Roles of neuronal nitric oxide synthase, oxidative stress, and propofol in N-methyl-D-aspartate-induced dilatation of cerebral arterioles

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Editor’s key points

- N-methyl-D-aspartate (NMDA) dilates blood vessels in the brain.
- In this study, using rat brain slices, the effect of propofol in the presence of NMDA was studied.
- NMDA induces nitric oxide release but also induces superoxide.
- Propofol reduced NMDA-induced dilation and decreased superoxide production.
- The effect of propofol on brain microvessels may be clinically relevant.

Background. It remains unclear whether N-methyl-D- aspartate (NMDA) receptors contribute to cerebral parenchymal vasodilatation, and any effects of clinically used anaesthetics on the dilatation. The present study was designed to examine whether NMDA induces neuronal nitric oxide synthase (NOS)-mediated dilatation, in the cerebral parenchymal arterioles, and whether propofol and superoxide modulate the dilatation in relation to the NMDA receptor activation.

Methods. The cerebral parenchymal arterioles within rat brain slices were monitored by a computer-assisted microscopy, and the vasodilatation in response to NMDA (10^{-7} to 10^{-5} M) was evaluated. Immunofluorescence analysis to neuronal and endothelial NOS and measurement of levels of superoxide and nitric oxide within the arteriole were simultaneously performed.

Results. Propofol, an NMDA receptor antagonist MK801, and a neuronal NOS antagonist S-methyl-L-thiocitrulline (SMT) reduced NMDA-induced dilation, whereas a superoxide inhibitor, Tiron, and NADPH oxidase inhibitor, gp91ds-tat, augmented NMDA-induced dilatation. Immunofluorescence analysis revealed distribution of neuronal NOS in both endothelial and smooth muscle cells in addition to neuronal cells. NMDA-induced superoxide and nitric oxide within the parenchymal arterioles. The increased superoxide within the arteriole was similarly inhibited by MK801, SMT, gp91ds-tat, propofol, and a neuronal NOS antagonist vinyl-L-NIO, whereas the level of nitric oxide was reduced by MK801, SMT, propofol, and vinyl-L-NIO, and it was augmented by gp91ds-tat.

Conclusions. NMDA dilates cerebral parenchymal arterioles possibly via neuronal NOS activation, whereas it produces superoxide via NADPH oxidase. In these arterioles, propofol reduces both the dilatation and superoxide production in response to NMDA.

Keywords: cerebral parenchymal arterioles; N-methyl-D-aspartate; propofol

Accepted for publication: 30 August 2011

N-methyl-D-aspartate (NMDA) causes dilatation of extraparenchymal arterioles in the brain resulting from activation of neuronal (type I) nitric oxide synthase (NOS). Indeed, the increased cerebral blood flow in response to NMDA and its receptor activation induced by glutamate has been documented. However, the role of NMDA receptor activation in cerebral parenchymal microvessels remains to be determined. Recent studies have demonstrated that NMDA receptor activation augments superoxide production in the brain via NADPH oxidase.
known as one of the important systems for superoxide production in vascular pathology. However, it has not been determined whether modulation of oxidative stress induced by NADPH oxidase in the brain parenchyma augments arteriolar dilation under activation of NMDA receptors.

Propofol is a clinically used i.v. anaesthetic, which maintains coupling with cerebral metabolic rate for oxygen and decreases intracranial pressure, allowing optimal intracranial pressure during neurosurgical operations, although its effect on the cerebral microcirculation remains unclear. A recent study documented an inhibitory effect of propofol on NMDA receptor subunit phosphorylation in cultured neurons, indicating possible neuronal receptor modulation by this anaesthetic. However, it has not been studied whether propofol together with oxidative stress affects regulation of cerebral microcirculation in relation to activation of neuronal receptors.

Therefore, the present study was designed to examine whether NMDA induces NOS-mediated dilation cerebral parenchymal arterioles, in addition to increased levels of superoxide induced by NADPH oxidase, and whether propofol and superoxide modulate the dilation in relation to NMDA receptor activation.

