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European Journal of Pharmacology 315 (1996) 297–303

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Prevention by the new Ca^{2+} channel antagonist, AJ-3941, of loss of endothelium-dependent relaxation after subarachnoid hemorrhage in rats

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Received 22 February 1996; revised 30 July 1996; accepted 2 August 1996

Abstract

AJ-3941 ((±)-(*E*)-1-(3-fluoro-6,11-dihydrodibenz[*b,e*]-oxepine-11-yl)-4-(3-phenyl-2-propenyl)-piperazine dimaleate; CAS No. 143110-70-7), a cerebrovascular-selective Ca^{2+} channel antagonist having anti-lipid peroxidative action, was reported to prevent cerebral vasospasm following subarachnoid hemorrhage in rats. The present study was undertaken to determine whether AJ-3941 protects the impairment of cerebroarterial endothelium-dependent relaxation which is concomitantly induced with cerebral vasospasm. Subarachnoid hemorrhage biphasically suppressed the response to acetylcholine in rat basilar artery, at 0.5 h ($n = 4$; $P < 0.06$) and 1 day ($n = 5$; $P < 0.05$) after subarachnoid hemorrhage. The reduction of the responses was correlated significantly to the degree of vasospasm determined angiographically. This reduction was accompanied by a 49% increase of arterial lipid peroxide contents. Endothelium-independent relaxation in subarachnoid hemorrhage rats was preserved in response to 3-morpholinosydnonimine, sodium nitroprusside and papaverine. AJ-3941 prevented ($n = 6–8$, $P < 0.05$) the suppression of the acetylcholine-induced response and the increase in lipid peroxide content in subarachnoid hemorrhage rats. These results suggest that AJ-3941 could exert its vasospasmolytic effect by preserving endothelial function through its anti-lipid peroxidative action, in addition to its inhibition of vasospasmogen-induced vasoconstriction related to intracellular Ca^{2+} mobilization.

Keywords: Subarachnoid hemorrhage; Endothelium-dependent relaxation; Lipid peroxidation; AJ-3941; Nimodipine; Ca^{2+} channel antagonist

1. Introduction

Cerebral vasospasm following subarachnoid hemorrhage is characterized by sustained constrictions caused by vasoconstrictive substances which are derived from blood clots, locally damaged vascular site or brain tissue, although details of the mechanism are still unclear (Kiwak and Heros, 1987). Recently, it has been proposed that experimental subarachnoid hemorrhage can impair endothelium-dependent relaxation (EDR) and the impairment may be an important event in the pathogenesis of cerebral vasospasm (Kim et al., 1988; Kanamaru et al., 1989). However, it remains controversial if the impairment of EDR after subarachnoid hemorrhage is a critical cause of cerebral vasospasm or a result of prolonged vasospasm. On the other hand, active oxygen species including free radicals were reported to play a pivotal role in the course of endothelial damage (Wedmore and Williams, 1981). However, it is unknown whether drugs that possess an anti-lipid peroxidative action prevent the development of cerebral vasospasm after subarachnoid hemorrhage by preserving endothelial function.

We have reported that AJ-3941 ((±)-(*E*)-1-(3-fluoro-6,11-dihydrodibenz[*b,e*]-oxepine-11-yl)-4-(3-phenyl-2-propenyl)-piperazine dimaleate; CAS No. 143110-70-7) is a novel Ca^{2+} channel antagonist that inhibits both constriction induced by activation of the voltage-dependent Ca^{2+} channels (Minato and Masuda, 1991) and protein kinase C (Hashizume et al., 1995). This compound has a cerebrovascular-selective vasospasmolytic action in isolated basilar arteries of rabbits and dogs (Minato and Masuda, 1991; Minato et al., 1993) and prevents the development of cerebral vasospasm after subarachnoid hemorrhage in rats (Honda et al., 1996). Furthermore, this compound inhibits Fe^{2+}-induced malonyldialdehyde formation in rat myocardial mitochondria (Masuda et al., 1990). The present study was performed to clarify the relation between alteration of EDR in cerebral artery and development of cerebral vasospasm and to examine whether AJ-3941 pre-

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PII S0014-2999(96)00632-2

vents the impairment of the basilar arterial EDR response in the rat subarachnoid hemorrhage model. The relationship between EDR and lipid peroxide level in the basilar artery was also studied.

