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Diazepam does not reduce infarct size in rats subjected to transient occlusion of the middle cerebral artery when normothermia is maintained

Johanna Kuhmonen, Jouko Lukkarinen, Olli Gröhn, Jukka Jolkkonen and Juhani Sivenius

Abstract

Activation of the γ-amino butyric acid (GABA)-ergic system might protect against the damage that occurs after cerebral ischaemia. We examined this hypothesis by administering diazepam to rats subjected to transient middle cerebral artery occlusion (MCAO) using the intraluminal thread method. Diffusion MRI (DWI) and perfusion imaging (PI) were acquired during MCAO to assess brain tissue status and haemodynamics, respectively. Rats were intraperitoneally injected with either 10 mg kg^{-1} diazepam (n = 5) or vehicle (n = 5) both 30 min and 90 min after the onset of MCAO. To exclude the possibility that neuroprotection was due to the hypothermic action of the drug, body temperature was maintained at 37–38°C for up to 7 h postischaemia with a feed-back controlled thermoregulatory unit. Infarct volumes quantified 2 days after MCAO from T_2-weighted images were similar in ischaemic control rats and in ischaemic rats treated with diazepam. We conclude that diazepam-induced enhancement of $GABA_A$ activity does not effectively protect against neuronal damage that occurs after transient MCAO in normothermic rats.

Introduction

Cerebral ischaemia rapidly depletes high-energy phosphate stores, causing membrane depolarization followed by increased extracellular levels of excitatory neurotransmitters, such as glutamate. The excessive release of glutamate (Benveniste et al 1984; Meldrum et al 1985) followed by an increase in intracellular calcium is thought to be the key trigger for ischaemic neuronal damage (Meldrum et al 1985; Siesjö & Bengtsson 1989). Consequently, there have been many attempts to develop neuroprotective drugs by antagonizing these effects of glutamate. An alternative strategy, enhancing inhibitory neurotransmission, has been proposed to reduce ischaemic brain damage (Sternau et al 1989; Johansen & Diemer 1991; Shuaib et al 1993, 1995; Schwartz et al 1994, 1995; Hall et al 1997; Schwartz-Bloom et al 1998).

γ-Aminobutyric acid (GABA), the main inhibitory neurotransmitter in the vertebrate central nervous system, counteracts the pathologic as well as the physiologic effects of excitatory amino acids (Roberts 1974). The majority of inhibitory effects of GABA are mediated through activation of $GABA_A$ receptors, resulting in chloride channel opening and subsequent membrane hyperpolarization (Olsen et al 1984). Benzodiazepines such as diazepam potentiate $GABA_A$ neurotransmission. They have been widely used clinically for decades because of their anticonvulsant, anxiolytic, sedative/hypnotic and muscle relaxant properties (Tallman et al 1980).

Previous studies indicate that GABA agonists (Sternau et al 1989; Shuaib et al 1993, 1995) and GABAergic activity-enhancing agents (Sternau et al 1989; Johansen & Diemer 1991; Schwartz et al 1994, 1995; Hall et al 1997; Schwartz-Bloom et al 1998) possess neuroprotective effects in rat and gerbil hippocampus after transient global cerebral ischaemia. Here, we studied the effects of diazepam on transient occlusion of the middle cerebral artery (MCA) produced using the intraluminal thread method

Department of Neuroscience and Neurology, University of Kuopio, Kuopio, Finland

Johanna Kuhmonen, Jukka Jolkkonen

NMR Research Group, AIVirtanen Institute, University of Kuopio, Kuopio, Finland

Jouko Lukkarinen, Olli Gröhn

Department of Neuroscience and Neurology and Brain Research and Rehabilitation Center Neuron, Kuopio, Finland

Juhani Sivenius

Correspondence: J. Sivenius, Department of Neurology, University Hospital of Kuopio, PO Box 1777, FIN-70210 Kuopio, Finland. E-mail: juhani.sivenius@fimnet.fi

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and evaluated infarct volumes using magnetic resonance imaging (MRI) (Allegrini & Sauer 1992; Busza et al 1992; Moseley et al 1990). Diazepam provokes substantial hypothermia, which is known to protect neurons under ischaemic conditions (Busto et al 1987). Thus, rats in this study were kept normothermic after drug treatment.

