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Endothelial dysfunction and altered bradykinin response due to oxidative stress induced by serum deprivation in the bovine cerebral artery

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Abstract

Oxidative stress plays a critical role in the pathogenesis of vasospasm after a subarachnoid hemorrhage. We demonstrate that 24-h incubation of the isolated bovine middle cerebral arteries in the serum-free media at 37 °C converted the response to bradykinin from relaxation to contraction, in a manner sensitive to free radical scavengers. In the freshly prepared strips, bradykinin induced an endothelium-dependent relaxation, while having no direct effect on the smooth muscle. However, in the strips treated in serum-free media, bradykinin failed to induce endothelium-dependent relaxation, but did demonstrate a direct contractile effect on smooth muscle. The addition of superoxide dismutase and ascorbic acid or 5% serum during the 24-h incubation in the serum-free media prevented the loss of endothelium-dependent relaxation and the development of a contractile response to bradykinin. SB203580 (4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole), a p38 mitogen-activated protein kinase inhibitor, and genistein (4′,5,7-Trihydroxyisoflavone), a tyrosine kinase inhibitor, also demonstrated a similar preventive effect. In conclusion, serum-deprivation induced endothelial dysfunction and the responsiveness of smooth muscle to bradykinin due to failure of eliminating oxidative stress. p38 mitogen-activated protein kinase and tyrosine kinase were suggested to play a critical role in this endothelial dysfunction.

Keywords: Serum-deprivation; Free radical scavenger; Endothelial dysfunction; Bradykinin; p38 MAP kinase; Tyrosine kinase

1. Introduction

Vasospasm is frequently associated with subarachnoid hemorrhage, and it is also one of the critical determinants in the prognosis of this disease (Longstreth et al., 1993). Elucidating the pathogenesis of vasospasm is thus fundamental for developing a new therapeutic strategy toward post-hemorrhagic vasospasm and to improve the prognosis of subarachnoid hemorrhage. The platelets products such as thromboxanes, oxyhemoglobin, and endothelin-1 have been reported to be possible spasmogens (Macdonald and Weir, 1991; Tosaka et al., 2001). However, the mechanism regarding the development of post-hemorrhagic vasospasm still remains to be elucidated. Oxidative stress has been suggested to contribute to the development of vasospasm (Gaetani et al., 1998). In particular, the superoxide anion has been reported to increase in the cerebrospinal fluid in SAH and to correlate to cerebral vasospasm (Mori et al., 2001). The superoxide dismutase was shown to reduce the vasospasm (McGirt et al., 2002; Watanabe et al., 2003). However, how oxidative stress contribute to development of vasospasm remains to be elucidated.

Oxidative stress causes functional alterations in both endothelial cells and smooth muscle, and thereby contributes to the pathogenesis of various vascular diseases, including not only subarachnoid hemorrhage (Kamii et al., 1999), but also hypertension (Cai and Harrison, 2000) and atherosclerosis (Jiang et al., 2000). Oxidative stress causes the rapid inactivation of nitric oxide (NO) (Nakazono et al., 1991), the reduction of NO production and down-regulation of endothelial nitric oxide synthase (eNOS) (Vaziri et al., 2002), and thereby reduces bioavailability of NO in the vascular tissue (Cai and Harrison, 2000). It has also been suggested to stimulate the proliferation and hypertrophy of smooth muscle cells (Griendling and Ushio-Fukai, 1998). Furthermore, it was shown to induce apoptosis and thereby

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cause total loss of cellular function in various types of cells including endothelial cells (de Bono and Yang, 1995; Ichijo et al., 1997). However, the effect of oxidative stress on the contractile responses of the cerebral artery as a consequence of these functional changes in vascular cells, and its cellular mechanism thus remains to be investigated.

The incubation of the rat ovarian follicle cells in the serum-free media was previously reported to induce oxidative stress (Tilly et al., 1992; Tilly and Tilly, 1995). The preliminary experiments demonstrated that the 24-h incubation of the isolated strips of the bovine middle cerebral artery in the serum-free media caused a loss of bradykinin-induced endothelium-dependent relaxation seen in the freshly prepared strips (Miyagi et al., 1996), which was prevented by scavengers. In the present study, we used this serum-deprivation protocol to apply oxidative stress to isolated strips, and investigated the functional alteration of the vascular response to bradykinin and its mechanism. Several kinases such as a p38 mitogen-activated protein (MAP) kinase and tyrosine kinase have been suggested to play a critical role in the oxidative stress-induced cellular dysfunction and apoptosis (Ichijo et al., 1997; Wang et al., 1999). We thus investigated the effects of various kinase inhibitors on the changes in the vascular response induced by incubation in a serum-free media.