2. Methods and materials

2.1. Animal preparation

Male Sprague-Dawley rats (Jcl:SD; Clea Japan, Tokyo, Japan) weighing between 300 and 450 g were used.

The surgical procedure was described previously (Honda et al., 1996). In brief, the animals were anesthetized with ether. A polyethylene cannula (SP-10; Natume Seisakusyo, Tokyo, Japan) filled with artificial cerebrospinal fluid (ACSF) was inserted into the cisterna magna for subsequent injection of blood. The composition of ACSF was as follows (mM): NaCl 126.5, $NaHCO_3$ 27.5, KCl 2.4, KH_2PO_4 0.5, $CaCl_2$ 1.1, $MgCl_2$ 0.85, Na_2SO_4 0.5, and glucose 5.9, pH 7.5. The distal end of the cannula was sealed, and its proximal part was blunted and attached to the atlanto-occipital muscle with sutures. One to 2 days after the implantation of the cannula, 0.3 ml homologous arterial blood was injected into the cisterna magna for 30 s through the cannula under ether anesthesia. The control group received ACSF instead of blood. During the injection of blood or the ACSF, the head was inclined by tilting the animal at about 40°.

2.2. Relaxation responses to acetylcholine, 3-morpholino-sydnonimine (SIN-1), sodium nitroprusside and papaverine in the basilar artery

The animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and killed by exsanguination from the carotid artery at 0.5 h, 3 h, 1 day and 5 days after the injection of blood. The basilar artery was isolated from the brain with the aid of a stereoscopic microscope (SZ6045; Olympus Kogaku, Tokyo, Japan), and a ring segment (approximately 1 mm long) was prepared from the middle portion of the artery. The segment was carefully suspended with L-shaped metal holders in an organ bath (Micro Organ Bath MOB-1; Technical Supply, Osaka, Japan) which was filled with an oxygenated modified Krebs bicarbonate solution (millimolar composition: NaCl 120, KCl 4.5, $MgSO_4$ 1.0, $NaHCO_3$ 27.0, KH_2PO_4 1.0, $CaCl_2$ 2.5, and glucose 10.0). The isometric tension was monitored with a transducer, and was recorded continuously with an ink recorder (FBR-252A; Toa Electronic, Tokyo, Japan) via an amplifier (Load Cell Converter LC210; Unipulse, Saitama, Japan). The Krebs solution was maintained at 37°C and was continuously aerated with a mixture of 95% O_2 and 5% CO_2. The resting tension of the segment was adjusted to 0.1 g. The EDR response was evaluated by measuring the extent of relaxation induced by acetylcholine (0.01–10 μM) in the segment constricted with prostaglandin $F_{2\alpha}$ (30–300 μM). The endothelium-independent relaxation response was measured as relaxations induced by SIN-1 (0.001–100 μM), sodium nitroprusside (0.001–100 μM) and papaverine (100 μM) in the segment constricted by prostaglandin $F_{2\alpha}$. The effects of AJ-3941 and nimodipine on the EDR responses were examined 1 day after blood injection.