Materials and Methods

Animals

All experimental procedures were conducted in accordance with the European Community Council directives 86/609/EEC. Non-fasted male Wistar rats, 220–280 g, were anaesthetized with 4% halothane (in 70% nitrous oxide and 30% oxygen), and maintained during the operation with 0.5–1% halothane via a nose cone. The rats were kept normothermic with a feedback-controlled thermoregulatory heating unit (Harvard Apparatus, Holliston, MA). The right femoral artery was cannulated for continuous blood pressure monitoring (CardioCap II, Datex, Finland) and for arterial blood gas sampling ($PaCO_2$, PaO_2), pH (ABL5, Radiometer, Denmark), and glucose concentration measurements (OneTouch II, Lifescan, USA).

Transient MCA occlusion (MCAO) was induced as described by Longa et al (1989). The right common carotid artery was exposed through a midline cervical incision under a surgical microscope and gently separated from the nerves. A microvascular clip was placed around the right common carotid artery to prevent bleeding during the insertion of the filament. The external common carotid artery was cut with microscissors and electrocoagulated. A nylon filament (Ø 0.25 mm, rounded tip) was inserted into the stump of the external common carotid artery. The filament was advanced 1.9–2.1 cm into the internal common carotid artery until resistance was felt. The filament was held in place by tightening the suture around the internal common carotid artery and placing a microvascular clip around the artery. After 90 min of MCAO, the filament was removed and the external carotid artery was permanently closed by electrocoagulation. Finally, the cervical incision was closed with silk sutures.

Magnetic resonance imaging

MRI was performed at 4.7 T using a Surrey Medical Imaging System scanner (Guildford, UK) with a single loop surface coil for signal transmission and reception. Ischaemia was verified by measuring images weighted by the trace of the diffusion tensor (D_{AV}), and the area of hypoperfusion during the occlusion was assessed from a coronal slice positioned through the middle of the MCA territory. Absolute D_{AV}-maps were computed from images acquired using a spin-echo sequence (TR 2000 ms, TE 60 ms, FOV = 35 mm, matrix 128×64, zero filled to 128×128, slice thickness 1 mm) with four bipolar gradients along all three orthogonal directions, developing b-values of 38, 611, and 1200 s mm^{-2} (Mori & van Zijl 1995). Perfusion imaging was performed with intravenous gadodiamide (Omniscan, Oslo, Norway) bolus tracking using a gradient echo-method (TR 9.5 ms, TE 4.5 ms, FOV = 35 mm, matrix 128×64, slice thickness 1.5 mm). The relative hyperintensity in the ipsilateral MCA territory was considered the area of hypoperfusion. The total infarct volume 48 h after MCAO was quantified from T_2-weighted MRI (TR 2500 ms, TE 60 ms, matrix 256×128, 15 consecutive slices of 1 mm thickness). The severity of oedema at the same time was assessed from T_2-maps, calculated based on images acquired using the same spin-echo sequence (TR 2000 ms, TEs 20, 50, 80 ms, FOV = 35 mm, slice thickness 1 mm), and D_{AV}-maps were calculated using the Matlab procedure (Mathworks, Nattick, MA). All image analyses were performed by an observer blind to the experimental groups.

Drug treatment

The rats were intraperitoneally injected with either diazepam (Diapam, Orion Pharma, Finland) 10 mg kg^{-1} (n = 5) or vehicle (33% polyethylene glycol in 0.9% NaCl) (n = 5) 30 and 90 min after the onset of MCAO. This diazepam dose has pharmacologic effects in rats (Shuaib et al 1993, 1995). To prevent diazepam-induced hypothermia, the rats remained connected to the thermoregulatory heating unit after the injections, and their core body temperature was maintained at 37–38°C for up to 7 h.

Statistics

Statistical differences in infarct volumes between experimental groups were analysed using Mann–Whitney U-test. Physiologic data were analysed using analysis of variance for repeated measures. The values are presented as means ± s.d.

Results

There were no significant differences in physiological parameters monitored between the two groups during transient MCAO (Table 1). Diffusion MRI (DWI) and perfusion imaging (PI) were performed 30 min after the onset of MCAO to verify that an acute ischaemic episode occurred (Figure 1). The sizes of the hypoperfusion areas in the coronal slice measured did not differ significantly between the two groups (vehicle, 38.7 ± 1.0 mm^2; diazepam, 37.7 ± 5.8 mm^2; $P = 0.74$). Additionally, D_{AV} measurements, either during the acute phase or 48 h after occlusion, were not significantly different between groups (Figure 1).

The rats injected with diazepam were drowsy and virtually inactive during the first 7 h after MCAO when the core body temperature was controlled. Core body temperature was similar in both groups until the end of the 48-h follow-up (Table 2).