2. Materials and methods

2.1. Tissue preparation

Bovine middle cerebral arteries were obtained at a local slaughterhouse and brought to the laboratory in ice-cold normal physiological salt solution (normal PSS). The segments of the middle cerebral artery 1–3 cm from the bifurcation of the internal carotid artery were excised. The arachnoid membranes were carefully dissected away under a microscope with minimal stretch to the smooth muscle, and the arteries were cut into circular strips (1.0 × 4.0 mm). To remove the endothelium, the inner surface of the arteries was rubbed with cotton swab as previously described (Hirano et al., 1990). The arterial segments or circular strips were kept at 4 °C in a glass vial filled with PSS pre-aerated with 5% CO_2 + 95% O_2.

2.2. Treatment of the isolated strips in the serum-free media

The incubation in the serum-free media was demonstrated to cause oxidative stress to the cultured rat ovarian follicles (Tilly and Tilly, 1995). This procedure was utilized to impose oxidative stress to the isolated strips. Namely, the strips were placed in serum-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 unit/ml penicillin and 100 μg/ml streptomycin, and incubated in a CO_2 incubater (Jujikagakukogyo, Tokyo, Japan) under aeration with humidified air + 5% CO_2 at 37 °C for 24 h. When the effects of superoxide dismutase, ascorbic acid, serum and kinase inhibitors on the responsiveness to bradykinin were examined, these agents were added to the media during the 24-h incubation.

2.3. Measurement of tension development

The strips were mounted vertically to a force transducer (TB-612T Nihon Kohden, Tokyo, Japan) in an organ bath, and equilibrated in PSS at 37 °C for at least 1 h before starting measurements, as described previously (Miyagi et al., 1996). During the 1-h equilibration period, the strips were stimulated with 118 mM K^+ every 15 min, and the resting load was increased in a stepwise manner and finally adjusted to 200 mg. This resting load was the minimal load necessary to obtain the maximal tension development with 118 mM K^+ depolarization. The tension was expressed as a percentage, assigning the value in normal PSS (5.9 mM K^+) and that at the steady state of contraction induced by 30 nM U46619 to be 0% and 100%, respectively.

2.4. Drugs and solution

The composition of normal PSS was as follows (mM): NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.25 and D-glucose 11.5. High K^+ PSS was prepared by replacing NaCl with equimolar KCl. PSS was bubbled with a mixture of 95% O_2 and 5% CO_2, with the resulting pH being 7.4. The reagents and venders used in the present study are as follows; bradykinin (Peptide Institute, Osaka, Japan); U46619 (9, 11-dideoxy9α, 11α-methanoepoxy prostaglandin F2α) (Funakoshi, Tokyo, Japan); superoxide dismutase, SB203580 (4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole), a p38 MAP kinase inhibitor (Cuenda et al., 1995), LY294002 (2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), a phosphatidylinositol 3-kinase inhibitor (Vlahos et al., 1994), GF109203X (2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide), a protein kinase C inhibitor (Han et al., 2002), genistein (4′,5,7-Trihydroxyisoflavone), a protein tyrosine kinase inhibitor (Migita et al., 1994) and Y27632 ((*R*)-(+)-*trans*-*N*-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide), a Rho kinase inhibitor (Uehata et al., 1997) (Calbiochem, San Diego, CA, USA); ascorbic acid (Jackson et al., 1998) (Wako, Osaka, Japan); PD98059 (2′-Amino-3′-methoxyflavone), a MAP kinase kinase inhibitor (Alessi et al., 1995) (Biomol Plymouth Meeting, PA, USA).