2.3. Measurements of lipid peroxide levels in blood and basilar artery

The levels of lipid peroxides in arterial blood and the basilar artery were measured by a fluorometric method (Yagi, 1976). The rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) at 0.5 h, 1 day and 5 days after blood injection. Arterial blood was taken from the abdominal aorta, and the basilar artery was immediately isolated from the brain with the aid of a stereoscopic microscope and soaked in ice-cold saline. The basilar artery was homogenized in 200 μl of 8.1% sodium lauryl sulfate solution. The homogenate, with the addition of 1.5 ml of 20% acetate buffer (pH 3.5), was heated with 1.5 ml of 2-thiobarbituric acid (0.8 mg/ml in 20% acetate buffer) for 60 min at 100°C. After cooling at room temperature, 5 ml of *n*-butanol-pyridine mixture (15 : 1, v/v) was added and mixed for exactly 20 s. After centrifugation at 3000 rpm for 15 min, the *n*-butanol-pyridine layer was taken off for fluorometric measurements (515 nm excitation and 553 nm emission; fluorescence spectrophotometer F-3000; Hitachi, Tokyo, Japan). The levels of lipid peroxides in blood were determined using a commercially available assay kit (Wako Pure Chemical Industries, Osaka, Japan) with 1,1,3,3-tetraethoxypropane, which yields 1 mol malonyldialdehyde/mol under these conditions, as a standard. The lipid peroxide levels in blood and the basilar artery were expressed as nmol/ml and pmol/artery, respectively.

2.4. Measurements of protein contents in basilar artery

Because the entire basilar artery was used for the measurement of lipid peroxide contents, the contents of protein in the artery were determined in a separate experiment. The basilar artery was isolated according to the method mentioned above (Section 2.3) on 1 day after blood injection. The basilar artery was homogenized in 200 μl of 8.1% sodium lauryl sulfate solution, and 100 μl of the homogenates was used for determining protein content. The protein contents were measured by colorimetry (750 nm), using a commercially available assay kit (Bio-Rad Laboratories, Richmond, CA, USA) with bovine serum albumin as a standard.

2.5. Drugs

AJ-3941 ((±)-(*E*)-1-(3-fluoro-6,11-dihydrodibenz[*b,e*]-oxepine-11-yl)-4-(3-phenyl-2-propen-

yl)-piperazine dimaleate) was synthesized in our laboratories. Nimodipine was purchased from Bayer (Leverkusen, Germany). For the intravenous administration, AJ-3941 was dissolved in ethanol at a concentration of 10 mg/ml and then diluted in 5% glucose solution. AJ-3941 was given intravenously in a volume of 1 ml/kg just before the injection of blood. For oral administration, AJ-3941 or nimodipine suspended in 0.5% tragacanth solution was given orally in a volume of 3 ml/kg, 2 h or 1 h, respectively, before the blood injection.

Acetylcholine, SIN-1, sodium nitroprusside and papaverine were purchased from Daiichi Pharmaceutical Co. (Tokyo, Japan), Funakoshi Chemical Co. (Tokyo, Japan), Sigma Chemical Co. (St. Louis, MO, USA) and Nacalai Tesque (Kyoto, Japan), respectively, and all were dissolved in the Krebs bicarbonate solution. Prostaglandin $F_{2\alpha}$, obtained from Funakoshi Chemical Co., was dissolved in ethanol at a concentration of 3 mM and diluted with distilled water.

2.6. Statistics

All data are presented as mean values ± S.E.M. The statistical significance of differences was analyzed using Student's *t*-test or Dunnett's multiple comparison test for comparison between two groups or among groups, respectively. P values lower than 0.05 were considered to be significant.

3. Results

3.1. Changes in acetylcholine-induced relaxation in basilar arteries

The ring segments of basilar arteries constricted with prostaglandin $F_{2\alpha}$ (30–300 μM) relaxed in response to acetylcholine (0.01–10 μM) in a concentration-dependent manner (Fig. 1). In the arteries from rats subjected to subarachnoid hemorrhage, however, the relaxation responses varied at different times after blood injection: the responses at 0.5 h ($n = 4$) and 1 day ($n = 5$) after the injection were smaller than those at 3 h ($n = 7$) and 5 days ($n = 4$) (Fig. 1). To avoid the influence of an increase in intracisternal pressure caused by blood injection, the maximum relaxation response to 10 μM acetylcholine was compared between blood-injected and ACSF-injected rats ($n = 4$–7) at the same time points after the injection, and a marked reduction of the response was found at 0.5 h (34.2 ± 15.5%; $P < 0.06$) and 1 day (34.6 ± 17.5%; $P < 0.05$), but not at 3 h (139 ± 41.7%) and 5 days (118 ± 19.1%) after blood injection (Fig. 2).

