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Short communication

NMDA receptor involvement in endothelin neurotoxicity in rat striatal slices

Yasufumi Kataoka [a,*], Shuichi Koizumi [a,1], Masami Kohzuma [a], Hirotomo Shibaguchi [a], Kazuto Shigematsu [b], Masami Niwa [c], Kohtaro Taniyama [a]

[a] *Department of Pharmacology 2, Nagasaki University School of Medicine, 1-12-4 Sakamoto, Nagasaki 852, Japan*
[b] *Department of Pathology 2, Nagasaki University School of Medicine, 1-12-4 Sakamoto, Nagasaki 852, Japan*
[c] *Department of Pharmacology 1, Nagasaki University School of Medicine, 1-12-4 Sakamoto, Nagasaki 852, Japan*

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Abstract

The high K^+-evoked dopamine release from rat striatal slices remained impaired by 50% up to 2 h after pulse exposure of the tissues to endothelin-3, under conditions of hypoglycemia/hypoxia. This striatal dysfunction was significantly improved by D-2-amino-5-phosphonopentanoic acid, a NMDA receptor antagonist, at a much lower concentration than that providing protection against NMDA-evoked dysfunction. In light of these findings, the important role of glutamatergic mechanisms, especially NMDA receptors, in mediating endothelin neurotoxicity warrants further attention.

Keywords: Endothelin; Neurotoxicity; NMDA receptor; Dopamine release; Striatal slice

1. Introduction

The endothelins are a family of three isopeptides (endothelin-1, -2 and -3), each with 21 amino acids and containing two intra-chain disulfide bridges (Yanagisawa et al., 1988; Masaki, 1993). Members of the endothelin family have a variety of biological activities, in both vascular and nonvascular tissues, including the modulation of neural functions (Masaki, 1993). Endothelins act on rat striatal slices to stimulate the release of dopamine (Koizumi et al., 1992, 1994a; Kataoka et al., 1994). We obtained evidence suggesting that endothelin-3 produces dopamine release through two distinct mechanisms: one is a direct stimulation of dopaminergic nerve terminals and the other is activation of interneurons which promote the release of glutamate (Koizumi et al., 1994a). Glutamate, in addition to mediating excitatory synaptic transmission, under conditions of excessive release causes neuronal damage and degeneration (Choi, 1988). There is evidence for a neurotoxic action of endothelins in the striatum (Kataoka et al., 1989) and hippocampus (Yamashita et al., 1993, 1994). Brief exposure to endothelins, under conditions of hypoglycemia/hypoxia, caused dysfunction of striatal dopaminergic neurons, an event evidenced by directly monitoring the release of dopamine (Kataoka et al., 1989). Taken together, these findings mean a possible involvement of glutamate in endothelin neurotoxicity.

Excessive Ca^{2+} entry, an etiological event of neuronal injury, occurs largely through Ca^{2+} channels gated by the NMDA receptor, a subtype of glutamate receptor, but also through voltage-gated Ca^{2+} channels (Choi, 1988). The endothelin-evoked striatal dysfunction is blocked by a 1,4-dihydropyridine Ca^{2+} channel antagonist, suggesting that voltage-gated Ca^{2+} channels function in the generation of endothelin neurotoxicity (Kataoka et al., 1989). We attempted to clarify the involvement of NMDA receptors in endothelin neurotoxicity in rat striatal slices. The striatal dysfunction evoked by *S*-(−)-1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-3-pyridinecarboxylic acid methyl ester (BAY K 8644, Ca^{2+} channel agonist) and *N*-methyl-D-aspartate (NMDA) was used as a model to

* Corresponding author. Tel. +81-958-47-2111 (ext. 2182), fax +81-958-49-0451.

[1] Present address: Division of Pharmacology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158, Japan.

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determine the concentration of the NMDA receptor antagonist, D-2-amino-5-phosphonopentanoic acid (D-AP5), required to provide specific protection against NMDA receptor-mediated neurotoxicity.

2. Materials and methods

2.1. Materials

Male Wistar rats (Kyudo, Kumamoto, Japan) 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 were maintained on a 12-h light-dark schedule (lights on 7:00 a.m.).

The chemicals used were as follows: endothelin-3 and BAY K 8644 were obtained from Peptide Institute (Osaka, Japan) and Research Biochemicals (MA, USA), respectively. NMDA, D-AP5, nifedipine HCl and ethyleneglycol-bis(β-aminoethyl ether) N,N,N',N'-tetra-acetic acid (EGTA) were from Sigma (MO, USA). All remaining reagents of analytical grade were purchased from Hayashi Purechemical Industries (Osaka, Japan).