2.5. Statistical analysis

The values are expressed as the mean ± S.E.M. Student's *t*-test was used to determine statistical significance between the two groups, and an analysis of variance was used to determine the effect of bradykinin. *P* values of less than 0.05 were considered to have statistical significance. All

data were collected using a computerized data acquisition system (MacLab; Analog Digital Instruments, Castle Hill, NSW, Australia, and Macintosh; Apple Computer, Cupertino, CA, USA).

3. Results

3.1. Effect of 24-h incubation in the serum-free media on the contractility of the bovine middle cerebral arterial strips

First, the effect of the 24-h incubation in the serum-free media with and without addition of superoxide dismutase, ascorbic acid, serum or various protein kinase inhibitors on the contractile response to 118 mM K^+ depolarization and 30 nM U46619, a stable analog of thromboxane A_2, was determined. The superoxide dismutase, ascorbic acid, serum or various protein kinase inhibitors were added to the media only during the 24-h incubation in the serum-free media, and no longer existed during the evaluation of the contractile responses. As summarized in Table 1, the contractile responses to both stimulations observed in the freshly prepared strips with or without endothelium did not differ significantly ($P>0.05$) from those seen after the 24-h incubation in the serum-free media with and without superoxide dismutase + ascorbic acid, 5% serum, SB203580 (a p38 MAP kinase inibitor), PD98059 (a MAP kinase kinase inhibitor), LY294002 (a phosphatidylinositol 3-kinase inhibitor), genestein (a tyrosin kinase inhibitor), GF109203X (a protein kinase C inhibitor) or Y27632 (a Rho kinase inhibitor). On the other hand, when added during the U46619-induced sustained contraction, Y27632 (Maeda et al., 2003) and GF109203X (unpublished observation) did inhibit the contraction.

Table 1
The tension developed by 118 mM K^+ and 30 nM U46619 in the strips treated as indicated

Treatment	Endothelium	118 mM K^+ (mg)	U46619 (mg)	n
Freshly prepared strips	+	277 ± 35	390 ± 27	7
	−	296 ± 22	402 ± 22	6
Serum free, 24 h at 37 °C	+	274 ± 33	361 ± 31	9
	−	272 ± 36	405 ± 24	6
+ SOD + ascorbic acid	+	267 ± 45	364 ± 12	3
	−	263 ± 29	363 ± 17	3
+ 5% serum	+	277 ± 44	381 ± 20	3
+ SB203580	+	272 ± 44	371 ± 10	3
+ PD98059	+	269 ± 22	372 ± 27	3
+ LY294002	+	278 ± 18	393 ± 29	3
+ genistein	+	276 ± 39	332 ± 16	3
+ GF109203X	+	303 ± 31	427 ± 43	3
+ Y27632	+	294 ± 20	441 ± 32	3

The strips (1.0 × 4.0 mm) were prepared as described in Materials and methods. The strips either with or without endothelium were incubated in the serum-free DMEM for 24 h at 37 °C, either with or without 500 unit/ml superoxide dismutase (SOD) + 1 mM ascorbic acid, 5% serum, 10 μM SB203580, 10 μM PD98059, 10 μM LY294002, 10 μM genistein, 10 μM GF109203X or 10 μM Y27632. After a 24-h incubation, the strips were mounted onto the force transducer and equilibrated in the normal PSS containing no superoxide dismutase, ascorbic acid, serum or protein kinase inhibitors, and the resting tension was adjusted to 200 mg. Thereafter, the response to 118 mM K^+ depolarization and 30 nM U46619 was evaluated in the absence of superoxide dismutase, ascorbic acid, serum or protein kinase inhibitors. The wet weight of the strips (0.96 ± 0.03 mg) did not significantly differ under the different conditions. The data are the mean ± S.E.M.