Methods

The Institutional Review Board of Wakayama Medical University approved this study. Male Wistar rats (16–20 weeks, n=41) were purchased from Charles River Japan Inc. (Yokohama, Japan). Rats were deeply anaesthetized with inhalation of 3% halothane with no response to initial surgical incision. After a midline thoracotomy, 50 ml saline was infused into the left ventricle, whereas a right atrial incision was made for blood drainage. Brains were rapidly removed and immersed with artificial cerebrospinal fluid (control solution) of the following composition (mM): NaCl 119, KCl 4.7, CaCl2 2.5, MgSO4 1.17, KH2PO4 1.18, NaHCO3 25, and glucose 5.5. Whole brains were cut into blocks containing the neocortex and thereafter sectioned into coronal slices (150 μm thick) with a tissue slicer (LEICA VT 1000S; Leica Instruments GmbH, Nussloch, Germany). Throughout the slicing procedure, brains were immersed in the control solution. Only one protocol was used for each slice.

Live computerized videomicroscopy

Each individual slice was then transferred to a recording chamber filled with control solution, which is attached to an inverted microscope (Olympus IX70, Tokyo, Japan). The recording system (10 ml) consisted of a recording chamber and a tubing part containing the perfusion chamber. The slices were superfused with control solution at a flow rate of 1.5 ml min⁻¹, bubbled with 93% O2+7% CO2 (PCO2=5.3 kPa, pH=7.4, 37°C in our condition). The internal diameter of an intraparenchymal arteriole (5.4–11.1 μm in internal diameter, 13.3–25.9 μm in external diameter) in the brain slice was continuously monitored with live computerized videomicroscopy comprising an inverted microscope, a 40X objective (Olympus), and a 2.25X video projection lens (Olympus). Identification of the arteriole in the brain slice is based on the previous studies demonstrating that the cerebral arteriole has a layer of smooth muscle cells, while the cerebral venule is composed of endothelial cells resting on a basal lamina. The internal diameter of an arteriole was defined as the distance between the internal margins of walls, and changes of internal diameter in cerebral arterioles were recorded on computer image files and then they were analysed by image analysis software (PhysioTech, Tokyo, Japan).

All experiments were performed during constriction to prostaglandin F2α (5×10⁻⁷ M), since we previously verified that the concentration induces ~70% vasoconstriction compared with the maximum produced by prostaglandin F2α (10⁻⁵ M) in the cerebral parenchymal arterioles. NMDA (10⁻⁷ to 10⁻⁵ M) was cumulatively applied to the brain slices. Propofol (3×10⁻⁷ and 10⁻⁶ M) was applied 15 min before the addition of prostaglandin F2α. Dizocilpine hydrogen maleate (MK801, 10⁻⁵ M), S-methyl-L-thiocitrulline (SMT, 10⁻⁵ M), gp91ds-tat (10⁻⁶ M), or 4,5-dihydroxy-1,3-benzene disulphonic acid (Tiron, 10 mM) was used 15 min before prostaglandin F2α to inhibit NMDA receptors, neuronal NOS activity, NADPH oxidase activity, and superoxide, respectively. The duration of each experiment was 3 h.

The amount of dilatation of the cerebral arteriole induced by vasodilators was normalized to the contraction by prostaglandin F2α (5×10⁻⁷ M) in each arteriole. The percentage dilatation or constriction was calculated by the following equation: % dilatation=100×[the diameter after application of NMDA – the diameter after application of prostaglandin F2α (5×10⁻⁷ M)][the diameter of control condition – the diameter after application of prostaglandin F2α (5×10⁻⁷ M)]⁻¹, % constriction to prostaglandin F2α=100×[the diameter after application of prostaglandin F2α (5×10⁻⁷ M) – the diameter of control condition][the diameter of control condition]⁻¹.

Immunofluorescence analysis

In the first set of experiments, the brain was fixed with 10% formalin solution buffered with phosphate-buffered saline (PBS, pH=7.2) and embedded in paraffin, followed by the preparation of 5 μm sections. After removing the paraffin, sections were incubated with PBS including 1% normal donkey serum and 1% bovine serum albumin to reduce non-specific reactions. Then the sections were exposed to pairs of rabbit anti-neuronal (type I) NOS polyclonal antibodies and mouse anti-endothelial (type III) NOS monoclonal antibody at each concentration of 1 μg ml⁻¹ at 4°C, overnight. After washing, the sections were incubated with Cy3-conjugated anti-rabbit IgG and FITC-labelled anti-mouse IgG secondary antibodies (15 μg ml⁻¹) at 24°C for 1 h. The sections were
examined using the FLUOVIEW FV300 laser scanning confocal microscope (OLYMPUS Inc., Tokyo, Japan). The negative control did not show any non-specific staining.