Table 1 Physiologic variables during transient occlusion of the middle cerebral artery in rats.

Variable	Before occlusion	30 min after occlusion	80 min after occlusion
PaO_2 (mmHg)			
Vehicle	137±22	108±12	102±14
Diazepam	147±13	127±12	112±18
$PaCO_2$ (mmHg)			
Vehicle	40±3	41±9	46±11
Diazepam	43±3	39±5	44±12
pH			
Vehicle	7.40±0.01	7.38±0.05	7.33±0.03
Diazepam	7.40±0.03	7.37±0.04	7.34±0.05
MAP (mmHg)			
Vehicle	93±6	103±9	92±9
Diazepam	96±5	100±8	93±5
Blood glucose (mM)			
Vehicle	6.5±0.3	5.6±0.5	6.1±0.7
Diazepam	6.2±0.3	5.7±0.4	6.3±0.6

PaO_2, arterial oxygen pressure; $PaCO_2$, arterial carbon dioxide pressure; MAP, mean arterial blood pressure. All values are represented as mean±s.d. There were no statistically significant differences between groups when analysed by analysis of variance for repeated measures.

Table 2 Core temperature (°C) in rats treated with vehicle or diazepam after transient occlusion of the middle cerebral artery.

Time after administration	Vehicle (n = 5)	Diazepam (n = 5)
0	37.2±0.5	37.4±0.3
1 h	37.3±0.6	37.5±0.9
2 h	37.4±0.5	37.1±0.2
3 h	37.2±0.3	37.0±0.1
4 h	37.2±0.3	37.1±0.2
5 h	37.1±0.2	37.0±0.2
6 h	37.0±0.2	37.0±0.2
7 h	37.1±0.1	37.1±0.1
24 h	38.0±0.3	37.9±0.3
48 h	37.6±0.5	37.4±0.3

Vehicle or drug was administered 30 min and 90 min after the onset of occlusion. All values are represented as mean±s.d. The number of rats is given in parentheses. There were no statistically significant differences between groups when analysed by analysis of variance for repeated measures.

Infarct volumes for the whole cerebral hemisphere or cortex were not different between experimental groups 48 h after MCAO (Table 3). There were no significant differences in T_2 relaxation times in either the ipsilateral or contralateral hemisphere between groups. In the ipsilateral hemisphere, the T_2 relaxation times were 86.2±5.2 ms (vehicle) and 88.2±5.3 ms (diazepam), and in the contralateral hemisphere, the values were 53.9±1.8 ms (vehicle) and 54.4±1.5 ms (diazepam).

Discussion

The results of this study indicate that postischaemic administration of diazepam (2×10 mg kg^{-1}) does not provide neuroprotection against focal cerebral ischaemia in rats. Transient occlusion of the MCA using the intraluminal thread model was used because of its similarity to stroke. The MCA is the most commonly affected vessel in stroke (Mohr et al 1986), and reperfusion occurs in up to 50% of stroke patients (Saito et al 1987). The severity of ischaemia, as assessed by DWI and PI (Moseley et al 1990; Busza et al 1992), was identical in both rat groups, which is a crucial

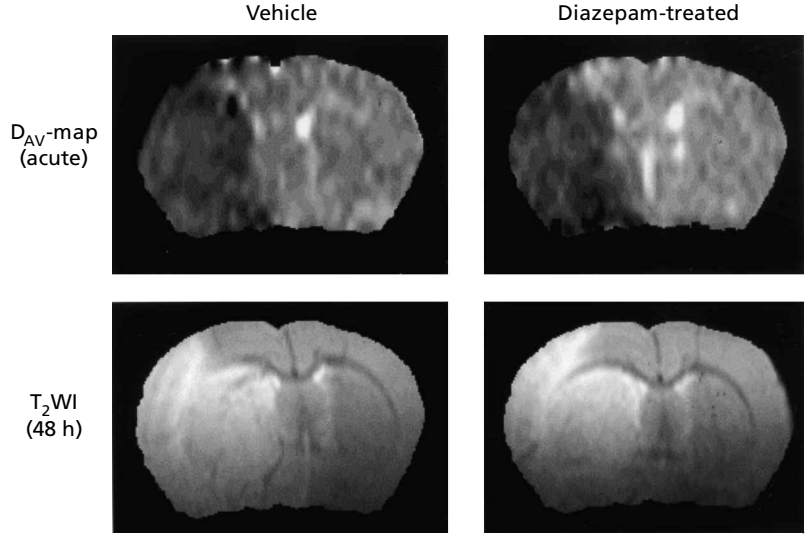

Figure 1 Representative D_{AV} images showing identical acute ischaemic damage in experimental groups, and T_2-weighted images showing no significant protection in diazepam-treated rats subjected to transient occlusion of the middle cerebral artery 48 h earlier.