3.2. Incubation in the serum-free media converted the response to bradykinin from relaxation to contraction in the bovine middle cerebral artery

In the freshly prepared strips of the bovine middle cerebral artery, 30 nM U46619 induced a sustained contraction both in the presence and absence of the endothelium (Fig. 1A). In both cases, the tension developed to a steady state level within 10 min and stayed at this level (390 ± 27 mg, $n=7$ in the presence of endothelium; 402 ± 22 mg, $n=6$ in the absence of endothelium) for more than 30 min. During the U46619-induced steady state of contraction and in the presence of endothelium, application of 100 nM bradykinin induced a transient relaxation, reaching a maximal relaxation (36.9 ± 7.8% of the level of the U46619-induced precontraction, $n=3$) at 0.89 ± 0.2 min ($n=3$) after the application of bradykinin. The tension returned to the precontraction level seen just prior to the application of bradykinin at 17.6 ± 1.8 min ($n=3$), and the level of tension at 20 min (100.3 ± 3.1%, $n=3$) was the same as the pre-application level. On the other hand, bradykinin did not induce any change in tension during the U46619-induced contraction in the absence of endothelium. As a result, in the freshly prepared strips, bradykinin induced an endothelium-dependent relaxation, but had no direct contractile or relaxing effect on the smooth muscle in the bovine middle cerebral artery. The bradykinin-induced endothelium-dependent relaxation similar to that seen in the freshly prepared strips was observed even after a 24-h storage of the strips at 4 °C in the normal PSS or DMEM without serum pre-aerated with 5% CO_2 + 95% O_2 (data not shown).

When the strips with endothelium were incubated in the serum-free DMEM in a CO_2 incubator at 37 °C for 24 h, both 118 mM K^+ and U46619 induced sustained contractions similar to those observed in the freshly prepared strips (Table 1). The level of the U46619-induced contraction was 361 ± 31 mg ($n=9$) in the presence of endothelium and 405 ± 24 mg ($n=6$) in the absence of endothelium. During the U46619-induced contraction, however, bradykinin did not induce relaxation but contraction in these strips (Fig. 1A). On the other hand, when the strips without endothelium were incubated in the serum-free DMEM at 37 °C for 24 h, bradykinin induced a transient contraction similar to that observed in the strips with endothelium treated in the serum-free media (Fig. 1A). The extent of the contraction obtained without endothelium (163.9 ± 7.0 %, $n=3$) did not significantly ($P>0.05$) differ from that obtained with endothelium (170.5 ± 14.1 %, $n=4$) (Fig. 1B). The 24-h incu-