In the second set of experiments, the brain was fixed for 1 h in 4% paraformaldehyde, immersed in PBS (pH 7.4), dipped into TissueTek O.C.T. compound (Sakura Finetech, Tokyo, Japan), and stored at −80°C. Twenty micrometre sections were exposed to PBS with 0.1% Triton X-100 and incubated with PBS containing 3% donkey serum albumin to reduce non-specific reactions. The sections were further incubated with pairs of rabbit anti-neuronal NOS polyclonal antibodies (4 μg ml−1) and goat anti-platelet/endothelial cell adhesion molecule-1 (PECAM-1) polyclonal antibodies (5 μg ml−1) at 37°C for 30 min. After washing, the sections were incubated with pairs of FITC-labelled anti-rabbit IgG secondary antibodies (2 μg ml−1) and Alexa Fluor 568-conjugated anti-goat IgG or anti-mouse IgG (5 μg ml−1) at 37°C for 30 min. Finally, Hoechst 33258 (1 μg ml−1) for 15 min was applied to stain nuclei. The fluorescence images were acquired using the FLUOVIEW FV300 laser scanning confocal microscope (OLYMPUS Inc.). The negative control did not show any non-specific staining.

**Measurements of in situ nitric oxide and superoxide production**

The membrane-permeant probe DAF-2DA or the oxidative fluorescent dye hydroethidine was used to determine levels of nitric oxide and superoxide in situ, respectively.10 23 24 In a light-protected chamber, 4,5-diaminofluorescein diacetate (DAF-2DA, 10−5 M) for 60 min and thereafter hydroethidine (2 × 10−6 M) in combination with or without NMDA (10−5 M) for 30 min was applied to each unfixed 150 μm thick brain slice in phosphate-buffered saline (PCO2=5.3 kPa, pH=7.4, 37°C). Images were obtained with a FLUOVIEW FV300 laser scanning confocal microscope (OLYMPUS Inc.) equipped with a krypton/argon laser. Fluorescence was detected with a 550 or 585 nm long-pass filter for nitric oxide and superoxide, respectively. Laser settings were identical for acquisition of images from all of the brain slices. In some experiments, MK801 (10−5 M), SMTC (10−5 M), gp91ds-tat (10−6 M), sgp91ds-tat (10−6 M), propofol (10−6 M), dimethyl sulphoxide (DMSO, 2 × 10−6 M), a selective neuronal NOS inhibitor N²-(1-imino-3-butenyl)-l-ornithine (vinyl-L-NIO, 5 × 10−7 M),25 or a selective inducible NOS inhibitor 1400 W26 was also applied. Brain slices, which exposed to DAF-2DA in combination with SMTC or hydroethidine in combination with Tiron (10 mM), served as the control for measurements of nitric oxide and superoxide, respectively.10 The fluorescence in the brain slice was expressed as a ratio using these control values.

**Drugs**

The following pharmacological agents were used: DMSO, MK801, NMBA, propofol, or SMTC (Sigma Aldrich Inc., St Louis, MO, USA), gp91ds-tat and sgp91ds-tat (Genemed Synthesis Inc., San Antonio, TX, USA), hydroethidine (Poly-science Inc., Warrington, PA, USA), vinyl-L-NIO and 1400 W (Cayman Chemical, Ann Arbor, MI, USA), rabbit anti-neuronal NOS polyclonal antibodies and goat anti-PECAM-1 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-endothelial NOS monoclonal antibody (BD Bioscience Pharmingen, San Jose, CA, USA), mouse anti-α-smooth muscle actin monoclonal antibody (LifeSpan Biosciences, Inc., Seattle, WA, USA), Cy3-conjugated anti-rabbit IgG (Millipore Corp., Billerica, MA, USA), FITC-labelled anti-mouse and anti-rabbit IgG secondary antibodies (Cosmo Bio Co. Ltd, Tokyo, Japan), Alexa Fluor 568-conjugated anti-goat IgG and anti-mouse IgG (Molecular Probes Inc., Eugene, OR, USA), and Hoechst 33 258 (Nacalai Tesque, Kyoto, Japan). GPA91ds-tat is a highly selective NADPH oxidase Nox2 inhibitor, whereas sgp91ds-tat is a scrambled peptide.20 Drugs were dissolved in distilled water such that volumes of <60 μl were added to the perfusion system. DMSO was used as the solvent for MK801, propofol, gp91ds-tat, sgp91ds-tat, vinyl-L-NIO, and 1400 W. The stock solution for each compound was dissolved with control solution to obtain the final DMSO concentration of <1.47 × 10−6 M since we previously demonstrated that DMSO (2 × 10−6 M) does not affect vasodilatation in the organ chamber study.27

