vertical from the bregma) revealed that isoelectricity occurred concomitantly in the cortex and the hippocampus (data not shown), we used only cortical electrodes to avoid hippocampal damage due to electrode implantation. Following release of the carotid clips, the rats were allowed to survive for 21 days.

Siagoside was prepared and purified in our laboratory according to the methods of Sonnino et al. Purity was assessed to be >99%. All solutions were made fresh immediately before administration.

We considered only the 58 rats showing cortical EEG flattening during the entire 30 minutes of occlusion. After the carotid clips were removed, 30 randomly selected rats were treated with 20 mg/kg i.v. siagoside. The remaining 28 rats were analogously treated with saline. Treatment was repeated intramuscularly 3 hours after release of the carotid clips, twice a day for 2 days, and then once a day for 18 days. We selected this treatment protocol based on pharmacokinetic studies indicating that such a protocol, as for GM1, results in a steady-state level of siagoside in the serum and based on pharmacodynamic studies demonstrating maximal GM1 efficacy when administered 0–2 hours following acute CNS injury.

The rats’ behavior was monitored through closed-circuit television. As previously reported, cortical EEG was monitored in 58 rats considered before, during, and for 30 minutes after occlusion using eight active electrodes implanted over the dura. A ground electrode and a common average reference electrode were also used. Cortical EEG during 30 minutes was also evaluated at intervals during the observation period (i.e., 3 and 24 hours and 3, 7, 14, and 21 days after occlusion) in 10 saline-treated and 11 siagoside-treated freely moving rats. We also recorded cortical EEG in four naive control rats. Each recording session was performed for each rat at the same time each day and was preceded by a 15-minute adaptation period. We never interrupted signal digitization during the recording session; unexpected signals were rejected. The cortical EEG was quantified by computing on-line the amplitude–phase spectrum of 4-second epochs (128 samples per second) for the entire 30 minutes of recording with a fast Fourier transformation algorithm. For this analysis, we defined the five frequency bands considered as the delta (0.75–3.75 Hz), theta (4.0–7.5 Hz), alpha1 (7.75–9.5 Hz), alpha2 (9.75–12.5 Hz), and beta (12.75–20 Hz) bands. Signal analysis was performed by means of an IBM PC AT/3 computer provided with a DIABASE program, an eight-channel/12-bit analog-to-digital converter, and a 32-bit digital signal processor board. By means of two-way analysis of variance for repeated measures using BMDP, we compared the pattern of posts ischemic cortical EEG activity in the two groups. Because the data were not normally distributed, we applied a logarithmic transformation.

After 21 days of recirculation the rats were anesthetized and their brains were perfusion-fixed with Bouin’s fixative via the ascending aorta after a brief washing with heparinized (0.1%) phosphate-buffered saline (pH 7.4). Brains were left in situ for 1–4 hours and then immersed in the same fixative. Paraaffin-embedded sections 8 μm thick were cut and stained with cresyl violet–Luxol fast blue. Four sections spaced at 100-μm distances throughout the rostro-caudal extent of the dorsal hippocampus (first section corresponding approximately to level 4,230 μm according to König and Klippel) were taken from both hemispheres. Integrity of the CA1 region was evaluated by light microscopy using a Leitz Orthoplan microscope (Wetzlar, FRG) equipped with a Vario-orthomath camera.

Computer-assisted morphometry of photographs of the sections printed at a final magnification of ×900 was conducted by dividing the entire CA1 region into subfields of constant area (30,000 μm2). We counted the total number of preserved neurons (cells not displaying typical ischemic changes, i.e., shrunken, pyknotic, and/or fragmented nuclei without recognizable cytoplasmic boundaries) in the CA1 region and divided it by the number of subfields considered, thereby obtaining the mean number of surviving neurons per subfield. All photographs were blindly evaluated. We compared the density of surviving neurons in the two groups using Student’s two-tailed t test for unpaired groups.

Results

Four rats (4%) died of respiratory failure very soon after occlusion. An additional 13 rats (14%) died during the 21 days of observation, with a concomitant severe reduction in body weight. We observed no difference in mortality (seven [25%] in the saline-
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FIGURE 1. Bar graphs of mean±SEM effect of siagoside treatment in rats on delta (A), theta (B), alpha_1 (C), and alpha_2 (D) cortical electroencephalographic (EEG) activity monitored using eight active electrodes implanted over dura 24 hours after electrode implantation and vertebral artery occlusion (baseline) (IMP), during 30-minute occlusion of carotid arteries (Car Occ), and 30 minutes (m), 3 and 24 hours (h), and 3, 7, 14, and 21 days (d) after carotid clip removal. Siagoside treatment significantly increased cortical EEG activity (p < 0.05 for A and D, p < 0.01 for B and C). Filled bars, saline-treated group; shaded bars, siagoside-treated group.

Compared with that in the four naive control rats, the baseline cortical EEG (recorded before carotid artery occlusion) of the 92 experimental rats was characterized by increased synchronous delta activity, most probably due to previous electrocauterization of the vertebral arteries. Immediately after carotid occlusion the cortical EEG amplitude began to decrease, and in 62 rats (68%) isoelectricity was observed in <2 minutes. Following 8-12 minutes of recirculation, slow, arrhythmic delta activity typically appeared in all 58 rats considered. Within the first 1-3 days of recirculation frequency and amplitude of all bands and in all derivations considered recovered, while the global cortical EEG activity declined during the 21 days.