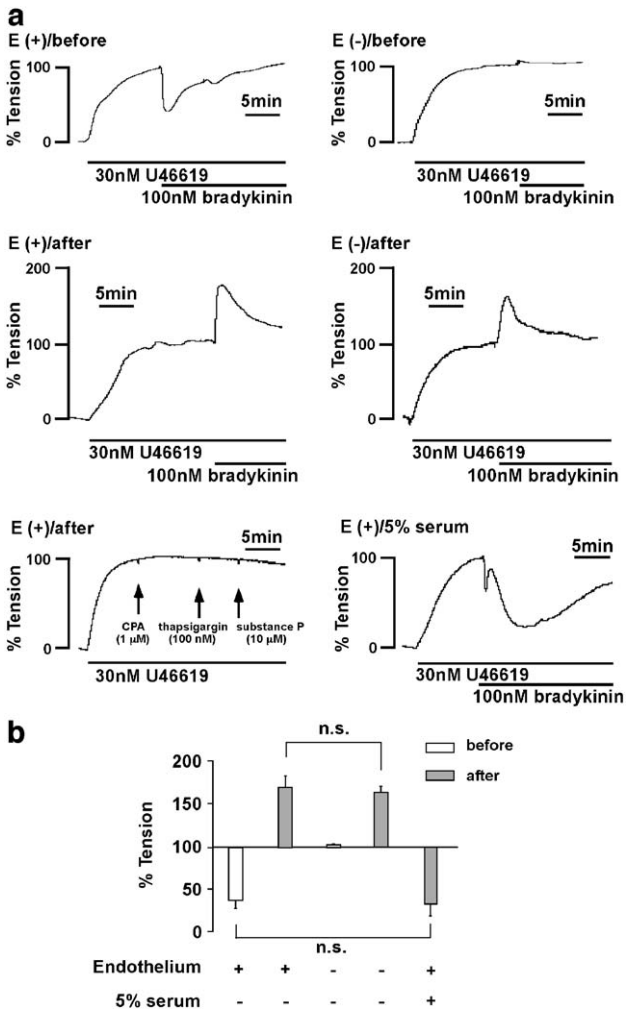

Fig. 1. The changes in the response mode to bradykinin after a 24-h treatment with the serum-free media in the bovine middle cerebral artery with and without endothelium. (A) Representative recordings showing the effects of 100 nM bradykinin on the U46619-induced contraction before (Traces on the top) and after (2nd traces) the 24-h incubation of the strips with (E(+)) and without (E(−)) endothelium in the serum-free media at 37 °C. The endothelium was removed before incubation in the serum-free media. The third trace; a representative recording showing the effect of 100 nM bradykinin on the U46619-induced contraction in the strips with endothelium treated with the media containing 5% serum (right), and a representative recording showing the effects of 10 μM substance P, 100 nM thapsigargin and 1 μM cyclopiazonic acid (CPA) on the U46619-induced contraction after the 24-h incubation of the strips with endothelium in the serum-free media at 37 °C (left). The levels of tension at rest and those at a steady state of contraction induced by 30 nM U46619 just prior to application of bradykinin were assigned to be 0% and 100%, respectively. (B) Summary of the response to bradykinin in the strips with and without endothelium and before and after incubation in the serum-free media and in the 5% serum-containing media at 37 °C for 24 h. Each column represents the level of tension obtained at the maximal response to bradykinin, or at 1 min after the application of bradykinin, in case no apparent response to bradykinin was observed, during the steady state contraction induced by 30 nM U46619. The data are the mean ± S.E.M. ($n = 3–5$).

bation in the serum-free media at 37 °C thus eliminated the bradykinin-induced endothelium-dependent relaxation, while inducing a direct contractile response of smooth muscle toward bradykinin. Substance P, thapsigargin and cyclopiazonic acid induced an endothelium-dependent relaxation in the freshly prepared strips, while these relaxations were all eliminated after 24-h incubation in the serum-free media, and no contractile effect on smooth muscle was observed with these stimulations (Fig. 1A). Serotonin induced no endothelium-dependent relaxation but only contraction in the freshly prepared strips. This contractile response was similarly observed in the strips treated in the serum-free (data not shown).

3.3. Prevention of the loss of the bradykinin-induced relaxation by serum supplementation during 24-h incubation of the bovine middle cerebral artery

When the strips with endothelium were incubated for 24 h at 37 °C in the 5% serum-containing DMEM, bradykinin induced a substantial relaxation (Fig. 1A). The extent of the maximal relaxation (31.2 ± 13.8%, $n = 3$) was similar ($P > 0.05$) to that obtained in the freshly prepared strips (Fig. 1B). However, the level of tension obtained at 20 min after the application of bradykinin (79.2 ± 10.2%) was significantly ($P < 0.05$) lower than the level obtained just prior to the application of bradykinin. On the contrary, addition of 5% serum did not demonstrate any preventive effects on the bradykinin-induced relaxation when it was applied to the strips after the 24-h treatment in the serum-free DMEM and just before starting the tension measurements (data not shown).

3.4. Prevention of the loss of the bradykinin-induced relaxation by superoxide dismutase and ascorbic acid after the 24-h incubation of the strips in the serum-free media

When the strips with endothelium were incubated for 24 h at 37 °C in the serum-free DMEM supplemented with 500 units/ml superoxide dismutase and 1 mM ascorbic acid, bradykinin induced a transient relaxation but no apparent contraction (Fig. 2A). On the other hand, when the strips without endothelium were incubated for 24 h at 37 °C under the same condition, bradykinin did not induce any contraction or relaxation (Fig. 2A). The maximal relaxation (32.5 ± 3.9%, $n = 3$) induced by 100 nM bradykinin in the strips with endothelium incubated with superoxide dismutase and ascorbic acid was similar ($P > 0.05$) to that observed in the freshly prepared strips (Fig. 2B). However, the level of tension obtained at 20 min after the application of bradykinin (76.0 ± 14.0%) was significantly ($P < 0.05$) lower than that of the level obtained just prior to the application of bradykinin. As a result, the duration of the relaxation observed in the strips treated with superoxide dismutase and ascorbic acid was longer than that seen with the freshly prepared strips. On the contrary, superoxide dismutase and ascorbic acid did not demonstrate any preventive effects on the bradykinin-induced relaxation when they were applied to the strips after the 24-h treatment in the serum-free