**Fig 1** Parenchymal arteriolar dilatation in response to cumulative addition of NMDA (10−7 to 10−5 M) in the rat brain slice in the presence or absence of MK801 (10−5 M) or SMTC (10−7 M). *Difference between the control arteriole and the arteriole treated with MK801 or SMTC is statistically significant (P < 0.05). Vasocostrictor responses to prostaglandin F2α (5 × 10−7 M) were −11.9 (−10.1 to −16.3, −9.7 to −18.3), −15.9 (−10.7 to −17.0, −10.1 to −18.1), or −15.9 (−10.4 to −17.1, −7.9 to −18.3)% for control arterioles and arterioles treated with MK801 or SMTC, respectively (median, inter-quartile ranges, and full range, not statistically significant). Data were from five rats.
shown in our previous study, application of propofol while propofol (3 \times 10^{-6} M) concentration-dependently reduced this dilatation (Fig. 2). As it was demonstrated dilatation in response to NMDA (10^{-7} M) for 15 min did not affect baseline internal diameters of cerebral parenchymal arterioles (data not shown).

**Fig 2** (a) Parenchymal arteriolar dilatation in response to NMDA in the rat brain slice in the presence or absence of gp91ds-tat (10^{-6} M) or 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron, 10 mm). *Difference between the control arteriole and the arteriole treated with gp91ds-tat or Tiron is statistically significant (P<0.05). Vasconstrictor responses to prostaglandin F_{2α} (5 \times 10^{-7} M) were −13.2 (−10.7 to −17.3, −9.7 to −19.4), −14.3 (−11.3 to −17.7, −9.5 to −18.3), or −14.1 (−10.3 to −18.3, −8.7 to −22.0)% for control arterioles and arterioles treated with gp91ds-tat or Tiron, respectively (median, inter-quartile ranges, and full range, not statistically significant). Data were from five rats. (a) Parenchymal arteriolar dilatation in response to NMDA in the presence or absence of propofol (3 \times 10^{-7} or 10^{-6} M). *Difference between the control arteriole and the arteriole treated with propofol is statistically significant (P<0.05). Vasconstrictor responses to prostaglandin F_{2α} (5 \times 10^{-7} M) were −18.1 (−14.5 to −19.4, −11.5 to −20.1), −14.2 (−9.8 to −17.0, −8.7 to −18.1), or −14.4 (−9.5 to −16.2, −9.3 to −23.0)% for control arterioles and arterioles treated with propofol 3 \times 10^{-7} M or 10^{-6} M, respectively (median, inter-quartile ranges, and full range, not statistically significant). Data were from five rats.

The concentrations of drugs are expressed as final molar concentration.

**Statistical analysis**

The data were expressed as median, inter-quartile, and full ranges. Each figure was built on data using the set of slices from the same animal. Data were evaluated using non-parametric statistical tests including the Mann–Whitney and Kruskal–Wallis tests. Differences were considered to be statistically significant when the P-value is <0.05.

**Results**

**Live computerized videomicroscopy**

NMDA (10^{-7} to 10^{-5} M) induced parenchymal arteriolar dilatation in a concentration-dependent manner, whereas MK801 (10^{-5} M) and SMTC (10^{-5} M) abolished this dilatation (Fig. 1). Tiron (10 mM) and gp91ds-tat (10^{-6} M) similarly augmented dilatation in response to NMDA (10^{-7} to 10^{-5} M), while propofol (3 \times 10^{-7} and 10^{-6} M) concentration-dependently reduced this dilatation (Fig. 2). As it was shown in our previous study, application of propofol (10^{-6} M) for 15 min did not affect baseline internal diameters of cerebral parenchymal arterioles (data not shown).