2.2. Preparation of striatal slices

The rats were decapitated and the brain was immediately removed and placed in oxygenated ice-cold Krebs-Ringer bicarbonate solution, pH 7.4 of the following composition (mM): NaCl 118.0, KCl 4.7, $CaCl_2$ 1.3, $MgCl_2$ 1.2, NaH_2PO_4 1.0, $NaHCO_3$ 25.0 and D-glucose 11.0. Coronal brain slices, each with a thickness of 450 μm, were prepared using a McIlwain tissue chopper, and then the striatal part of the slices was dissected in ice-cold Krebs-Ringer buffer.

2.3. Direct monitoring and assay of released dopamine

The monitoring system was fundamentally composed of a tissue perfusion component and an electrochemical detector component, as described previously (Kataoka et al., 1989). In brief, three striatal slices sandwiched between two membrane filters (pore size 3.0 μm, Nucleopore, CA, USA) were placed in a chamber (250-μl inner volume) and perfused with oxygenated (95% O_2/5% CO_2) Krebs-Ringer solution at a flow rate of 500 μl/min at 37°C. These slices were subjected to pulsatile stimulation by injecting chemical stimuli into the flow stream in a volume of 500 μl through a loop injector (Rheodyne, CA, USA). The perfusate from the tissue chamber was directly introduced into the electrochemical detector monitoring component (LC-4B and TL-5A, Bioanalytical System, IN, USA). The electrode potential was set at +450 mV versus an Ag/AgCl reference electrode to detect oxidizable substances released from the striatal slices. In several experiments, the amounts of dopamine released in each individual response to the stimuli were determined by collecting the corresponding aliquot of the perfusate and using high-performance liquid chromatography with electrochemical detector, as described (Koizumi et al., 1994a). There was a high correlation between the peak amplitude of the output current obtained by the on-line system and the amount of dopamine released with each response to the stimuli ($r^2 = 0.945$), although 3,4-dihydroxyphenylacetic acid also contributed to the output current (Koizumi et al., 1992). Therefore, the current-time curves can be interpreted as real-time dynamics of dopamine release from the slices. The high K^+-evoked response was expressed as the area of the current-time curve. In the experiment with extracellular Ca^{2+} depletion, 1.3 mM $CaCl_2$ in Krebs-Ringer solution was replaced with 1 mM EGTA (Ca^{2+}-depleted Ringer).

2.4. Endothelin neurotoxicity in rat striatal slices

As previously described (Kataoka et al., 1989), tissues were stimulated 3 times (S1–S3) at 20-min intervals by application of 40 mM KCl after a 60-min preincubation period. The tissue was then perfused with Krebs-Ringer solution containing D-sucrose as the substitute for D-glucose and bubbled with 95% N_2/5% CO_2 (D-G-free Ringer). Under conditions of hypoglycemia/hypoxia, the tissue was exposed twice at 5-min intervals to 500 μl of various reagents as follows: endothelin-3 (4 μM), Bay K 8644 (50 μM), and NMDA (100 μM). NMDA was dissolved in D-G-free Ringer depleted of Mg^{2+} (D-G-free/Mg^{2+}-free Ringer) and then given under perfusion with this Ringer to avoid the blockade of NMDA receptor-operated channels by Mg^{2+} (Watkins et al., 1990). Following the termination of hypoglycemia/hypoxia, the test stimulation with KCl (40 mM) (S4–S6) was carried out 3 times at 20-min intervals, under conditions of reperfusion with normal Ringer solution. D-AP5 and nifedipine were applied during the perfusion with D-G-free Ringer or D-G-free/Mg^{2+}-free Ringer. Dopamine release was expressed as the relative response, that is, the ratio of the peak amplitude of the current-time curve for the high K^+-evoked response to that for the standard response to S3. The influence of various treatments during the 20-min period of hypoglycemia/hypoxia was taken as a percentage of the relative response of S6 to S3 (S6/S3) in the control experiment, in which no treatment was given during hypoglycemia/hypoxia. The protective actions of drugs were, in some cases, expressed as percentage of recovery calculated by the following equation: [S6/S3 after application of drug with stimuli − S6/S3 after application of stimuli alone] ÷ [control − S6/S3 after application of stimuli alone] × 100.