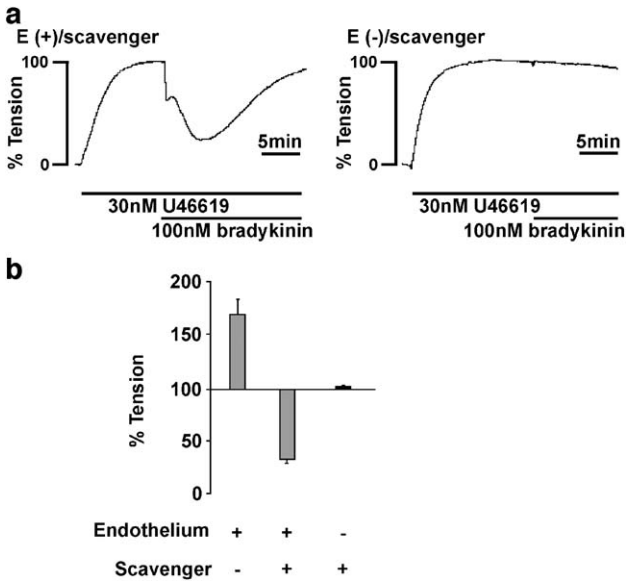

Fig. 2. Effects of superoxide dismutase and ascorbic acid on the alteration of the response to bradykinin after the 24-h incubation in the serum-free media. (A) Representative recordings showing the effect of 100 nM bradykinin on the U46619-induced contraction in the strips with (E(+)) and without (E(−)) endothelium treated with the serum-free media containing 500 units/ml superoxide dismutase and 1 mM ascorbic acid (scavenger). The levels of tension at rest and those at a steady state of contraction induced by 30 nM U46619 just prior to the application of bradykinin were assigned to be 0% and 100%, respectively. (B) Summary of the effects of 500 units/ml superoxide dismutase and 1 mM ascorbic acid (scavenger) on the alteration of the response to bradykinin after 24-h incubation in the serum-free media in the strips with and without endothelium. Each column represents the level of tension obtained at the maximum response to bradykinin, or at 1 min after the application of bradykinin, in case no apparent response to bradykinin was observed, during the steady state contraction induced by 30 nM U46619. The data are the mean ± S.E.M. ($n = 3$).

DMEM and just before starting the tension measurements (data not shown).

3.5. Effects of kinase inhibitors on the arterial responsiveness to bradykinin after the 24-h incubation in the serum-free media

To elucidate the protein kinases involved in conversion of the relaxing response to bradykinin to the contractile response, we investigated the effects of kinase inhibitors on the response to bradykinin in the strips treated with the serum-free media (Fig. 3). The strips were treated with the inhibitors during 24-h incubation in the serum-free DMEM, and the inhibitors were no longer present during the tension measurement. Among the inhibitor tested, 10 μM SB203580 and 10 μM genistein prevented a loss of the bradykinin-induced relaxation and induction of a contractile response in the strips with endothelium (Fig. 3A). The extent of the maximal relaxation obtained in the strips treated with SB203580 (43.3 ± 2.4%, $n = 3$) and genistein (56.8 ± 10.9%, $n = 3$) was similar ($P > 0.05$) to that obtained with the freshly prepared strips (Fig. 1B) or with the strips treated with either superoxide dismutase plus ascorbic acid or serum (Fig. 2B). However, the level of tension obtained at 20 min after the application of bradykinin in the strips treated with SB203580 (87.4 ± 2.2, $n = 3$) but not genistein (96.3 ± 7.2, $n = 3$) was significantly ($P < 0.05$) lower than that of the level obtained

