DOCTORAL THESIS

Study on the pharmacological characteristics of the basilar artery

（脳底動脈の薬理学的特徴に関する研究）

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Contents

ABSTRACT

The present thesis was designed to study the responsiveness of the mouse basilar artery (MBA) and porcine basilar artery (PBA) to intrinsic vasoactive substances in physiological and some induced pathological conditions.

In MBA, acetylcholine (ACh), bradykinin (BK), noradrenaline (NA), 5-hydroxytryptamine (5-HT), histamine (His) and angiotensin (Ang) II-induced vascular responses were characterize with their related receptor subtypes *in vitro*. Functional studies of the isolated arteries to vasoactive substances were performed by micro-organ-bath system. ACh and BK induced endothelium-dependent relaxation. Atropine (a non-selective muscarinic antagonist) and a nitric oxide (NO) synthase inhibitor, *Nω*-nitro-L-arginine (L-NNA) but not muscarinic M_1, M_2 and M_3 antagonists shifted the concentration-response curve for ACh to the right. B_2 antagonist and L-NNA shifted the concentration-response curve for BK to the right, whereas B_1 antagonist and indomethacin (a cyclooxygenase inhibitor) had no significant effect. NA failed to produce any vasomotor action. His and Ang II induced concentration-dependent contraction. H_1 but not H_2 antagonist shifted the concentration-response curve for His to the right. AT_1 but not AT_2 antagonist shifted the concentration-response curve for Ang II to the right. These results suggest that the H_1 and AT_1 receptor subtypes play an important role in arterial contraction, whereas muscarinic receptors apart from M_1, M_2 and M_3, and B_2 receptors modify these contractions to relaxations.

Methylmercury (MeHg) increased the risk of hypertension and cardiovascular diseases. Therefore, in the subsequent study, I investigated the responsiveness of the MBA to Ang II and ACh in *in vivo* MeHg exposed mice. Mice were exposed to MeHg (40 ppm) in drinking water for 21 days. Blood pressure was measured in conscious mice by an indirect tail-cuff method. Systolic and mean blood pressures were significantly increased after 2 and 3 weeks of treatment with MeHg, respectively. Ang II-induced contraction in an isolated basilar artery, which is mediated via Rho-kinase activation, was increased in MeHg-treated mice. ACh-induced relaxation, which is dependent on NO production from the endothelial cells, was decreased in MeHg-treated mice. However, alterations of vascular responses to Ang II and ACh were not observed in the isolated thoracic aorta. Concomitant treatment with tempol, a reactive oxygen species (ROS) scavenger, suppressed MeHg-induced increase in blood pressure, enhanced Ang II-induced contraction and decreased ACh-induced relaxation in MeHg-treated mice. These results suggest that *in vivo* MeHg exposure increases blood

pressure and causes alterations in the cerebrovascular reactivity in response to Ang II and ACh through ROS generation in mice.

In PBA, BK induced relaxation and contraction via activation of endothelial B_2 receptors. Previously, NO and prostaglandin (PG) H_2 were suggested the relaxing and contracting factors respectively, but PGH_2 has different functionally active isoforms, including PGD_2, PGE_2 and $PGF_{2\alpha}$. Therefore, I attempted to quantify NO and specific PG production from cultured porcine basilar arterial endothelial cells (PBAECs). The cultured PBAECs produced NO spontaneously, and BK enhanced this production in a concentration-dependent manner. In a functional study, PGD_2, PGE_2 and $PGF_{2\alpha}$ induced concentration-dependent contractions in isolated PBA rings. The cultured PBAECs produced PGD_2, PGE_2 and $PGF_{2\alpha}$ spontaneously, and BK significantly enhanced the production of $PGF_{2\alpha}$, but not that of PGD_2 and PGE_2. The B_2, but not B_1, antagonist completely abolished the BK-enhanced production of both NO and $PGF_{2\alpha}$. These results suggest that BK induces production of NO and $PGF_{2\alpha}$ simultaneously from PBAECs via B_2 receptor activation.

Ang II is known to promote vascular disease and hypertension by its effect on vascular endothelium and affect vascular activity to endothelial dependent vasoactive substances. Hence, this study also evaluated the effect of intravenous infusion of Ang II (200 ng. kg^{-1} min^{-1} for 14 days) on the basilar arterial responsiveness to the endothelium-dependent agonist, in microminipig (MMPig). BK-induced relaxation was abolished and contraction was significantly enhanced in isolated basilar artery from Ang II-infused MMPig. Then the PBAECs were cultured and stimulated with Ang II (10^{-7} M) or vehicle for 48 hours. BK-induced NO production was significantly decreased but $PGF_{2\alpha}$ production was increased in Ang II stimulated PBAECs. These results suggest that the decreased NO and increased $PGF_{2\alpha}$ productions from the endothelial cells are the causes of altered BK response of the isolated basilar artery in Ang II treated MMPig.

In conclusion, *in vivo* exposure of MeHg and Ang II cause endothelial dysfunction of the MBA and PBA respectively. The endothelium-derived NO is decreased and the Rho-kinase activity of the vascular smooth muscle is augmented in MeHg exposed mice. On the other hand, the production of BK-induced endothelium-derived NO (relaxing factor) is decreased and endothelium-derived $PGF_{2\alpha}$ (contracting factor) is increased by Ang II in PBAECs. ROS generation might play an important role in the dysfunction of the endotheial cells caused by MeHg and Ang II exposed mice and MMPig.

General introduction

The brain is critically dependent on a continuous and well-regulated blood supply to support its dynamic needs for oxygen and glucose and to remove metabolic by-products of brain activity [83]. Cerebral blood flow (CBF) is highly regulated, involving multiple coordinated mechanisms. This regulation includes the integration of both regional and segmental changes in vascular tone, as well as major interactions between different cell types. Maintaining adequate supply of oxygen and energy to the brain in order to meet the metabolic demands despite the varying neuronal activity and circulatory alterations of everyday life is a key requirement for the regulation of CBF. The close interplay of flow and function is demonstrated by the fact that most, if not all, brain diseases are associated with changes in CBF, either as a cause or as a consequence of brain dysfunction. CBF is provided by two main pairs of arteries: the vertebral arteries, and the internal carotid arteries. The vertebral arteries arise from the subclavian arteries and unite to form the basilar artery.

The basilar artery runs along the ventral aspect of the medulla oblongata and bifurcated terminally as the posterior cerebral arteries, which connect to the internal carotid arteries by posterior communicating arteries and supplies oxygenated blood to the cerebellum, brain stem, and occipital lobes. Studies of basilar artery is important because this artery is one of the major resistance vessels in the brain [29, 30].The responsiveness of this artery seems to reflect changes in CBF and local microvascular pressure. In addition, this artery is segment of the vasculature where some of the most important clinical complications of vascular disease occur. Interruption of the blood flow through the basilar artery can lead to severe brain damage, organ malfunction, or death. The unique features of the cerebral circulation make it difficult to extrapolate findings from peripheral blood vessels. The cerebral arteries are more productive and greatly influenced by vasoactive substances than others which make them vulnerable to pathological condition. Several endogenous factors with strong vasoregulatory properties produced either locally or carried by blood to the basilar artery have been implicated in the local control of CBF. In the response to a number of pathophysiologic conditions including atherosclerosis, strokes or hypertension, alteration of vasoregulatory properties of endogenous factor have been reported [24, 25, 121].

Mice are widely considered to be a prime model of inherited human disease and share

99% of their genes with humans [116]. Their genetic, biological and behavioral characteristics are closely resembled to those of humans, and many symptoms of human conditions can be replicated in mice. They are the most commonly used vertebrate species, because of their availability, size, low cost, ease of handling, high reproduction rate, the ability to compare well-documented genetic backgrounds, and the ability to delete or overexpress specific genes. The routine availability of mouse models of various cerebral circulatory disorders like hypertension, Alzheimer's disease, migraine and stroke [7] requires pharmacological characterization of the regulation of basilar arterial tone in physiological condition which would helpful in management of pathological condition.