**Immunofluorescence analysis**

Immunofluorescence analysis revealed distribution of neuronal NOS at the arteriolar wall and neuronal cells. The neuronal enzyme was partly localized in the distinct portion of the arteriolar wall from endothelial NOS (Fig. 3). Co-staining for the endothelial cell marker PECAM-1 or the smooth muscle cell marker α-smooth muscle actin in addition to neuronal NOS revealed co-localization of the neuronal enzyme and endothelial or smooth muscle cells, respectively (Fig. 4). The immunoreactivity was not recognizable when in the primary antibody absence.

**Measurements of in situ nitric oxide and superoxide production**

Application of NMDA (10^{-5} M) to the brain slice induced production of superoxide and nitric oxide within parenchymal arterioles (Fig. 5). The increased superoxide within the arteriole was similarly inhibited by MK801 (10^{-5} M), SMTC (10^{-5} M), gp91-ds-tat (10^{-6} M), propofol (10^{-6} M), or vinyl-L-NIO (5 \times 10^{-7} M). Nitric oxide was decreased by MK801, SMTC, propofol, or vinyl-L-NIO, but it was augmented by gp91ds-tat (Fig. 5). In the preliminary experiments, sgp91ds-tat did not affect increased production of nitric oxide or as superoxide.
within parenchymal arterioles treated with NMDA (10^{-5} M). Relative superoxide and nitric oxide production in the brain slices treated with NMDA (10^{-5} M) or NMDA in combination with sgp91ds-tat (10^{-6} M) was 20.3 (13.8–24.4) and 23.6 (12.8–24.6) for superoxide and 4.0 (3.4–5.1) and 3.7 (3.1–4.6) for nitric oxide, respectively [median (range), n=5 (NS)].

**Discussion**

MK801 and the selective neuronal NOS inhibitor SMTC similarly abolished NMDA-induced dilatation, indicating the possible involvement of nitric oxide production via neuronal NOS in response to activation of NMDA receptors in cerebral parenchymal arterioles. This possibility was further suggested by the immunohistochemical analysis showing distribution of neuronal NOS at the arteriolar wall in addition to neuronal cells. The neuronal enzyme was partly localized in the distinct portion of the arteriolar wall and was distinct from endothelial NOS. Therefore, we also used co-staining with an endothelial cell marker PECAM-1 or a smooth muscle cell marker α-smooth muscle actin in addition to neuronal NOS, which revealed co-localization of the neuronal enzyme with endothelial or smooth muscle cells, respectively. In addition, MK801, SMTC, and the selective neuronal NOS inhibitor vinyl-L-NIO, but not a selective inducible (type II) NOS inhibitor, 1400W, similarly reduced levels of nitric oxide in the arteriolar wall after the addition of NMDA, indicating that the nitric oxide production by NMDA is probably via neuronal NOS. Indeed, previous studies demonstrated that NOS-immunoreactive fibres innervate cerebral arterial wall and that nitric oxide derived from neuronal NOS in the perivascular region is capable of acting on microvascular smooth muscle cells. Our model adds to previous studies in isolated microvessels and whole animals in terms of evaluating the cerebral microcirculation when neuronal–vascular interactions are preserved. However, the results might be affected by our study design using brain slice preparation. Our study together with previous studies may support a role of nitric oxide from neuronal NOS as a critical link between NMDA receptor-activation and cerebral blood flow in vivo. Indeed, previous in vivo studies also demonstrated that NMDA (10^{-7} to 10^{-5} M), similarly to glutamate, induces pial arteriolar dilatation via neuronal NOS. However, we cannot completely rule out the involvement of endothelial
NOS in the dilation to NMDA in our study, although neuronal NOS inhibitors used in the current study are highly selective towards the neuronal enzyme.\textsuperscript{19, 25}

In the current study, gp91ds-tat or Tiron similarly augmented NMDA-induced arteriolar dilatation and gp91ds-tat increased levels of nitric oxide, whereas both compounds inhibited superoxide within the cerebral arteriole. Tiron is a superoxide antagonist,\textsuperscript{22} and gp91ds-tat is a highly selective NADPH oxidase inhibitor, especially towards Nox2.\textsuperscript{8, 20, 21}