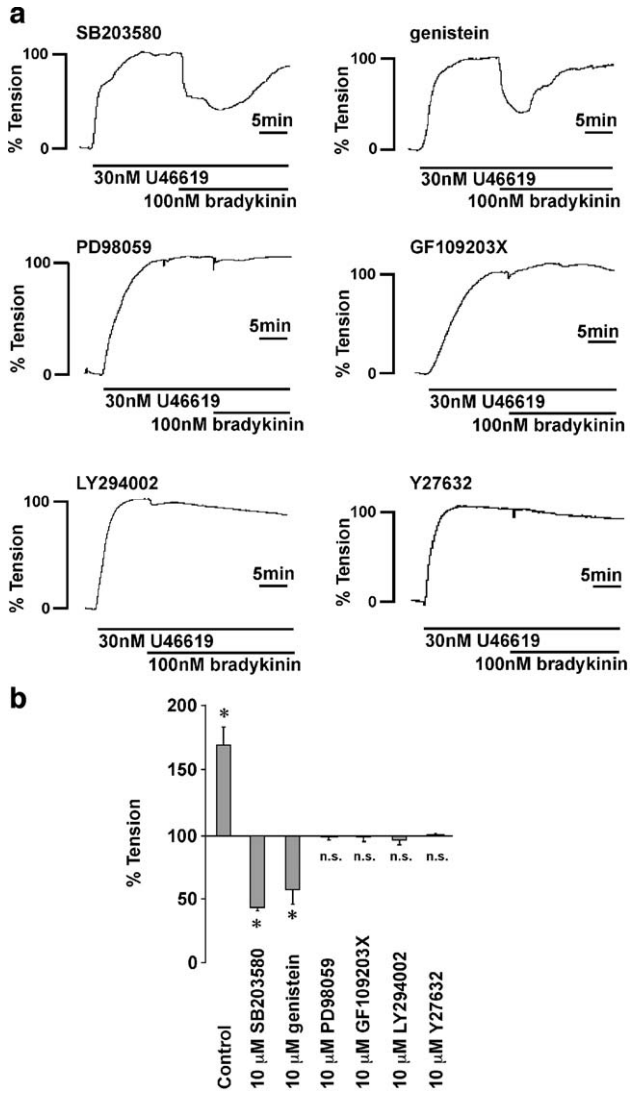

Fig. 3. Effects of kinase inhibitors on the alteration of the response to bradykinin after the 24-h incubation in the serum-free media. (A) Representative recordings showing the effect of 100 nM bradykinin on the U46619-induced contraction in the strips with endothelium treated in the serum-free media containing 10 μM SB203580, 10 μM genistein, 10 μM LY294002, 10 μM GF109203X, 10 μM PD98059 and 10 μM Y27632 for 24 h at 37 °C. The levels of tension at rest and those at steady state of contraction induced by 30 nM U46619 just prior to application of bradykinin were assigned to be 0% and 100%, respectively. (B) Summary of the effects of kinase inhibitors on the alteration of the response to bradykinin after the 24-h incubation in the serum-free media in the strips with and without endothelium. Each column represents the level of tension obtained at the maximum response to bradykinin or at 1 min after the application of bradykinin, in case no apparent response to bradykinin was observed. The data are the mean ± S.E.M. ($n = 3$). *, significantly ($P < 0.05$) different from the 100% level; n.s., not significantly ($P > 0.05$) different from the 100% level.

just prior to the application of bradykinin. In the strips treated with the other inhibitors at 10 μM, bradykinin did not induce any significant (P>0.05) relaxation or contraction both in the strips with endothelium (Fig. 3B) and those without endothelium (data not show). All the inhibitors used in the present study were dissolved in dimethyl sulfoxide, and the final concentration of dimethyl sulfoxide was no higher than 0.1%. Treatment with 0.1% dimethyl sulfoxide had no effect on the loss of bradykinin-induced relaxation and induction of the contractile response to bradykinin seen in the strips exposed to the serum-free media (data not shown).

4. Discussion

We herein demonstrated that the 24-h incubation of the bovine middle cerebral artery with endothelium in the serum-free media abolished the endothelium-dependent relaxation in response to bradykinin and instead induced contractile response to bradykinin. The loss of endothelium-dependent relaxation was prevented, and no contractile response was induced, when scavenger (superoxide dismutase and ascorbic acid) or serum was added during the 24-h incubation in the serum-free media. Inhibition of either p38 MAP kinase or tyrosine kinase prevented the loss of the endothelium-dependent relaxation and inhibited the induction of the contractile reponse to bradykinin. It is thus suggested that the 24-h incubation in the serum-free media caused oxidative stress, which converted the relaxing response to the contractile response toward bradykinin in the bovine middle cerebral artery. The exposure to the serum-free media has been reported to cause oxidative stress as well as a depletion of mitogenic stimuli (Tilly and Tilly, 1995). To our best knowledge, this is the first report demonstrating that oxidative stress due to serum-deprivation induced the conversion of the vascular response to a same stimulus from relaxation to contraction.