Similarly an appropriate non-rodent animal model that reproduces human physiology and pathology would also be ideal for investigating human diseases. Swine represent a potentially useful non-rodent animal model because, their anatomy, genetics, physiology and habits of feeding and sleep are very similar to those of human. Most evolutionary models favor closer phylogenetic relationship between pigs and humans. Pigs and humans shared a common ancestor 90 million years ago [39]. Porcine protein has the same amino acid that is implicated in a human disease such as obesity, diabetes, Parkinson`s disease and Alzheimer`s diseases [39]. The microminipig (MMPig) has recently been established as an experimental animal. This is the world's smallest pig, which has very close similarities to human with respect to lipid metabolism and atherogenesis [58]. It is well known that porcine models often relate well to human conditions, [71] and of the major cerebral arteries in non-primates; the basilar artery is perhaps most like that in humans [37].

Therefore, the present thesis characterize the responsiveness of basilar artery in a rodent (mice) and a non-rodent (pig) animal which share a close evolutionary and genetic similarities with human to intrinsic vasoactive substances.

Acetylcholine (ACh), bradykinin (BK), 5-hydroxytryptamine (5-HT), noradrenaline (NA), angiotensin (Ang) II, histamine (His), nitric oxide (NO) and prostaglandins (PGs) are intrinsic vasoactive substances which play important role in maintaining vascular homeostasis. Moreover, it has been reported that the responsiveness of this artery to intrinsic vasoactive substances varied among the different species of animals.

Species differences in basilar arterial responsiveness to intrinsic vasoactive substances have been reported. Some of the species specific responses are very unique and

characteristic. For example, NA, a well-known vasoconstrictor, induces contraction of the basilar artery in dogs [107], monkeys [103], guinea pigs [19] and rabbits [28], whereas it induces relaxation in that of cattle [6] and pigs [79]. The intensity of relaxation in pigs is much greater than that in cattle, and this larger relaxation induced by NA is one of the characteristics of PBA. In other case, BK, which is a well-known vasorelaxing factor, induces relaxation in human basilar artery, but induces very strong contraction in equine basilar artery [106]. The contraction induced by BK is greater than that induced by NA, His or 5-HT, and this is also one of the characteristics of the equine basilar artery. Basilar arterial responsiveness to these vasoactive substances in one species of animal has never been similar to that of other species.

On the other hand, many acute and chronic medical conditions such as hypertension, diabetes, obesity, atherosclerosis and toxic substances such as MeHg or metallic mercury and lead are associated with impaired or lost vascular regulatory function, eventually leading to impaired functionality. Among them, the present thesis aimed to study the effect of MeHg in mice and Ang II-induced hypertension in porcine basilar artery.

MeHg is a potent environmental toxic pollutant that is generated by methylation of inorganic mercury. It is a well-documented neurotoxicant in both humans and experimental animal models [15]. The central nervous system is the main target organ of MeHg toxicity, especially when exposure occurs during the early stages of brain development. Even though the developing brain has been considered the critical target organ of MeHg toxicity in children, recent evidence indicates that the cardiovascular system may be a sensitive organ in adults [21]. It has also been reported that, in humans, cardiovascular outcomes of MeHg exposure include myocardial infarction [90], heart rate variability, atherosclerosis, coronary heart disease, and hypertension [111].

The harmful effects of mercury are mediated by the excessive release of reactive oxygen species (ROS) [128] and the vascular endothelium is highly sensitive to oxidative stress [121]. Oxidative stress caused by mercury exposure decreases the bioavailability of NO and alters the expression of endothelial NO synthase, which result in increased vasoconstriction and the reduction of the endothelial vasodilator response [34, 121]. ROS is also able to activate vascular Rho-kinase which is an important regulator of several vasoconstrictors [31]. In spontaneously hypertensive rats, augmented contribution of Rho-kinase activity via ROS generation in cerebral vasculature has been reported [22].

Together, these studies suggest that ROS plays an important role in the regulation of vascular constriction and relaxation through augmentation of Rho-kinase activity and a decrease in NO availability.

So the chapter 1, study 2 of this thesis, investigated the effect of MeHg exposure on the cardiovascular system, by analyzing blood pressure and vascular responses to Ang II and ACh, using mice treated with MeHg *in vivo*. Ang II is a potent vasoconstrictor that induces contraction via activation of the Rho-kinase signaling pathway [31] and ACh-induced endothelium-dependent and NO-mediated relaxation [25, 50].

In the porcine basilar artery (PBA), BK is an important intrinsic vasoactive substance which potentially contribute to cerebral autoregulation, as BK induces relaxation followed by contraction via B_2 receptors on endothelial cells [74, 75]. We previously reported that the main EDCF of BK in the PBA might be PGH_2 and that the endothelium-derived relaxing factor (EDRF) may be NO [75]. However, there is no direct or quantitative evidence of BK-induced NO production from cultured basilar arterial endothelial cells from animals of any species. Moreover, PGH_2 is converted to biologically active vasoconstrictive PGE_2, PGD_2, $PGF_{2\alpha}$ and TXA_2, and there is no information to indicate which of these specific PGs evokes contraction in response to BK. Therefore, the goal of the chapter 2, study 1 of this thesis was to confirm quantitatively that BK enhances NO production as an EDRF, and to quantify and specify the PG isoform responsible for BK-induced contraction using cultured PBAECs. It was anticipated that quantitative and specific analysis of NO and PGs might provide a useful index of physiological, pharmacological and pathological mechanisms operating in the cerebral circulation.

On the other hand, BK is a proinflammatory peptide that mediates a variety of physiological and pathophysiological responses [38, 63]. BK increases during inflammation, because of augmented release from inflammatory cells, including macrophages, neutrophils, and monocytes [62] and might disrupt vascular homeostasis.

The renin-angiotensin system, and particularly Ang II underlie many of the changes in vascular structure and function that occur in animal's models of hypertension as well as patients with hypertension. It produces cerebrovascular inflammation and remodeling, and impairs CBF regulation [27]. Intravenous infusion of Ang II acutely increases blood pressure, levels of ROS [13, 14], and impairs vascular function including endothelium

dependent responses in brain [13, 14] or enhancement of vasoconstrictor response to different agonists [110]. Moreover, It has also reported that Ang II stimulate the activity of plasma prekallikrein and release of BK from the vascular endothelium [131]. So the chapter 2, study 2 of the present thesis hypothesized that Ang II would alter endothelium-dependent biphasic response of BK in PBA by altering BK production or BK induced NO and $PGF_{2\alpha}$ production from the basilar artery endothelial cells.

Chapter 1

Study 1

Vasomotor effects of acetylcholine, bradykinin, noradrenaline, 5-hydroxytryptamine, histamine and angiotensin II on the mouse basilar artery

1. ABSTRACT

We investigated the responsiveness of the mouse basilar artery to acetylcholine (ACh), bradykinin (BK), noradrenaline (NA), 5-hydroxytryptamine (5-HT), histamine (His) and angiotensin (Ang) II in order to characterize the related receptor subtypes *in vitro*. ACh and BK induced endothelium-dependent relaxation of precontracted arteries with U-46619 (a thromboxane A_2 analogue). Atropine (a non-selective muscarinic receptor antagonist) and *Nω*-nitro-L-arginine (a NO synthase inhibitor, L-NNA) shifted the concentration-response curve for ACh to the right, whereas pirenzepine, methoctramine and pFHHSiD (muscarinic M_1, M_2 and M_3 antagonists, respectively) had no significant effect. L-NNA and HOE140 (a B_2 antagonist) shifted the concentration-response curve for BK to the right, whereas des-Arg^9-[Leu^8]-BK (a B_1 antagonist) and indomethacin (a cyclooxygenase inhibitor) had no significant effect. NA failed to produce any vasomotor action. His and Ang II induced concentration-dependent contraction. Diphenhydramine (a H_1 antagonist) shifted the concentration-response curve for His to the right, whereas cimetidine (a H_2 antagonist) had no significant effect. Losartan (an AT_1 antagonist) shifted the concentration-response curve for Ang II to the right, whereas PD123319 (an AT_2 antagonist) had no significant effect. These results suggest that the H_1 and AT_1 receptor subtypes might play an important role in arterial contraction, whereas muscarinic receptor subtypes apart from M_1, M_2 and M_3, and B_2 receptors on the endothelium, might modify these contractions to relaxations.