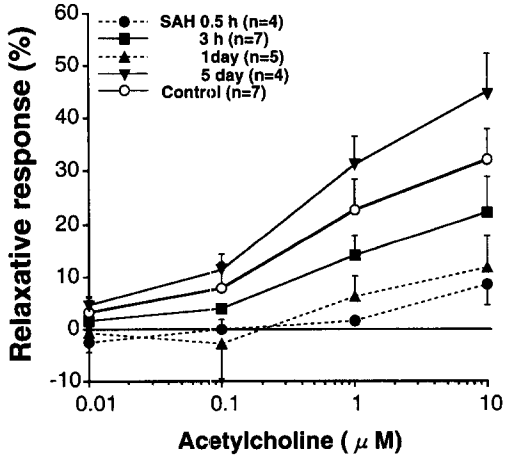

Fig. 1. Concentration-response curves of acetylcholine-induced relaxation in rat basilar arteries at various times following subarachnoid hemorrhage. The arterial rings were first constricted with prostaglandin $F_{2\alpha}$ (30–300 μM) and then acetylcholine was added cumulatively. Values represent means ± S.E.M. from 4–7 animals.

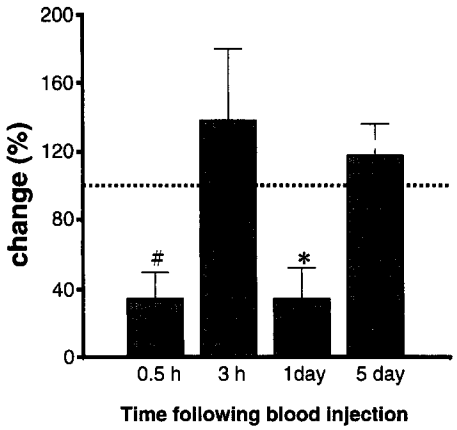

Fig. 2. Time course changes of acetylcholine-induced relaxation in basilar arteries of subarachnoid hemorrhage rats. The relaxation response to acetylcholine (10 μM) in each subarachnoid hemorrhage group was expressed as percentage of the respective reference group (ACSF was injected cisternally instead of blood). Values represent means ± S.E.M. from 4–7 animals. # $P < 0.06$ and * $P < 0.05$, vs. respective reference group.

3.2. Changes in relaxation induced by SIN-1, sodium nitroprusside and papaverine in basilar arteries

In the blood- and ACSF-injected rats, SIN-1 (0.001–100 μM) and sodium nitroprusside (0.001–100 μM) caused a concentration-dependent relaxation of the arteries constricted with prostaglandin $F_{2\alpha}$ (30–300 μM) (Fig. 3). The difference in the relaxation responses with SIN-1 (Fig. 3a) or sodium nitroprusside (Fig. 3b) between the two groups was not statistically significant. Papaverine (100 μM) fully relaxed the arteries up to the resting level and its potency was similar in the arteries from the ACSF- and the blood-injected animals (Fig. 3b).

3.3. Preventive effect on impairment of acetylcholine-induced relaxation in basilar arteries following subarachnoid hemorrhage

AJ-3941 (0.3 mg/kg, p.o.) significantly prevented ($P < 0.05$) the reduction of acetylcholine-induced relaxation

(1 and 10 μM) on 1 day after blood injection. The extent of the relaxation responses in AJ-3941-treated rats was almost equal to those in the control rats without the blood injection (Fig. 4a). When AJ-3941 (0.1 mg/kg) was given intravenously just before the blood injection, it also prevented ($P < 0.07$) the impairment of acetylcholine-induced relaxation (0.1–10 μM) (Fig. 4b). However, the treatment with nimodipine (0.3 mg/kg, p.o.) had no effect on the impairment of acetylcholine-induced relaxation (Fig. 4a).