The contractile response to bradykinin observed after a 24-h incubation in the serum-free media was not dependent on endothelium, but was due to the direct effect on smooth muscle. However, the contractile responses of smooth muscle to 118 mM K^+ and U46619 (Table 1) and serotonin were not altered by the serum-deprivation. The relaxing response to substance P was lost in the strips treated with serum-free media, while no contractile response to substance P was observed. Therefore, the induction of contractile response appeared to be specific to bradykinin. It is thus most likely that the induction of the contractile response to bradykinin was due to the induction of bradykinin receptor in smooth muscle. However, the precise mechanism regarding the induction of contractile response in smooth muscle remains to be determined.

On the other hand, the loss of endothelium-dependent relaxation is not only observed with bradykinin but also with substance P, thapsigargin and cyclopiazonic acid. The endothelium-dependent relaxations induced by bradykinin, substance P, thapsigargin and cyclopiazonic acid were shown to be mediated by not only NO but also hyperpolarization (Higuchi et al., 1996; Kuroiwa-Matsumoto et al., 2000; Ohnishi et al., 2001). The endothelial function to produce relaxing factors thus appeared to be totally lost after 24-h incubation with serum-free media. The observation that the scavenger prevented the loss of endothelium-dependent relaxation suggests the involvement of oxidative stress in the endothelial dysfunction. Oxidative stress has been reported to decrease the bioavailability of NO in vascular tissue (Cai and Harrison, 2000). The decline in NO availability is mainly due to the enhanced metabolism of NO by radicals such as O_2^- (Nakazono et al., 1991). Furthermore, oxidative stress is considered to oxidize tetrahydrobiopterin, a cofactor of eNOS, and cause the ‘‘uncoupling of eNOS’’ (Rubanyi and Vanhoutte, 1986). In this situation, the production of NO by eNOS is impaired, and the production of O_2^- and H_2O_2 is augmented (Katusic, 2001; Pou et al., 1992). The uncoupling of eNOS is thus suggested to cause not only the endothelial dysfunction but also an augmentation of oxidative stress. However, in the present study, the endothelium-dependent relaxation was totally lost, thus indicating that the production of not only NO but also other relaxing factors was lost.

The prevention of the endothelial function by scavenger, p38 MAP kinase inhibitor and tyrosine kinase inhibitor suggested p38 MAP kinase and tyrosine kinase to contribute to the oxidative stress-induced total loss of endothelial function. On the other hand, oxidative stress was reported to cause apoptosis in many cell types including endothelial cells via an apoptosis signal-regulating kinase 1 (ASK1)-p38 MAP kinase pathway (de Bono and Yang, 1995; Ichijo et al., 1997). The total loss of the endothelial function due to oxidative stress is thus consistent with the apoptotic effect of oxidative stress. However, this possibility remains to be examined.

It is noteworthy that the bradykinin induced a greater relaxation in the strips incubated in the serum-containing media than that seen in the freshly isolated strips. The similar enhancement was observed in the strips treated with free radical scavenger and p38 kinase inhibitor. These observations suggest that the endothelial function including production of the endothelium-derived relaxing factors and their bioavailability, or viability of the endothelial cells of the freshly prepared strips were somewhat impaired by the basal oxidative state. The scavenger, serum, or p38 kinase inhibitor might have eliminated the basal oxidative stress, improved the endothelial function, and thus enhanced the bradykinin-induced relaxing response. However, the precise mechanism of this enhancement remains to be determined.

In conclusion, we herein demonstrated that the 24-h incubation of the bovine middle cerebral artery in the serum-free media caused a total loss of endothelium-dependent relaxation, while also inducing a contractile response to bradykinin, in a manner sensitive to scavenger or serum. It is suggested that serum-deprivation induced the oxidative

stress, and thereby caused endothelial dysfunction and up-regulated bradykinin receptor in smooth muscle. p38 MAP kinase and tyrosin kinase is suggested to play a critical role in the endothelial dysfunction. As a result, the vascular response to bradykinin was converted from relaxation to contraction by serum-deprivation in the bovine middle cerebral artery.

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