2. INTRODUCTION

Because of the very small internal diameter (0.09–0.14 mm) and length (4–5 mm) of the mouse basilar artery (MBA), little information has been available regarding its reactivity to intrinsic vasoactive substances, such as acetylcholine (ACh), bradykinin (BK), noradrenaline (NA), 5-hydroxytryptamine (5-HT), histamine (His) and angiotensin (Ang) II *in vitro* and the receptor subtypes involved.

The basilar artery runs along the ventral aspect of the medulla oblongata and supplies the brain with blood in reptiles [125], birds [87] and mammals. The responsiveness of this artery seems to reflect changes in cerebral blood flow and local microvascular pressure. Species differences in the responsiveness of this artery to intrinsic vasoactive substances have been reported, and some are very unique and characteristic. For example, NA, a well-known vasoconstrictor, induces contraction of the basilar artery in dogs [107], monkeys [103], guinea pigs [19] and rabbits [28], whereas it induces relaxation in that of cattle [6] and pigs [79]. The intensity of relaxation in pigs is much greater than that in cattle, and this larger relaxation induced by NA is one of the characteristics of porcine basilar artery. In other case, BK, which is a well-known vasorelaxing factor, induces relaxation in human basilar artery, but induces very strong contraction in equine basilar artery [106]. The contraction induced by BK is greater than that induced by NA, His or 5-HT, and this is also one of the characteristics of the equine basilar artery.

Differences in responsiveness to these vasoactive substances might be dependent on differences in the distribution of their receptor subtypes on smooth muscle or endothelial cells. To our knowledge, basilar arterial responsiveness to these vasoactive substances in one species of animal has never been similar to that of other species. Therefore, characterization of basilar arterial reactivity in different species of animal would appear to be useful for investigating evolutionary relationships among animals.

Mice are widely considered to be a prime model of inherited human disease and share 99% of their genes with humans [116]. They are the most commonly used vertebrate species, because of their availability, size, low cost, ease of handling and high reproduction rate. The routine availability of mouse models of various cerebral circulatory disorders like Alzheimer's disease, migraine and stroke [7] requires characterization of the regulation of basilar arterial tone. A study of basilar artery is

important because it is one of the major resistance vessels in the brain.

In the present study, therefore, we attempted to clarify in detail the responsiveness of isolated mouse basilar arteries to ACh, BK, NA, 5-HT, His and Ang II and the receptor subtypes involved.

3. MATERIALS AND METHODS

3.1. Tissue preparation

Adult male mice (ICR, weight: 40 ± 5 g, age: 4 months ± 15 days) were decapitated under diethylether anesthesia. The basilar arteries were then gently isolated from the brain and transferred to ice-cold physiological saline (119 mM NaCl, 4.7 mM KCl, 1.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4 and 10 mM glucose), pH 7.4, aerated with carbogen (95% (v/v) O_2, 5% (v/v) CO_2). Each artery was immediately dissected free of adherent tissues under a stereomicroscope. All experiments were performed in accordance with the Kagoshima University Guidelines for Animal Experimentation.

3.2. Reagents

We used the following reagents and final concentrations:

NA (10^{-9}–10^{-5} M), His hydrochloride (10^{-6}–10^{-3} M), diphenhydramine hydrochloride (10^{-7}–10^{-4} M), cimetidine (10^{-5} M), Ang II acetate salt (10^{-9}–10^{-5} M), losartan potassium (10^{-7} and 10^{-6} M), PD123319 ditrifluoroacetate salt (10^{-6} M), BK acetate salt (10^{-9}–10^{-6} M), des-Arg^9- [Leu^8]-BK (10^{-5} M), methoctramine hydrate (10^{-6} M), *Nω*-nitro-L-arginine (L-NNA; 10^{-4} M) and sodium nitroprusside (SNP; 10^{-4} M) (Sigma-Aldrich, St. Louis, MO, U.S.A.). 5-HT (serotonin)-creatinine sulfate (10^{-9}–10^{-5} M; Merck, Darmstadt, Germany), HOE140 (10^{-7} and 10^{-6} M; Peptide Institute, Osaka, Japan), indomethacin (10^{-5} M; Nacalai tesque, Kyoto, Japan), ACh chloride (10^{-9}–10^{-5} M; Daiichi Sankyo, Tokyo, Japan), pirenzepine dihydrochloride (10^{-6} M; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), hexahydro-sila-difenidol hydrochloride, p-fluoro analog (pFHHSiD, 10^{-5} M; Research Biochemicals, Natick, MA, U.S.A.) and U-46619 (10^{-7} M; Cayman Chemical Company, Ann Arbor, MI, U.S.A.). All drugs were dissolved in distilled water.

3.3. Functional studies

Two rings approximately 2 mm long were cut from each MBA. Each ring was mounted horizontally between two L-shaped stainless steel holders (outer diameter 0.02 mm), with one part fixed to an isometric force transducer (TB-611T, Nihon Kohden Kogyo, Tokyo, Japan), and immersed in a 4-ml water-jacketed micro tissue organ bath (UMTB-1, Unique Medical Co., Ltd, Tokyo, Japan) containing oxygenated salt solution at 37°C (pH 7.4). Each suspended ring was left to equilibrate for at least 120 min under a resting tension of 0.03 g. This tension was chosen, because it allowed us to induce maximum contractions in the basilar artery. KCl (60 mM) was applied every 30 min until the amplitude of the contraction reached a constant value. Changes in the KCl concentration of the physiological saline were compensated for by equimolar adjustment of the NaCl concentration. The isometric tension was recorded with an amplifier (AP-621G, Nihon Kohden Kogyo, Tokyo, Japan), digitized with an analogue-digital converter (PowerLab/8SP, ADInstruments Co., Castle Hill, NSW, Australia) and stored on the hard disk of a personal computer. The cumulative concentration-response curve of each agonist was obtained by adding a solution of agonist directly to the fluid in the bath. Antagonists were added to the bathing media 30 min before the agonist. The antagonists had no effect on the resting vascular tone. The log concentration-ratio of EC_{50} values (i.e., concentration producing half-maximum response) in the absence or presence of antagonist was calculated and plotted against the logarithm of antagonist concentration to obtain the pA_2 values [5].

3.4. Statistical analysis

Results are expressed as means ± SEM. Statistical analyses were performed by Student's *t*-test or the Bonferroni test after one-way analysis of variance (Stat View J-4.5, Abacus Concepts Inc., Berkeley, CA, U.S.A.). Significance was established when the probability level was equal to or less than 5%.

4. RESULTS

4.1. Responsiveness to ACh, BK, NA, 5-HT, His and Ang II

We generated concentration-response curves for ACh, BK, NA, 5-HT, His and Ang II using isolated mouse basilar arteries (Fig. 1). Contractile response was measured under the resting tone of normal artery, whereas the relaxation response was measured under

contraction with U-46619 (a thromboxane A_2 analogue). ACh and BK induced relaxation in a concentration-dependent manner (Fig. 1B). NA did not induce any changes in the vascular tone. His and Ang II induced contraction in a concentration-dependent manner. 5-HT induced infrequent contraction (9 of 36 cases) (Fig. 1A).

4.2. Responsiveness to L-NNA and indomethacin

L-NNA (a NO synthase inhibitor, 10^{-4} M) induced contraction (13.4 ± 1.8% to 60 mM KCl) under resting tension, and indomethacin (a cyclooxygenase inhibitor, 10^{-5} M) induced relaxation (5.8 ± 0.5% to 10^{-4} M SNP) under contraction with L-NNA.