3.4. Effect on lipid peroxide levels in blood and basilar artery following subarachnoid hemorrhage

The content of lipid peroxides in the basilar arteries of blood-injected rats (89.8 ± 17.8 pmol/artery) was 49%

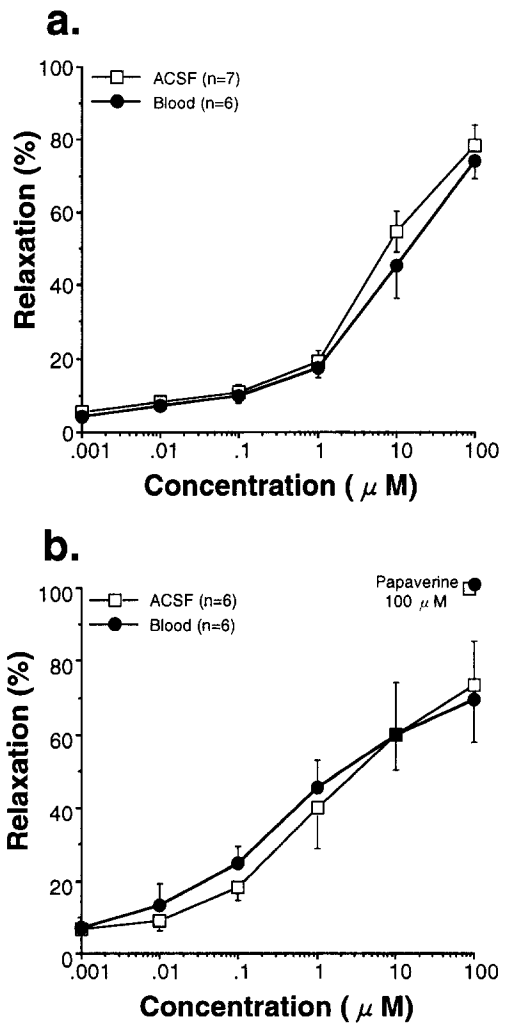

Fig. 3. Concentration-response curves of relaxation responses induced by SIN-1 (a), sodium nitroprusside and papaverine (b) in basilar arteries from ACSF- or blood-injected rats. The relaxation responses induced by SIN-1, sodium nitroprusside or papaverine were examined 1 day after the injection. The arterial rings were first constricted with prostaglandin $F_{2\alpha}$ (30–300 μM) and then SIN-1 or sodium nitroprusside was added cumulatively. Papaverine (100 μM) was finally applied after the addition of sodium nitroprusside. Values represent means ± S.E.M. from 6–7 animals.