Table 3 Infarct volumes (mm^3) quantified from T_2-weighted MRI 48 h after transient occlusion of the middle cerebral artery in rats.

	Hemisphere	Cortex
Vehicle (n = 5)	260.9±81.3	132.1±97.2
Diazepam (n = 5)	294.7±114.5	126.9±59.2

Values are expressed as mean±s.d. The number of rats is given in parentheses. There were no statistically significant differences between groups.

factor in preclinical drug screening studies. The efficacy of diazepam treatment was evaluated from T_2-weighted images, which is a means of assessing drug effects in-vivo (Allegrini & Sauer 1992).

Previous studies suggested that GABAergic activity-enhancing agents might attenuate neuronal damage following global forebrain ischaemia. These agents include pentobarbital, valproic acid, No-328, diazepam, PNU-101017, imidazenil, muscimol and clomethiazole (Sternau et al 1989; Johansen & Diemer 1991; Shuaib et al 1993, 1995; Schwartz et al 1994, 1995; Hall et al 1997; Schwartz-Bloom et al 1998). Furthermore, $GABA_A$ receptor agonists reduce infarct volumes after transient (Sydserff et al 1995b; Lyden 1997) and permanent (Sydserff et al 1995a) MCAO in the rat. GABAergic downregulation (Verheul et al 1993; Qü et al 1998) and glutamate-induced excitotoxicity (Roberts 1974) are putative target(s) through which diazepam acts. Based on the above findings, we were surprised not to find diazepam-induced neuroprotection in rats subjected to transient MCAO. The mechanism(s) leading to neuronal damage in the transient MCAO model might be different from that in global ischaemia, however, thus explaining why there was no neuroprotection observed in this study. In particular, the severely necrotic core typical to the MCAO model might be difficult to protect. Furthermore, benzodiazepines enhance GABA function only when there is endogenous GABA present (Tallman et al 1980). This might not be the case after massive GABA release induced by cerebral ischaemia (Hillered et al 1989; Uchiyama-Tsuyuki et al 1994), which might be followed by feedback inhibition, down-regulation of the GABA system and decreased GABA release (Green et al 1992).

One possible explanation for the results of the previous studies is the alteration in body temperature that occurs after diazepam administration. Diazepam (10–20 mg kg^{-1}) induces a substantial hypothermia (2–3°C) (Elliot & White 2001), which might provide secondary protection of neurons from ischaemic damage (Busto et al 1987). Although postischaemic body temperature is increased for up to 48 h in the transient MCAO model, possibly due to hypothalamic damage and increasing neuronal damage (Li et al 1999; Reglodi et al 2000), it is possible that diazepam counteracts the hyperthermia (if not controlled) and reduces infarct volumes. Recent data obtained using the global ischaemia model support this notion. When post-ischaemic temperature was regulated, histologic and behavioural protection provided by diazepam was abolished (Dowden et al 1999). The role of hypothermia as a sole mechanism by which diazepam provides neuroprotection remains to be confirmed, because diazepam is reported to have neuroprotective efficacy even after direct infusion into the hippocampus (Schwartz et al 1995) and in hippocampal slices (Galeffi et al 2000).

These data are clinically relevant with respect to the ongoing Early GABAergic Activation Study In Stroke (EGASIS) (Lodder 2002). A feasibility study in 104 stroke patients demonstrated that diazepam at a dose of 10 mg twice daily for 3 days is well tolerated despite drowsiness in some patients (Lodder et al 2000). As the EGASIS is intended to be a large and simple trial, body temperature is not monitored or controlled (Lodder, personal communication) and this factor might contribute to the final results of this multicenter effort.

The possibility of an easily administered and safe drug acting as a neuroprotectant in acute brain infarct is attractive. Experimental studies revealed promising neuroprotective effects with glutamate antagonists against cerebral ischaemia, but clinical trials so far have been disappointing, mainly because of the occurrence of severe side effects (Muir & Lees 1995). Thus, enhancement of GABAergic activity would be a possible alternative strategy for this purpose. The ongoing EGASIS study will reveal whether diazepam provides protection to such an extent that stroke patients can benefit from its administration.

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