4.3. Maximal responses and pEC_{50} values for ACh, BK, NA, 5-HT, His and Ang II

Table 1 shows the maximal responses and pEC_{50} values for the agonists examined. BK was the most sensitive relaxing agent (pEC_{50}= 6.84 ± 0.09) and induced the most potent maximum relaxation (-65.5 ± 4.3%), whereas Ang II was the most sensitive contracting agent (pEC_{50}= 6.81 ± 0.08) and induced the most potent maximum contraction (57.9 ± 4.7%) of the MBA.

4.4. Effect of endothelial denudation, L-NNA, atropine, pirenzepine, methoctramine and pFHHSiD on ACh-induced relaxation

We investigated the effects of endothelial denudation, L-NNA, atropine, pirenzepine (a M_1 receptor antagonist), methoctramine (a M_2 receptor antagonist) and pFHHSiD (a M_3 receptor antagonist) on the concentration-response curve for ACh. Atropine at 10^{-7} M and 10^{-6} M shifted the concentration-response curve for ACh to the right and at 10^{-5} M largely abolished the ACh-induced relaxation (Fig. 2A). The calculated pA_2 value for atropine was 8.02 ± 0.06 and its slope was 0.86 ± 0.05, which was not significantly different from unity (Fig. 2C). Figure 2B shows the effects of endothelial denudation, L-NNA, pirenzepine, methoctramine and pFHHSiD on ACh-induced relaxation under the contraction induced by U-46619. ACh-induced relaxation was completely abolished in endothelial denudated artery and significantly inhibited by L-NNA. None of the three antagonists had any significant effect on the ACh-induced relaxation.

4.5. Effects of endothelial denudation, L-NNA, indomethacin, and B_1 and B_2 receptor antagonists on BK-induced relaxation

Endothelial denudation had completely abolished BK-induced relaxation, and the NO synthase inhibitor L-NNA partially inhibits it. The cyclooxygenase inhibitor indomethacin had no significant effect on BK-induced relaxation (Fig. 3A). To characterize the BK receptor subtypes, the arteries were pretreated with B_1 and B_2 receptor antagonists. Figure 3B shows the effect of des-Arg^9-[Leu^8]-BK (a B_1 receptor antagonist) on BK-induced relaxation of the MBA. Des-Arg^9-[Leu^8]-BK (10^{-5} M) did not significantly affect BK-induced relaxation. This figure also shows the effect of HOE140 (a B_2 receptor antagonist) on BK-induced relaxation. HOE140 shifted the BK-induced concentration-response curve to the right. The calculated pA_2 value for HOE140 was 7.53 ± 0.12 and its slope was 1.03 ± 0.14, which was not significantly different from unity (Fig. 3C).

4.6. Effect of 5-HT on isolated mouse basilar artery

5-HT induced concentration-dependent contraction in 9 of 36 mouse basilar arteries, the second-time 5-HT response being significantly lower than the first (Fig. 4). Endothelial denudation or inhibition of NO synthase by L-NNA had no effect on this phenomenon (data not shown).

4.7. Effects of diphenhydramine and cimetidine on His-induced contraction

We investigated the effects of diphenhydramine (a H_1 receptor antagonist) and cimetidine (a H_2 receptor antagonist) on the concentration-response curve for His. Diphenhydramine (10^{-7}–10^{-4} M) shifted the concentration-response curve for His to the right in parallel; cimetidine (10^{-5} M) had no significant effect (Fig. 5A). The calculated pA_2 value for diphenhydramine was 6.62 ± 0.11 and its slope was 0.81 ± 0.19, which was not significantly different from unity (Fig. 5B).

4.8. Effects of losartan and PD123319 on Ang II-induced contraction

We examined the effects of losartan (an AT_1 receptor antagonist) and PD123319 (an AT_2 receptor antagonist) on the concentration-response curve for Ang II (Fig. 6). Losartan (10^{-7} and 10^{-6} M) shifted the concentration-response curve for Ang II to the right in

parallel. The calculated pA_2 value for losartan was 8.12 ± 0.10 and its slope was 0.79 ± 0.03, which was significantly different from unity. PD123319 had no significant effect.

5. DISCUSSION

To our knowledge, this is the first study to have demonstrated the responsiveness of the isolated MBA to ACh, BK, NA, 5-HT, His and Ang II. Although some of these vasoactive substances have been investigated by pressure myograph system previously [7], the receptor subtypes have not yet been described.

ACh is an endogenous substance producing endothelium-dependent vasorelaxation via NO, prostacyclin and/or endothelium-derived hyperpolarizing factor (EDHF). In the present study, ACh-induced relaxation was completely abolished by endothelial denudation and significantly inhibited by L-NNA as shown in Fig. 2B. These results suggested that ACh induces endothelium-dependent and NO-mediated relaxation in MBA. Three types of muscarinic receptors appear to be involved in the relaxation or contraction of arteries [10], but in the present study, atropine shifted the concentration-response curve for ACh to the right with a pA_2 value of 8.02 as shown in Fig. 2A. There is no report regarding the pA_2 value of atropine in ACh-induced relaxation on mouse artery or other tissues. But, our calculated pA_2 value is similar to that reported for the rabbit aorta (8.14) [53], but lower than that reported for the rat mesenteric artery (9.78) [117]. The differences might be due to the differences in artery and species of animals studied. M_1, M_2 and M_3 receptor antagonists had no significant effect. These results differed from those obtained using chicken basilar arteries, where atropine (non-selective muscarinic receptor antagonist) and pFHHSiD (selective M_3 receptor antagonist), but not pirenzepine (selective M_1 receptor antagonist) and methoctramine (selective M_2 receptor antagonist), shifted the concentration-response curve for ACh to the right [65]. Our results suggest that some other receptor subtypes apart from M_1, M_2 and M_3 receptors might be responsible for ACh-induced relaxation. Molecular cloning studies have revealed the existence of five molecularly distinct ACh receptor subtypes (M_1-M_5) [16, 118]. Studies of the expression of the cloned M_5 receptor gene in cultured mammalian cells have shown that the encoded receptor protein is functional and efficiently couples to G proteins of the Gq family, similarly to the M_1 and M_3 receptor subtypes [10, 64]. As no M_5 antagonist is commercially available, we were unable to characterize the receptor, although a previous

study observed that ACh-mediated dilation of cerebral arteries and microvessels was virtually abolished in M_5 receptor-knockout mice [122].

BK induced relaxation of mouse basilar arteries precontracted with U-46619, and this effect was abolished in arteries after endothelial denudation as shown in Fig. 3A. This result was consistent with the previous findings by [91], which used light-dye or laser-dye techniques to show that cerebral vasodilator responses to BK *in vivo* were abolished after injury to endothelial cells in mice. Pretreatment with L-NNA partially shifted the concentration-response curve for BK to the right, and indomethacin had no significant effect on it. These results suggest that BK-induced relaxation might be partly mediated by NO, but not by arachidonic acid metabolites. Some other EDHF might also be involved in BK-induced relaxation of MBA. The dilative action of BK on small pial arteries is reportedly mediated by release of hydroxyl radicals in mouse [91] and cat [61]. In line with this, one previous study has verified that BK-induced relaxation of human forearm resistance vessels did not involve NO or a vasodilator prostanoid, this effect being mediated by hyperpolarization of the vascular wall [48]. Thus, it seems that the vasorelaxing pathways involved in BK-induced relaxation vary depending on the vascular bed studied. In the present study, the relaxing effect of BK was significantly inhibited by HOE140, but not by the B_1 receptor antagonist des-Arg^9- [Leu^8]-BK as shown in Fig. 3B. These data indicate that the dilative effect of BK on the MBA is mediated by the B_2 receptor, and not by the B_1 receptor. B_1 receptor-mediated responses are generally not observed under normal physiological conditions [113]. The calculated pA_2 value of HOE140 is 7.53, which is identical to the value for the human isolated umbilical artery, i.e. 7.50 [1].

In the MBA, NA had no effect on resting vascular tone. This result was similar to that obtained by a previous study [19], which showed that NA had no effect on the rat basilar artery.