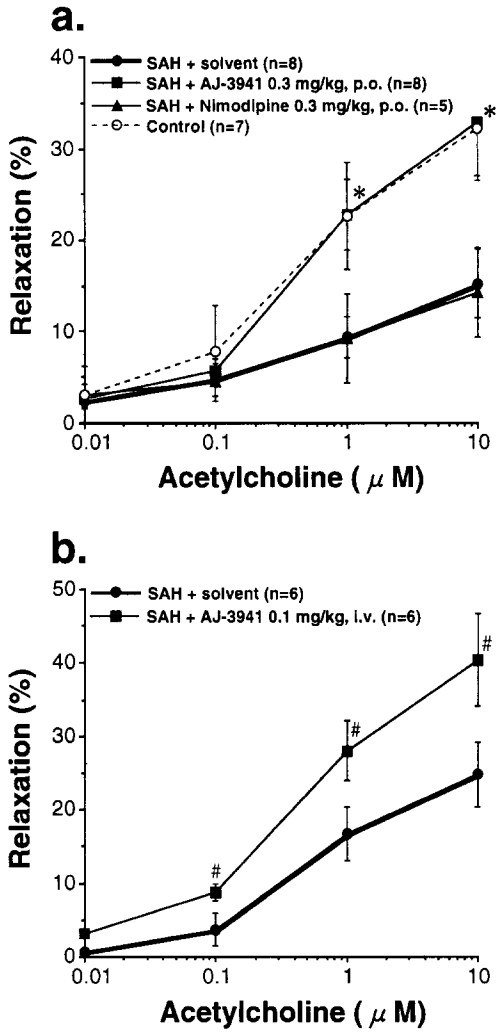

Fig. 4. Effect of AJ-3941 and nimodipine on impairment of acetylcholine-induced relaxation in basilar arteries of subarachnoid hemorrhage rats. The effect on impairment of acetylcholine-induced relaxation was examined 1 day after subarachnoid hemorrhage. (a) For the oral experiment, AJ-3941 or nimodipine was administered p.o. at 2 h or 1 h, respectively, before the injection of blood. (b) For the intravenous experiment, AJ-3941 was administered just before the blood injection. Values represent means ± S.E.M. from 5–8 animals. # $P < 0.07$ and * $P < 0.05$, vs. solvent-treated group.

higher than those of the control rats (60.2 ± 5.5 pmol/artery) on 1 day after blood injection, although there was no significant difference (Table 1, Fig. 5a). However, the elevation of lipid peroxides was not observed at 0.5 h and 5 days after blood injection (Table 1). AJ-3941 (0.3 mg/kg, p.o.) completely inhibited ($P < 0.05$) the increase in arterial lipid peroxide contents, but nimodipine (0.3 mg/kg, p.o.) did not (Fig. 5a). The contents of protein in the basilar artery on 1 day after blood injection were not significantly different between these groups: control (36.4 ± 2.7 μg/artery, $n = 5$), blood injection + vehicle (38.6 ± 2.9 μg/artery, $n = 4$), blood injection + AJ-3941 0.3 mg/kg, p.o. (35.7 ± 1.8 μg/artery, $n = 6$) and blood injection + nimodipine 0.3 mg/kg, p.o. (33.9 ± 4.0

Table 1
Time course of changes in lipid peroxide contents in basilar artery of subarachnoid hemorrhage rats

Groups	Time after subarachnoid hemorrhage		
	0.5 h	1 day	5 days
Control	76.5±9.8 (8)	60.2±5.5 (10)	43.7±5.2 (8)
Subarachnoid hemorrhage	69.0±4.4 (8)	89.8±17.8 (10)	47.9±8.5 (9)
% Change (vs. control)	90.2	149.2	109.6

Subarachnoid hemorrhage was induced experimentally by injection of 0.3 ml homologous arterial blood into the cisterna magna. Numbers in parentheses indicate number of animals used. Lipid peroxide contents were expressed as pmol/artery. Values represent means±S.E.M.