Postischemic changes in the quantitative cortical EEG of the saline-treated group are exemplified in Figure 1. Typically, within the first 24 hours of recirculation, the delta frequency and amplitude reached values comparable to those at baseline and were organized in synchronized episodes of mixed fast (3-3.25 Hz) and slow (1-1.25 Hz) delta waves. In contrast, recovery of the theta and alpha waves was incomplete; their amplitudes 24 hours after occlusion were lower than those at baseline, and desynchronized signals were evident. Subsequently, delta activity decreased markedly to approximately 35% of baseline values at day 21. Activities of the other bands, although slightly increased, still remained below baseline values between days 1 and 7 and then declined further to approximately 40% of baseline values. At day 7, bursts of hypersynchronized fast (12-14 Hz) activity lasting a few seconds were evident, generally at the end of a synchronized episode (data not shown). Alternations between sleeping and waking reappeared by day 3 and subsequently declined to a nearly continuous state of wakefulness by day 21.

Figure 1 also reports postischemic changes in the quantitative cortical EEG of the siagoside-treated group. Although the pattern of electrophysiologic changes was similar to that observed in the saline-
treated group, the subsequent decline in cortical EEG activity was either absent or markedly reduced. Accordingly, while no difference was found in beta activity (data not shown), delta and theta activities were significantly higher in almost all derivations considered. Likewise, the alpha1 and alpha2 activities were also consistently more elevated. In addition, at these times the siagoside-treated group showed longer periods of synchronized sleep.

All coronal sections examined were well perfusion-fixed. No residual intravascular blood was present. Fixation artifacts (e.g., dark cells or dropical cells) were absent, and neuronal morphology was well preserved.

Thirty minutes of near-complete forebrain ischemia consistently resulted in morphologic manifestation of neuronal damage in all rats considered. Although we also observed damage in the cerebral cortex and dorsolateral thalamus, the most consistently affected area was the CA1 sector of the hippocampus. The CA1 pyramidal neurons were, in most cases, severely reduced in number or had virtually disappeared (Figure 2). When present, most CA1 pyramidal neurons displayed a normal morphology; a few showed features typical of neuronal damage (i.e., shrinkage and triangulation) but not microvacuolation or hyperchromasia of the cell body and nucleus. Thus, we graded hippocampal damage as the density of surviving CA1 pyramidal neurons.

Figure 3 reports the density of surviving CA1 pyramidal neurons in the naive control, saline-treated, and siagoside-treated rats. While most saline-treated rats displayed a loss of >50% CA1 pyramidal neurons, this amount of damage occurred less frequently among the siagoside-treated rats. Furthermore, comparing the means, the siagoside-treated group showed a significantly higher density of surviving neurons than the saline-treated group (p<0.05).

Discussion

Transitory forebrain ischemia in rats produced by the four-vessel occlusion method resulted in a relatively circumscribed loss of most pyramidal neurons in the hippocampal CA1 region. The rats also displayed neurophysiologic signs of temporally evolving cortical damage after ischemia. Although the mean quantitative cortical EEG power spectrum recovered partially during the first 1–3 days of reperfusion, within 21 days cortical EEG activity of all bands in all derivations considered declined. Such secondary deterioration of cortical EEG activity suggests a delayed impairment of previously recovered neuronal transmission in this region. This suggestion is further supported by the observation that the initial recovery of sleep–wake alternation patterns seen after 3 days of reperfusion also disappeared, leaving a nearly continuous state of wakefulness by day 21. The neurophysiologic dysfunction of the cortical circuitry observed at relatively long reperfusion times most
transient hypoxia-ischemia in rats; morphologic damage and neurologic deficits subsequent to transient middle cerebral artery occlusion in cats; and neurologic impairment after global cerebral ischemia in monkeys.

The molecular mechanisms underlying the capability of siagoside to limit neuronal damage after a cerebral hypoxic/ischemic insult remain to be fully defined. When added to cultured neuronal cells, monosialogangliosides are stably incorporated into the outer layer of the cell membrane in a temperature-, time-, and concentration-dependent manner. A similar phenomenon occurs in the CNS following the systemic administration of monosialogangliosides in vivo. Furthermore, gangliosides, including the monosialogangliosides, reduce glutamate-related toxicity in cultured CNS neurons. Likewise, systemically administered monosialogangliosides are efficacious in reducing excitotoxin-induced brain damage in both neonatal and adult rats. Thus, it is conceivable that the siagoside effects that we report reflect the ability of this agent to reduce excitatory amino acid–related neurotoxicity in ischemic brain.

Also noteworthy is that the antiexcitotoxic effect of gangliosides observed in vitro has been attributed solely to inhibition of the downstream consequences of excessive excitatory amino acid–receptor activation. In addition, gangliosides are capable of reducing glutamate neurotoxicity at 37°C when given as long as 30 minutes after glutamate exposure. In contrast, agents such as phencyclidine, known to act at the NMDA receptor level, are effective only when administered concurrent with or immediately after glutamate. These aspects differentiate gangliosides from NMDA receptor antagonists.

Another, not mutually exclusive, possibility concerns the relation between monosialogangliosides and neuronotrophic factors. Recent studies indicate that nerve growth factor may protect hippocampal CA1 pyramidal neurons against ischemic injury in both gerbils and rats. Because GM1 potentiates the action of neuronotrophic factors both in vitro and in vivo, we cannot exclude a potentiation of endogenous neuronotrophic factors as contributing to the action of siagoside in reducing neuronal damage after ischemia.

Recent studies have shown that early treatment with GM1 may ameliorate outcome in patients affected by acute cerebrovascular insufficiencies. This evidence, together with our results, supports the continuation of clinical trials to assess further the efficacy of treatment, not only with GM1 but also with its inner ester derivative, siagoside.

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References


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