In MBA, 5-HT induced contraction with an intensity of 18.1%, whereas in most species of animal, the intensity ranges from 40% to 100% [126]. The frequency of 5-HT-induced contraction was 25% among all experimental cases (9 of 36), and furthermore second-time responses were significantly lower than the initial ones and absent in some cases. After first application, a possible desensitization or internalization and/or down regulation of the 5-HT receptor might interfere second-time responses. A similar

phenomenon has been reported in rabbit middle cerebral artery [101]. Therefore, we were unable to carry out subsequent experiments to characterize the receptor subtypes involved in the 5-HT-induced contraction. This brings into question the usefulness of the mouse as a model for studies of migraine and stroke, conditions in which 5-HT is thought to play important roles [84, 119].

The H_1 receptor antagonist diphenhydramine shifted the concentration-response curve for His to the right, whereas the H_2 receptor antagonist cimetidine had no significant effect on the His-induced contraction as shown in Fig. 5A. These results suggest that activation of the H_1 receptor induces contraction of the MBA. Contraction of resting vascular tone in response to His has also been reported in guinea pigs [19], pigs [80], humans [95], horses and cattle [78]. The calculated pA_2 value for diphenhydramine was 6.62, which is lower than the values reported for bovine (7.61) and porcine (7.77) basilar arteries [78, 80]. A further study is needed to clarify the differences in pA_2 values among species.

The effects of Ang II occur via activation of two receptor subtypes, AT_1 and AT_2. The vasocontractile effects of Ang II are generally considered to result from activation of the AT_1 receptor. The selective AT_1 receptor antagonist losartan shifted the concentration-response curve for Ang II to the right, whereas PD123319 (an AT_2 receptor antagonist) had no effect as shown in Fig. 6A. These results suggest that the Ang II-induced contraction in MBA is mediated by activation of AT_1 receptors. A previous study showed that vasoconstriction of the cerebral artery in response to Ang II was markedly reduced in genetic AT_{1A}-deficient mouse [31]. The calculated pA_2 value for losartan is 8.12, which is similar to that reported in canine mesenteric (8.15) and pulmonary (7.96) artery [42].

L-NNA induced contraction and indomethacin induced relaxation through inhibition of NO synthase and cyclooxygenase, respectively. These results suggest that the resting tone balance of the MBA is also maintained by spontaneous release of NO and thromboxane A_2.

Small rodents, such as mice and rats are frequently used in preclinical cerebrovascular research, mice being particularly useful, because an increasing number of transgenic models are becoming available. Mice are often used as small animal models of brain ischemia, venous thrombosis or vasospasm, and Alzheimer's disease. The majority of novel therapeutic approaches are tested in small animal models of human disease,

especially those involving mice, prior to clinical testing. A variety of murine models of cerebrovascular disease are available, from which a number of molecular and structural elements of cerebral disorders have been clarified.

In summary, we have investigated the responses of the MBA to a number of pharmacological agents that are modulators of cerebrovascular circulation in both normal and pathophysiological states. We have demonstrated that the MBA is responsive to ACh and BK with relaxation, and to 5-HT, His and Ang II with contraction, but is unresponsive to NA. These response characteristics are unique to the MBA.

Table 1. pEC_{50} values and maximal responses to agonists

Agonists	pEC_{50}	Max (%)
Bradykinin	6.84 ± 0.09	-65.5 ± 4.3[a]
Acetylcholine	6.76 ± 0.06	-46.4 ± 4.4[a]
Angiotensin II	6.81 ± 0.08	57.9 ± 4.7[b]
5-Hydroxytryptamine	6.64 ± 0.10	18.1 ± 2.3[b]
Histamine	4.58 ± 0.02	41.6 ± 2.4[b]
Noradrenaline	–	No response

[a]Relaxation induced by 10^{-4} M SNP (0.011 ± 0.002 g) was taken as 100%.

[b]Contraction induced by 60 mM KCl (0.038 ± 0.004 g) was taken as 100%.

Each point represents the mean ± SEM of 8–12 mice.

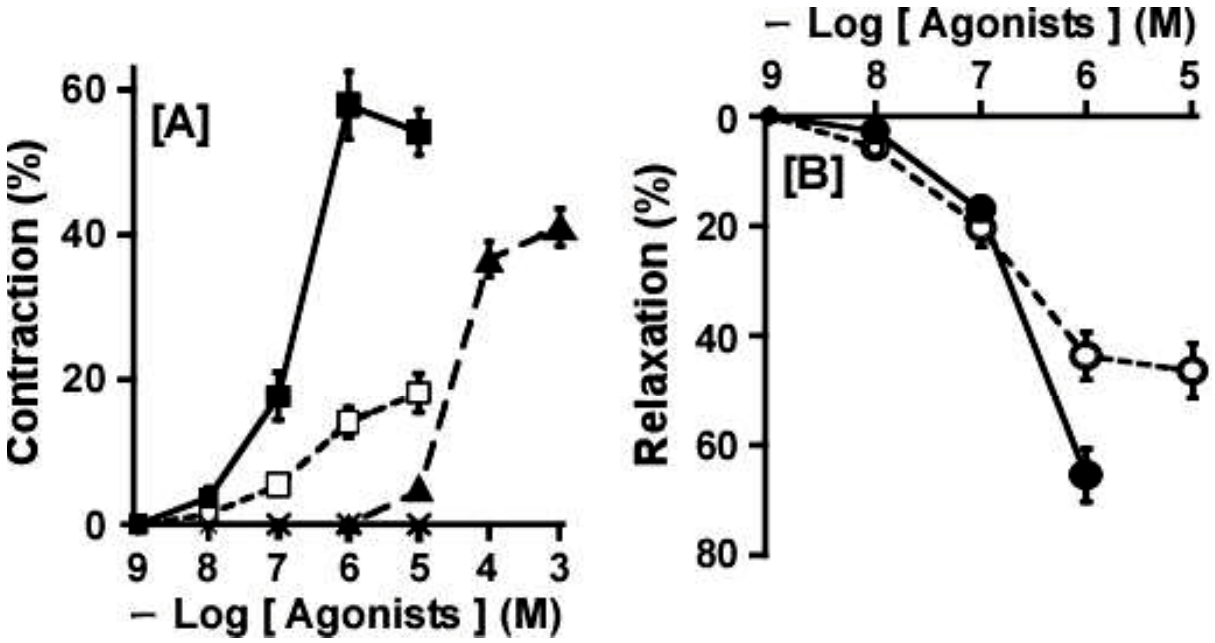

Fig. 1. Responsiveness of isolated mouse basilar artery to angiotensin II (Ang II: ■), 5-hydroxytryptamine (5-HT: □), histamine (His: ▲), noradrenaline (NA: ×) [A], acetylcholine (ACh: ○) and bradykinin (BK: ●) [B]. Relaxation in response to ACh and BK was investigated under precontraction with U-46619 (10^{-7} M). Contraction responses were compared with 60 mM KCl response, and relaxation responses were compared with 10^{-4} M SNP response. Absolute values of KCl-induced contraction and SNP-induced relaxation were 0.038 ± 0.004 g and 0.011 ± 0.002 g, respectively. Each point represents the mean ± SEM of 8–12 mice.