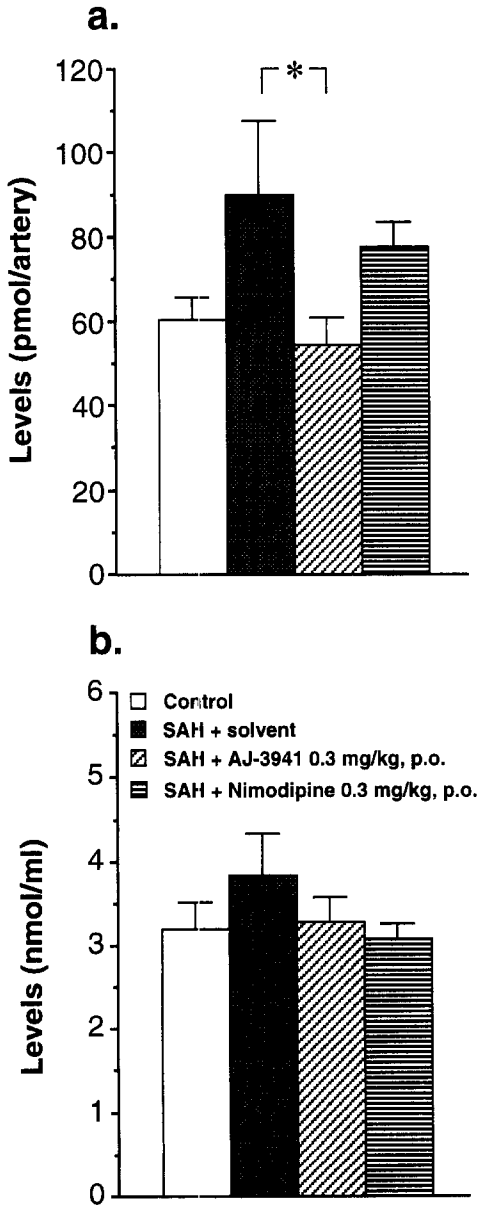

Fig. 5. Effect of AJ-3941 and nimodipine on lipid peroxide levels in basilar artery (a) and arterial blood (b) of subarachnoid hemorrhage rats. The levels of lipid peroxides in blood and basilar artery were measured 1 day after blood injection. Values represent means±S.E.M. from 10 animals. * $P < 0.05$, vs. control group.

μg/artery, $n = 5$). There was no difference in lipid peroxide levels in arterial blood between the groups (Fig. 5b).

4. Discussion

We have recently reported that, in isolated perfused canine basilar artery, intraluminal perfusion of N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of nitric oxide (NO) synthase, markedly amplified the vasoconstriction induced by extraluminal application of potassium chloride or prostaglandin $F_{2\alpha}$. The effect of L-NMMA was completely inhibited by intraluminal perfusion of L-arginine, but not of D-arginine, and was abolished by removal of the endothelium (Minato et al., 1995). These findings suggest that endothelium-derived NO may have great significance for the response to extraluminal vasoactive substances in cerebral artery.

The present study demonstrated that, in the rat basilar artery, experimental subarachnoid hemorrhage suppressed the acetylcholine-induced relaxation response, but failed to inhibit the relaxation responses induced by NO donors such as SIN-1 and sodium nitroprusside, and the phosphodiesterase inhibitor, papaverine, which are well-known as endothelium-independent relaxants. These results are strong indications that the existence of blood or blood clots in the subarachnoid space impairs the endothelium-dependent relaxation (EDR) response in the cerebral artery, and that the impairment is caused by reduction of NO synthesis in the endothelium. Similar results have been reported for cerebral arteries of rabbits (Hongo et al., 1988; Vorkapic et al., 1990), dogs (Kim et al., 1988, 1989, 1992; Katusic et al., 1993) and monkeys (Kanamaru et al., 1989).

The most interesting aspect was that the EDR response was biphasically suppressed by the subarachnoid hemorrhage treatment, with its maximum at 0.5 h and 1 day after blood injection and recovery on day 5. The result was in good agreement with our previous result, where the cerebral vasospasm in the rat subarachnoid hemorrhage model showed a biphasic pattern, which reached its maximum at 10 min and 1 day after blood injection, and diminished on day 5 (Honda et al., 1996). Taking into account these findings and our results for the isolated perfused canine artery, it seems conceivable that the loss of EDR following subarachnoid hemorrhage may be one of the crucial factors in the pathogenesis of cerebral vasospasms.

It has been proposed that one of the endothelium-damaging factors in vessels is an active oxygen species including free radicals (Wedmore and Williams, 1981). In the present study, the contents of lipid peroxide and protein in basilar artery were estimated by microassay and compared with the EDR response. The elevation of lipid peroxide contents in the artery was observed on 1 day after subarachnoid hemorrhage, but not at 0.5 h and day 5. Thus, the impairment of EDR on day 1 was, at least partly,

associated with an increase in lipid peroxide contents in the basilar artery of subarachnoid hemorrhage rats, although the mechanism for the impairment of EDR at 0.5 h is still unclear. These results indicate that one of the mechanisms underlying cerebral vasospasm involves enhancement of the constrictions by suppression of NO synthase activity through endothelial damage, in addition to the sustained constrictions caused by vasospasmogens derived from blood clots. Recently, transient complete cerebral ischemia in rats was shown to induce a biphasic breakdown of blood-brain barrier function, at around 1 h and 6 h to 24 h post-ischemia, because of impairment of endothelial cells by released proteases or oxygen metabolites (Pluta et al., 1994). Accordingly, the increase in arterial lipid peroxide contents after subarachnoid hemorrhage may have resulted from transient ischemia accompanying the cerebral vasospasm.