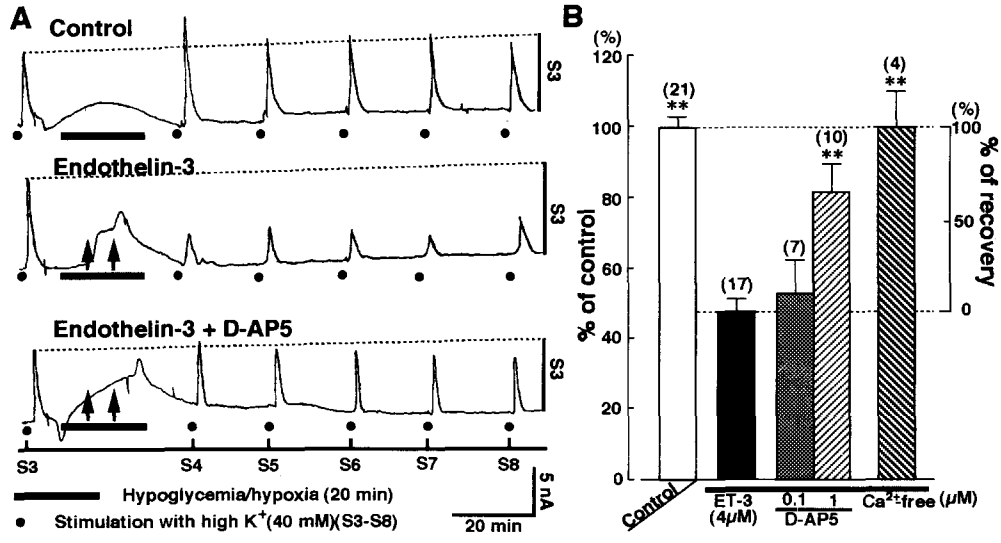

Fig. 1. (A) Traces showing typical current-time curves of dopamine release from rat striatal slices evoked by high K^+. Tissues were exposed to a 20-min period of hypoglycemia/hypoxia, without application of endothelin-3 (control, top panel). Tissues were exposed to endothelin-3 (4 μM, twice) in the absence (middle) and presence (bottom) of D-AP-5 (1 μM), under conditions of hypoglycemia/hypoxia. Vertical bar on the right of each panel indicates the peak amplitude of the S3-evoked response. (B) Effects of D-AP-5 and Ca^{2+} depletion on endothelin-3-evoked striatal dopaminergic dysfunction. Data are the means ± S.E.M. for the number of experiments shown in the top of each column. Values indicate percentage of the relative ratio of S6 to S3 in the control experiment. ** $P < 0.01$ vs. endothelin-3 alone.

2.5. Statistical analysis

Values represented means ± S.E.M. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. Differences were regarded as statistically significant at $P < 0.05$.

3. Results

When striatal slices were exposed to a 20-min period of hypoglycemia/hypoxia without the application of various reagents, the relative response of S6 to S3 (S6/S3) was 0.85 ± 0.06 (control, $n = 21$) (Fig. 1A). This value was identical to that in the experiment without exposure to hypoglycemia/hypoxia (0.86 ± 0.04, $n = 11$). The striatal response to test stimulation (S4–S6) was inhibited by 40–50% after the pulsatile application of various reagents, under conditions of hypoglycemia/hypoxia (Table 1 and Fig. 1). When these stimuli were applied during perfusion with normal Ringer solution, inhibition of the S6-evoked response was not observed (data not shown). The concentrations and number of applications of reagents produced almost the same degree of inhibition. Exposure to endothelin-3 (4 μM), BAY K 8644 (50 μM) and NMDA (100 μM) significantly decreased the S6-evoked response to 47.4 ± 3.7% ($n = 17$), 48.6 ± 3.3% ($n = 8$) and 59.9 ± 2.4% ($n = 8$) of control, respectively ($P < 0.01$ vs. control). These striatal responses remained impaired for up to 2 h after the termination of the hypoglycemic/hypoxic period (Fig. 1A).

D-AP5 (100 μM) provided significant protection against the dysfunction of striatal dopaminergic neurons induced by NMDA (88.3 ± 11.4% of recovery, Table 1). D-AP5, even at a high concentration of up to 100 μM, failed to influence the BAY K 8644-evoked striatal dysfunction (Table 1). In contrast to D-AP5, nifedipine at a concentration of 1 μM significantly

Table 1
Effects of D-AP5, nifedipine and Ca^{2+} depletion on the striatal dopaminergic dysfunction evoked by NMDA and BAY K 8644

Stimuli	In the absence of drug	+D-AP5 (μM)		+Nifedipine (μM)			Ca^{2+}-free
		10	100	0.1	1	10	
NMDA (100 μM)	59.9 ± 2.4 (8) [b]	70.7 ± 7.7 (4) [b]	95.4 ± 4.5 (4) [c]	n.o.	75.1 ± 8.8 (7) [a]	100.2 ± 9.2 (15) [c]	88.7 ± 7.4 (3) [c]
BAY K8644 (50 μM)	48.6 ± 3.3 (8) [b]	52.9 ± 10.6 (4) [b]	52.6 ± 8.5 (4) [b]	44.0 ± 4.7 (6) [b]	83.0 ± 9.3 (7) [c]	n.o.	79.6 ± 11.4 (10) [c]