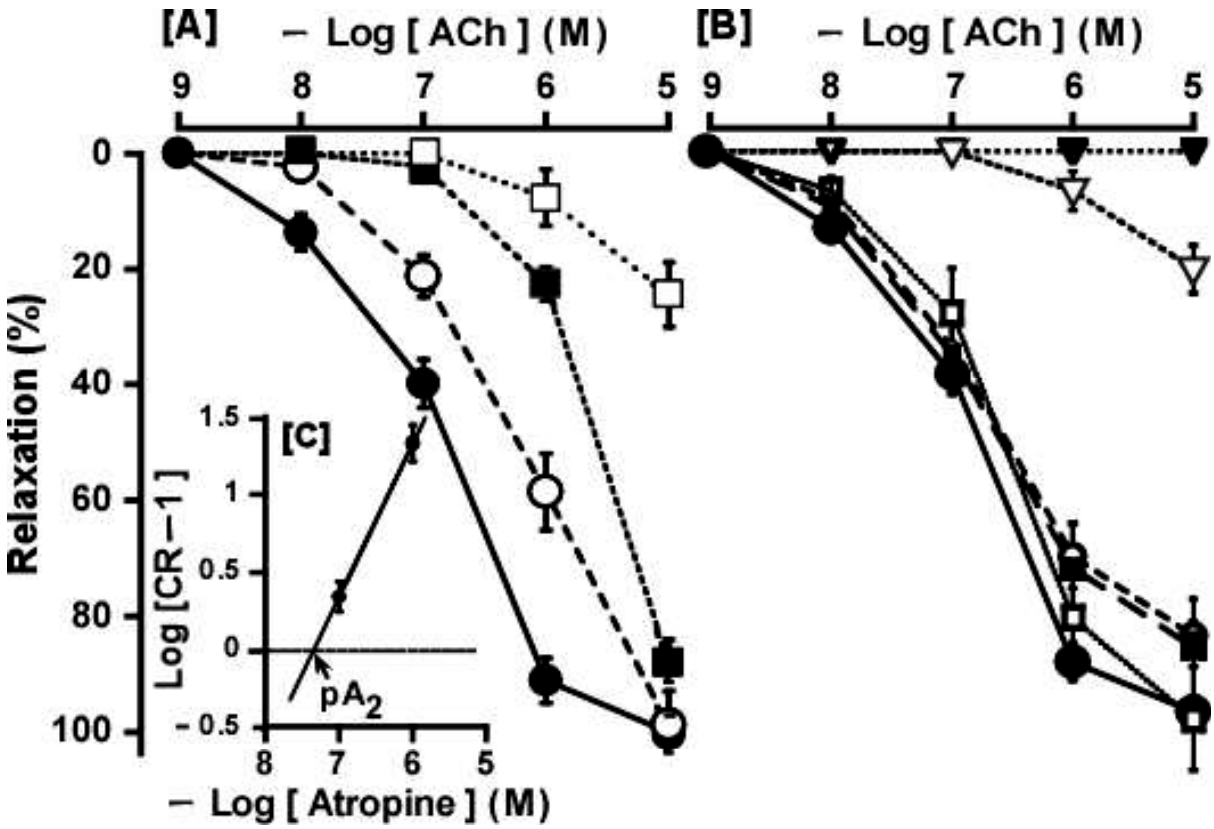

Fig. 2. Effect of the non-selective muscarinic-antagonist atropine (○,10^{-7} M, ■,10^{-6} M and □, 10^{-5} M) on acetylcholine (ACh)-induced relaxation (●) **[A]** and effects of the endothelial denudation (▼), L-NNA (▽, 10^{-4} M), M_1 receptor antagonist pirenzepine (○, 10^{-6} M), the M_2 receptor antagonist methoctramine (■,10^{-6} M) and the M_3 receptor antagonist pFHHSiD (□,10^{-5} M) on ACh-induced relaxation (●) **[B]** and Schild plot of atropine **[C]** for the isolated mouse basilar artery. The maximum relaxation induced by ACh in the absence of antagonist was taken as 100%. Each point represents the mean ± SEM of 6–10 mice. CR: equieffective ACh concentration ratio [concentration producing 50% maximal (EC_{50}) in the presence of atropine/EC_{50} in the absence of atropine].

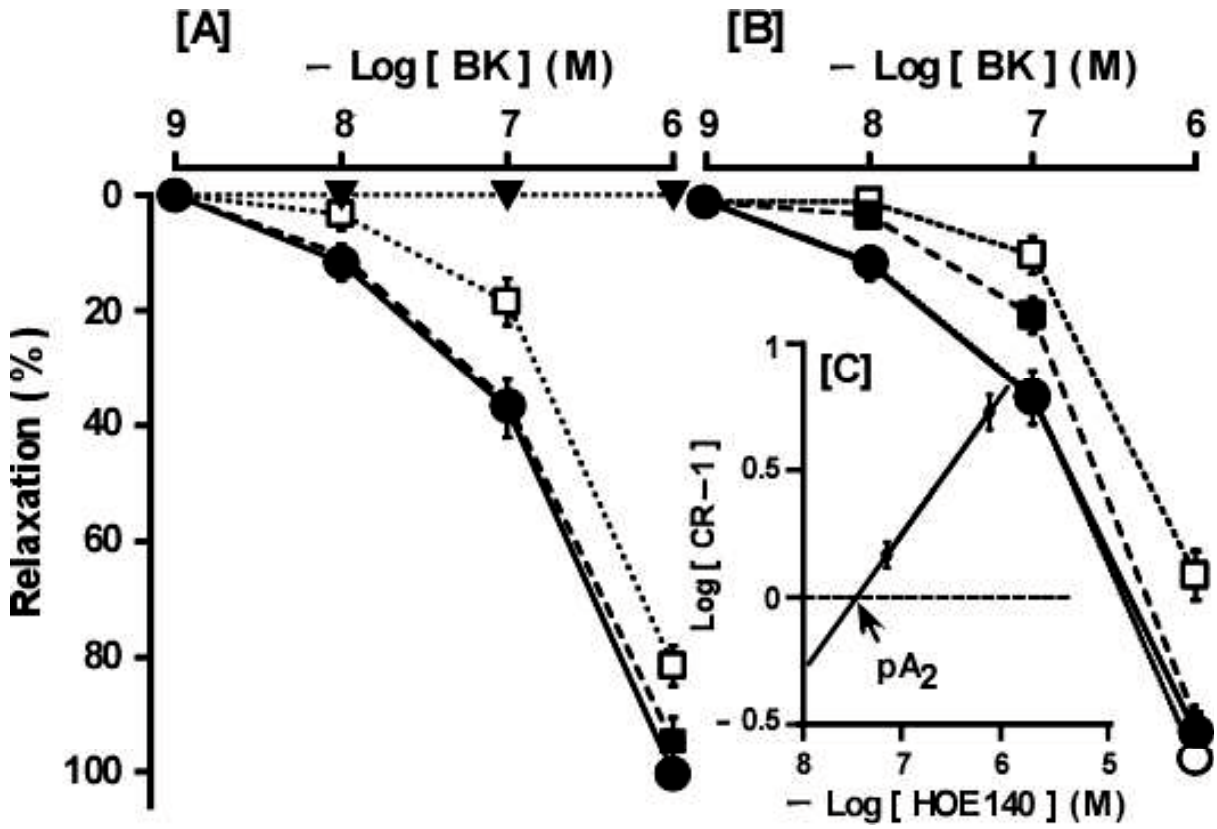

Fig. 3. Effects of endothelial denudation (▼), L-NNA (□) (a nitric oxide synthase inhibitor) and indomethacin (■, 10^{-5} M, a cycloxygenase inhibitor) on bradykinin (BK)-induced relaxation (●) **[A]**, effects of the B_1 receptor antagonist des-Arg^9-[Leu^8]-BK (○, 10^{-5} M) and the B_2 receptor antagonist HOE140 (■, 10^{-7} M and □, 10^{-6} M) on BK-induced relaxation (●)**[B]** and Schild plot of HOE140 **[C]** for the isolated mouse basilar artery. The maximum relaxation induced by BK in the absence of antagonist was taken as 100%. Each point represents the mean ± SEM of 8 mice. CR: see Fig. 2.