Therefore, these results suggest that NMDA augments superoxide production in the brain parenchymal arterioles via NADPH oxidase as it was shown by previous studies using neuronal tissues.\textsuperscript{4–7} In addition, there is a possibility that superoxide captures a portion of nitric oxide produced by NOS under activation of NMDA receptors, reducing dilatation of the brain parenchymal arteriole.\textsuperscript{33} Our study also indicates a possible role of Nox2 in the superoxide production induced by NMDA in the cerebral microcirculation. Indeed, activation of Nox2 via NMDA receptors has been confirmed in the brain in recent studies.\textsuperscript{5–7}

Propofol attenuated NMDA-induced dilatation in accordance with decreased production of nitric oxide in the cerebral parenchymal arteriole. A recent study has documented an inhibitory effect of propofol on NMDA receptor subunit phosphorylation in cultured neurones, indicating possible receptor regulation by this anaesthetic in the brain.\textsuperscript{12} However, it is unclear whether the inhibition of phosphorylation on NMDA receptor subunits contributes to the inhibitory effect of propofol on cerebral vasodilatation. Further studies are needed to clarify this point. It is also crucial to note that in our study, propofol decreased the level of nitric oxide to a similar extent to that seen in arterioles treated with SMT or vinyl-l-NIO. Propofol probably reduces production of nitric oxide related to neuronal NOS activation via NMDA receptors, resulting in impairment of dilatation of cerebral parenchymal arterioles. In humans, plasma concentrations of propofol in the clinical practice were reportedly up to $6 \times 10^{-5}$ M.\textsuperscript{34–36} Therefore, effective concentrations of propofol to reduce arteriolar dilatation in response to NMDA in the current study are within the clinical range given the plasma-free concentrations calculated from its plasma protein binding (up to 98%).\textsuperscript{35} In this study, propofol reduced levels of superoxide when NMDA was added and this is in agreement with our recent study demonstrating that propofol lowers levels of superoxide as a NADPH oxidase inhibitor in the brain slice preparations.\textsuperscript{10, 11}

However, propofol's effect
on cerebral arteriolar tone by the reduction of oxidative stress seems negligible under activation of NMDA receptors, since it appears to be an inhibitor of the NMDA receptor-related pathway.12

Augmented expression of NMDA receptors corresponding with increased superoxide production in the brain has been shown after cerebral ischaemia of animal models.7 33 The involvement of NMDA receptor activation in adverse neurological outcome was also documented in some surgical patients.38 Therefore, it can be concluded that oxidative stress via NMDA receptors is one of the important determinants to predict neurological outcome after a brain insult. It is also crucial to note the conclusions from previous studies using gene-disrupted mice that neuronal NOS activity is critical to induce brain damage after focal and global cerebral ischaemia, although the activity plays a role in increased cerebral capillary blood flow response to hypoxia.39 40 In addition, previous studies documented that propofol is neuroprotective in animal models of cerebral ischaemia, both in vitro and in vivo.41 These results, together with our study, may support the current concept that propofol reduces cerebral blood flow but maintains coupling with cerebral metabolic rate for oxygen, and that it decreases intracranial pressure, resulting in optimal intraoperative conditions during neurosurgical operations.9

Conclusions

NMDA dilates the cerebral parenchymal arterioles possibly via neuronal NOS activation, whereas it produces superoxide via NADPH oxidase. In these arterioles, propofol within clinically relevant concentrations reduces both dilatation and superoxide production in response to NMDA. NMDA receptors appear to play a role in cerebral microvessel dilatation mediated by neuronal activation, whereas propofol and oxidative stress may modulate this dilatation in the clinical condition. However, the clinical significance of the modulation in cerebral vascular function by the use of propofol is
still unclear and further studies are warranted to clarify this point.

Acknowledgement

The authors thank Takahito Hayashi, MD, Department of Forensic Medicine, Wakayama Medical University, Wakayama, Japan, for his technical assistance.

Declaration of interest

None declared.

Funding

This work was supported in part by Grant-in-Aid, 19390409 (H.K.) for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Tokyo, Japan.

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