Data are the means ± S.E.M. for the number of experiments shown in parentheses. Values indicate percentage of the relative ratio of S6 to S3 in the control experiment (0.86 ± 0.06 = 99.7 ± 4.1%, $n = 21$). n.o., not observed. [a] $P < 0.05$, [b] $P < 0.01$, significantly different from control. [c] $P < 0.01$, significantly different from NMDA or BAY K 8644 alone.

blocked the striatal impairment induced by BAY K 8644 but not that induced by NMDA (Table 1). The latter was significantly improved by nifedipine at a concentration of 10 μM. The impairment of the evoked response to S6 was not observed after treatment with NMDA and BAY K 8644 during perfusion with Ca^{2+}-depleted D-G-free/Mg^{2+}-free Ringer and Ca^{2+}-depleted D-G-free Ringer solution, respectively (Table 1).

D-AP5 at 1 μM produced a significant recovery from endothelin-3-evoked dysfunction of $62.1 \pm 16.6\%$ ($P < 0.01$, Fig. 1). Nifedipine (0.1 and 1 μM) also produced a recovery from endothelin-3-evoked striatal dysfunction of $31.3 \pm 5.4\%$ ($n = 3$) and $80.5 \pm 5.4\%$ ($n = 3$, $P < 0.01$), respectively. The impairment of the evoked response to S6 was not observed after treatment with endothelin-3 during perfusion with Ca^{2+}-depleted D-G-free Ringer solution (Fig. 1B).

4. Discussion

The endothelin-evoked dopaminergic dysfunction in rat striatal slices was significantly improved by D-AP5, a competitive NMDA receptor antagonist (Watkins et al., 1990), given at 100-fold lower concentration than that producing protection against NMDA-induced striatal dysfunction. This concentration of D-AP5 did not protect against BAY K 8644-evoked dysfunction. These observations suggest that D-AP5 protects striatal slices from endothelin neurotoxicity by specifically inhibiting NMDA receptors, one of the target sites of glutamate released by endothelin (Koizumi et al., 1994a). The predominant effect of D-AP5 on the endothelin-3- rather than the NMDA-evoked dysfunction may be interpreted as being due to a different efficacy in blocking NMDA receptors activated by endogenous glutamate and exogenous NMDA. It is widely accepted that brain damage is closely associated with neuronal Ca^{2+} overload caused by an excessive influx of extracellular Ca^{2+} (Choi, 1988). In the present study, no apparent striatal dysfunction occurred following the pulse exposure of tissues to various stimuli during perfusion with Ca^{2+}-depleted D-G-free Ringer. Therefore, a neuroprotective action of D-AP5 against endothelin neurotoxicity appears to result from a decrease in the inappropriate Ca^{2+} entry into neurons through NMDA receptor-gated Ca^{2+} channels.

Transient forebrain ischemia remarkably increases endothelin-like immunoreactivity and the number of endothelin ET_B receptors in the CA1 subfield of rat hippocampus (Yamashita et al., 1993, 1994). A link between neuronal Ca^{2+} overload and increased level of glutamate causes neuronal damage and cell death in the brain (Choi, 1988). We have reported that endothelins increase intracellular Ca^{2+} concentrations in cultured neurons and slices of the rat hippocampus (Koizumi et al., 1994b) and rat striatal slices (Kataoka et al., 1994). Endothelin-3 stimulates the release of glutamate from striatal slices (Koizumi et al., 1994a). As a consequence of these findings, it is considered that endothelin-accelerated ischemic brain damage is closely associated with activation of glutamatergic mechanisms. Astrocyte-mediated glutamate release was found to be one of the astrocyte-neuron signaling pathways (Parpura et al., 1994). The observation that endothelins elevated cytosolic Ca^{2+} in striatal astrocytes (Marin et al., 1991) led to the suggestion that endothelins stimulate glutamate release from astrocytes, in a manner which closely resembles that found with bradykinin (Parpura et al., 1994). We obtained evidence suggesting that endothelin ET_B rather than ET_A receptors are preferentially involved in stimulatory and neurotoxic actions of endothelins on striatal dopaminergic neurons (Kohzuma et al., 1994). All these findings may mean that endothelins stimulate endothelin ET_B receptors of neurons and/or astrocytes to enhance the release of glutamate, leading to striatal dopaminergic damage through NMDA receptors which are probably located on dopaminergic nerve terminals in the striatum (Wang, 1991). The possibility that other glutamate receptor subtypes such as non-NMDA ionotropic receptors and metabotropic receptors are involved in endothelin neurotoxicity needs to be taken into account.

In conclusion, glutamatergic mechanisms, especially NMDA receptors, have a role in mediating endothelin-related neurotoxicity.

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