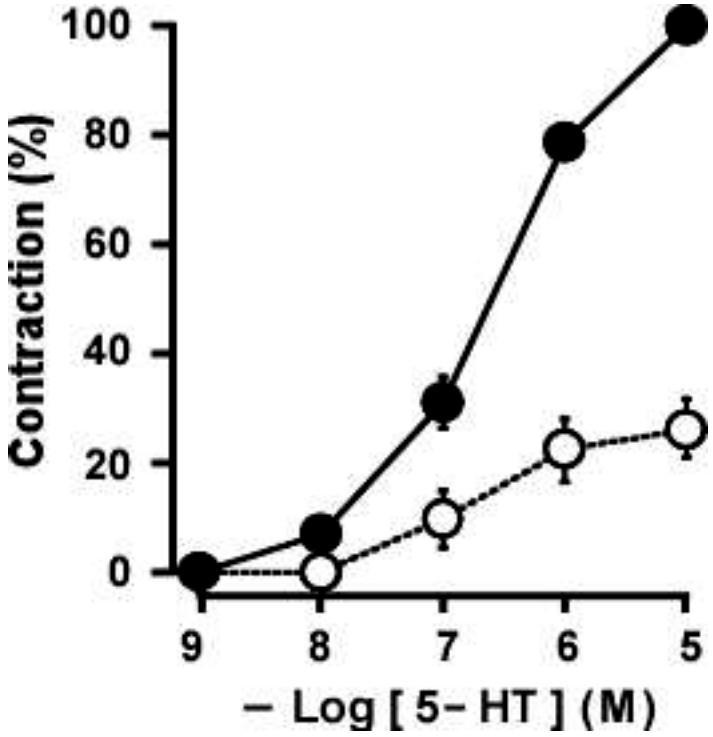

Fig. 4. Effect of repeated application of 5-hydroxytryptamine (5-HT) on the isolated mouse basilar artery (●, 1st response, ○, 2nd response). The maximum contraction induced by the first application of 5-HT was taken as 100%. Each point represents the mean ± SEM of 9 mice.

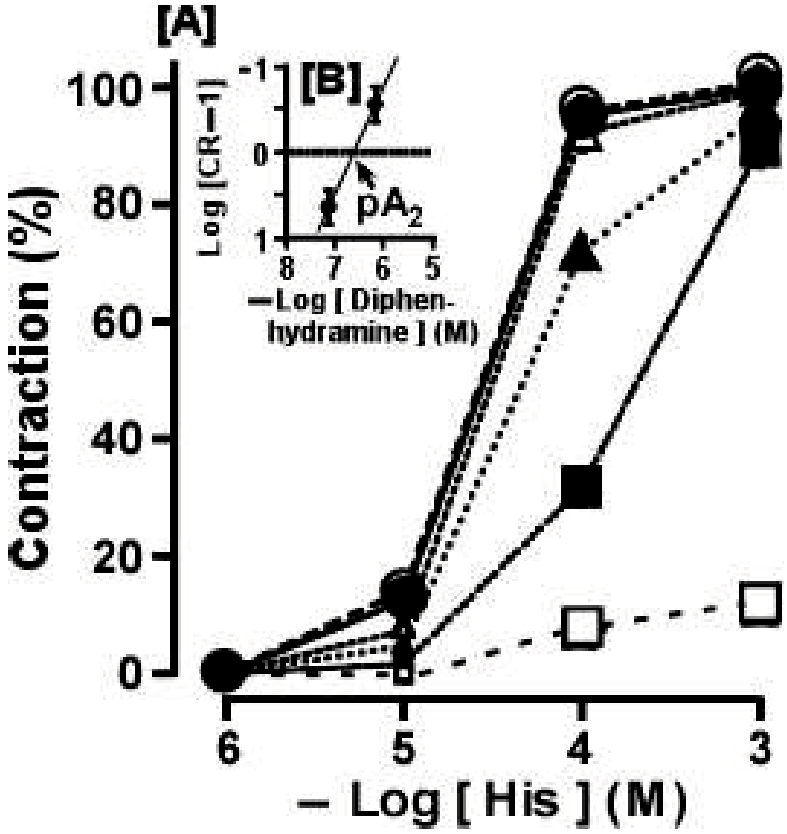

Fig. 5. Effect of the H_1 receptor antagonist diphenhydramine (△ 10^{-7} M, ▲ 10^{-6} M, ■ 10^{-5} M, □ 10^{-4} M) and the H_2 receptor antagonist cimetidine (○ 10^{-5} M) on histamine (His)-induced contraction (●) [A] and Schild plot of diphenhydramine **[B]** for the isolated mouse basilar artery. The maximum contraction induced by His in the absence of antagonist was taken as 100%. Each point represents the mean ± SEM of 6–10 mice. CR: see Fig. 2.

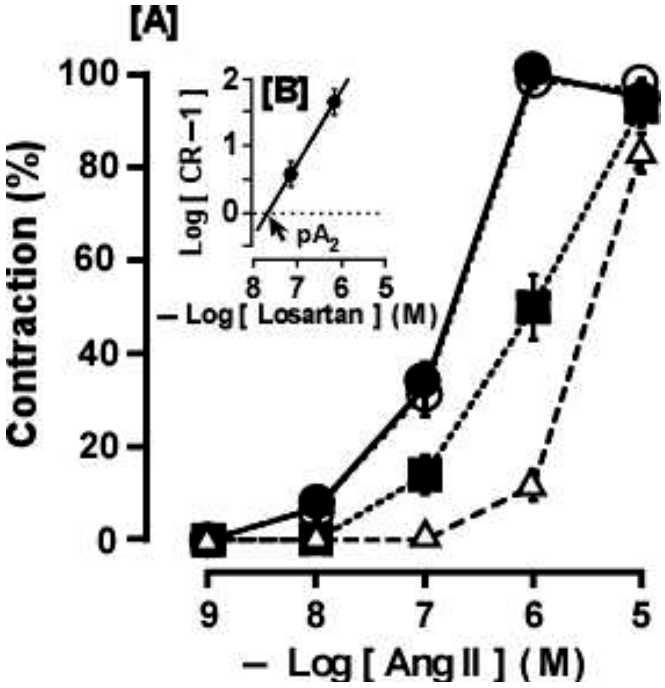

Fig. 6. Effect of the AT_1 receptor antagonist losartan (■ 10^{-7} M, △ 10^{-6} M) and the AT_2 receptor antagonist PD123319 (○, 10^{-6} M) on angiotensin (Ang) II-induced contraction (●) **[A]** and Schild plot of losartan **[B]** in the isolated mouse basilar artery. The maximum contraction induced by Ang II in the absence of antagonist was taken as 100%. Each point represents the mean ± SEM of 7 mice. CR: see Fig. 2.

Chapter 1

Study 2

Methylmercury affects cerebrovascular reactivities to angiotensin II and acetylcholine via Rho-kinase and NO pathways

1. Abstract

Increased risk of hypertension and cardiovascular diseases after methylmercury (MeHg) exposure has been suggested. In this study, we aimed to investigate whether the *in vivo* exposure of MeHg in mice affects blood pressure and basilar arterial responses to angiotensin II (Ang II) and acetylcholine (ACh), which are important modulators of cerebrovascular autoregulation. Mice were exposed to MeHg (40 ppm) in drinking water for 21 days. Blood pressure was measured in conscious mice by an indirect tail-cuff method. Functional studies of the isolated arteries' response to vasoactive substances were performed using a micro-organ-bath system. Systolic and mean blood pressures were significantly increased after 2 and 3 weeks of treatment with MeHg, respectively. Ang II-induced contraction in an isolated basilar artery, which is mediated via Rho-kinase activation, was increased in MeHg-treated mice. ACh-induced relaxation, which is dependent on NO production from the endothelial cells, was decreased in MeHg-treated mice. However, alterations of vascular responses to Ang II and ACh were not observed in the isolated thoracic aorta. Concomitant treatment with tempol, a reactive oxygen species (ROS) scavenger, suppressed MeHg-induced increase in blood pressure, enhanced Ang II-induced contraction and decreased ACh-induced relaxation in MeHg-treated mice. These results suggest that *in vivo* MeHg exposure increases blood pressure and causes alterations in the cerebrovascular reactivity in response to Ang II and ACh through ROS generation in mice.

Keywords: Cerebral artery, acetylcholine, angiotensin II, reactive oxygen species, Rho-kinase, tempol, methylmercury, hypertension, nitric oxide.