The treatment with AJ-3941 prevented both the increase in lipid peroxide contents and the reduction of the EDR response in the basilar artery of subarachnoid hemorrhage rats. In connection with lipid peroxidation, we have reported (Masuda et al., 1990) that AJ-3941 exerts a potent anti-lipid peroxidative action (IC_{50} 7.8 μM), as evaluated from the ability to inhibit Fe^{2+}-induced formation of malonyldialdehyde in rat myocardial mitochondria. However, AJ-3941 has no scavenging action on experimentally generated free radicals in a cell-free system (authors' unpublished data). Therefore, the lipid peroxidation-inhibitory effect of AJ-3941 in the subarachnoid hemorrhage rats would result in its direct protection of cell membrane peroxidation. AJ-3941 at the doses used (0.3 mg/kg p.o. and 0.1 mg/kg i.v.) in this study was also reported to prevent the development of cerebral vasospasm in the subarachnoid hemorrhage rats (Honda et al., 1996). These results suggest that the increase in lipid peroxides in the basilar artery has an important role in the impairment of endothelial function, i.e., the EDR response, and concurrently in the genesis of the late vasospasm.

We previously reported (Honda et al., 1996) that nimodipine (0.3 mg/kg, p.o.) prevented the development of cerebral vasospasm in the subarachnoid hemorrhage rats, but that its potency was weaker than that of AJ-3941 (0.3 mg/kg, p.o.). Since the Ca^{2+} blocking activity of AJ-3941 in vitro was reported to be weaker than that of nimodipine (Minato and Masuda, 1991; Minato et al., 1993), the difference between AJ-3941 and nimodipine may be explained by the fact that nimodipine inhibited neither the reduction of the EDR response nor the increase in lipid peroxide content in the basilar artery of subarachnoid hemorrhage rats. Therefore, AJ-3941 may exert its preventive effect on vasospasm through both its anti-lipid peroxidative action and the inhibition of intracellular Ca^{2+} mobilization, while nimodipine's effect may come only from its voltage-dependent Ca^{2+} channel blocking action. However, the possibility remains that inhibition of intracellular Ca^{2+} mobilization through the protein kinase C-dependent pathway by AJ-3941 contributes to the difference in the effect on the cerebral vasospasm of AJ-3941 and of nimodipine (Hashizume et al., 1995).

In conclusion, the EDR response in the basilar artery was biphasically suppressed in the rat subarachnoid hemorrhage model and its time course corresponded to the development of cerebral vasospasm following subarachnoid hemorrhage. These results suggest strongly that the loss of EDR is one of crucial factors in the development of cerebral vasospasm after subarachnoid hemorrhage. The latter suppression of EDR was accompanied by an increase in the arterial lipid peroxide contents. AJ-3941, a cerebrovascular-selective Ca^{2+} channel antagonist having an anti-lipid peroxidative action, prevented not only the suppression of basilar arterial EDR responses but the increase in the contents of arterial lipid peroxides following subarachnoid hemorrhage. Consequently, the vasospasmolytic effect of AJ-3941 in subarachnoid hemorrhage rats could be based on the protective action of endothelium through the anti-lipid peroxidative action, in addition to its inhibitory action of vasospasmogen-induced vasoconstriction related to intracellular Ca^{2+} mobilization. AJ-3941 would be of more benefit in the treatment of cerebral vasospasm than a pure Ca^{2+} channel antagonist because of its multiple mechanisms.

Acknowledgements

We thank Dr. T. Karasawa, Director of Discovery Research Laboratories I, Dainippon Pharmaceutical Co., Ltd., for helpful advice and encouragement.

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