2. Introduction

Methylmercury (MeHg) is a potent environmental toxic pollutant that is generated by methylation of inorganic mercury. It is a well-documented neurotoxicant in both humans and experimental animal models [15]. For example, prenatal MeHg intoxication has been associated with neurodevelopmental disorders such as mental retardation and motor and cognitive dysfunction [32, 56]. Experimental animal studies likewise showed deficits resulting from prenatal MeHg exposure in corresponding behavioral domains including deficits in learning, discrimination/transition reversal and working memory, increased perseverative behavior, and increased behaviors interpreted as anxiety [17, 123]. The central nervous system is the main target organ of MeHg toxicity, especially when exposure occurs during the early stages of brain development. Even though the developing brain has been considered the critical target organ of MeHg toxicity in children, recent evidence indicates that the cardiovascular system may be a sensitive organ in adults [21]. It has also been reported that, in humans, cardiovascular outcomes of MeHg exposure include myocardial infarction [90], heart rate variability, atherosclerosis, coronary heart disease, and hypertension [111].

Clinical and experimental studies indicate that oxidative stress contributes to the development of hypertension in humans [118] and animals [109]. It has been reported that the harmful effects of mercury are mediated by the excessive release of reactive oxygen species (ROS) and the reduction of antioxidant defenses, resulting in inactivation of important enzymes that are responsible for the body's defenses, including glutathione reductase, glutathione peroxidase, superoxide dismutase, catalase, and glutathione (GSH) in different organs [128].

In the cardiovascular system, the vascular endothelium is highly sensitive to oxidative stress [121], and is recognized as a fundamental homeostatic organ for the regulation of vascular tone and structure. Under physiologic conditions, endothelial stimulation induces the production and release of nitric oxide (NO), which diffuses to the surrounding tissues and cells. NO exerts its cardiovascular protective role by relaxing smooth muscle cells, preventing leukocyte adhesion and migration into the arterial wall, muscle cell proliferation, platelet adhesion and aggregation, and adhesion molecule expression [108]. Oxidative stress caused by mercury exposure decreases the

bioavailability of NO and alters the expression of endothelial NO synthase, which result in increased vasoconstriction and the reduction of the endothelial vasodilator response [34, 121].

Conversely, activation of Rho-kinase is thought to be a key mechanism of calcium sensitization that regulates vascular responses to several vasoconstrictors [31]. ROS activate Rho-kinase by activation of Rho in the rat aorta *in vitro* [55], and also increase Rho-kinase activity via a redox-regulated, positive-feedback mechanism in human and rabbit ductus arteriosus [57]. In spontaneously hypertensive rats, augmented contribution of Rho-kinase activity to cerebral vascular tone has been reported [22]. In addition, increased Rho activity and Rho-kinase expression in cultured aortic smooth muscle cells from spontaneously hypertensive rats have been reported [82]. Together, these studies suggest that ROS play an important role in the regulation of vascular constriction and relaxation through augmentation of Rho-kinase activity and a decrease in NO availability both *in vitro* and *in vivo*.

In the present study, we investigated the effect of MeHg exposure on the cardiovascular system, by analyzing blood pressure and vascular responses to angiotensin II (Ang II) and acetylcholine (ACh), using mice treated with MeHg *in vivo*. Ang II is a potent vasoconstrictor that induces contraction via activation of the Rho-kinase signaling pathway [31] and ACh-induced endothelium-dependent and NO-mediated relaxation [25, 50]. In addition, the effect of combined treatment with tempol, a ROS scavenger, and MeHg were observed.

3. Materials and Methods

3.1. Animal preparation

Adult male ICR mice (3.5 months old) were obtained from Kyudo (Kumamoto, Japan) and maintained under controlled environmental conditions (12-h light/dark cycle, 23±2°C) with access to food and water *ad libitum*. Mice were divided into four groups: 1) Control (no treatment); 2) MeHg; 3) MeHg plus tempol; and 4) tempol. MeHg (40 ppm; [33]) and tempol (2 mM; [115]) were administered with the drinking water for 21 days. The body weight and water intake were measured every second day. Significant differences in body weight and visible clinical signs, such as abnormal locomotion or hind limb paralysis, were not observed in the four groups during the 21-day treatment period.

Blood pressure was measured each week in conscious mice using an indirect tail-cuff method (Indirect Blood Pressure Meter BP-98A; Softron™, Tokyo, Japan). All animal experiments were performed in accordance with the guidelines of Kagoshima University for the care and use of laboratory animals.

3.2. Tissue preparation

After the treatment period, mice were sacrificed by decapitation under anesthesia. The basilar artery and thoracic aorta were then gently isolated and transferred to ice-cold physiological saline (119 mM NaCl, 4.7 mM KCl, 1.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, and 10 mM glucose, pH 7.4) that was aerated with carbogen (95% (v/v) O_2, 5% (v/v) CO_2). Each artery was immediately dissected free of adherent tissues under a stereomicroscope.

3.3. Reagent

We used the following reagents: Ang II acetate salt, [Sar^1, Thr^8] (Ang II), Nω-nitro-L-arginine (L-NNA), Y27632, 4-hydroxy-Tempo (tempol), sodium nitroprusside (SNP) (Sigma-Aldrich, Saint Louis, MO, USA); ACh chloride (Daiichi Sankyo, Tokyo, Japan); MeHg (Kanto Chemical, Tokyo, Japan); fasudil (Wako Pure Chemical Industries Ltd, Osaka, Japan), and U-46619 (Cayman Chemical Company, Ann Arbor, MI, USA). Tempol was dissolved in sterile ethanol and further diluted in the drinking water to a concentration of 2 mM. The final concentration of ethanol in drinking water was 0.02%. All other drugs were dissolved in distilled water.

3.4. Vascular reactivity studies

The mouse basilar arterial reactivity study was described previously [50]. We also followed the same procedure for thoracic aorta reactivity studies. However, we adjusted the resting tension to 0.3 g for the thoracic aorta in this study. This tension was chosen because it allowed us to induce maximum contractions in this artery. After the equilibration period, contraction induced by Ang II was measured under resting tension conditions and calculated as the percentage of 60 mM KCl-induced contraction. To examine responses to ACh, basilar arteries were precontracted submaximally (50–60% of the response to 60 mM KCl) using the thromboxane mimetic U-46619 at concentrations

varying from 1×10^{-7} M to 4×10^{-7} M. For ACh vasodilator responses, results are expressed as the percent dilation (% of induced tone), with 100% representing the difference between the resting value under basal conditions and the constricted value in response to U-46619. Y27632 or fasudil were administered 30 min before the addition of Ang II.

3.5. Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed using the Student's *t* test or the Bonferroni test after a one-way analysis of variance. $P < 0.05$ was considered significant.

4. Results

4.1. MeHg induced an increase in blood pressure

We observed the effect of *in vivo* MeHg exposure on blood pressure and heart rate in mice. Exposure to MeHg significantly increased both systolic blood pressure (SBP) at 14 and 21 days and mean blood pressure (MBP) at 21 days after exposure compared with those in control mice (Fig. 7). The MeHg-induced increase in SBP and MBP was suppressed by concomitant treatment with tempol, although the tempol alone had no significant effect on blood pressure. There was no significant difference in diastolic blood pressure among the groups. However, heart rate in the MeHg-treated group was slightly but significantly lower than that in the control group.

4.2. Effect of MeHg on Ang II-induced contraction

We then examined the alteration of the vasoconstriction response against Ang II using an isolated basilar artery and thoracic aorta from mice treated with MeHg. *In vivo* MeHg exposure significantly augmented the vasoconstrictor response of isolated basilar arterial rings against Ang II compared with that in control (Fig. 8A), while the response to 60 mM KCl was not changed (Fig. 8B). Combined treatment with tempol and MeHg suppressed the augmented contractile response induced by Ang II (Fig. 8C) in the MeHg-treatment group. Tempol treatment did not affect the Ang II response in the absence of MeHg treatment. In contrast, no significant difference in vascular reactivity to Ang II in thoracic aorta was observed between the MeHg-treated and control groups (Fig. 8D).

4.3. Involvement of Rho-kinase in the enhanced Ang II responses caused by MeHg

Because Rho-kinase has an important role in Ang II-induced vasoconstriction [31], we analyzed involvement of Rho-kinase in the MeHg-induced increase in vasoconstriction using a specific Rho-kinase inhibitor, Y27632. Y27632 treatment inhibited the vasoconstriction response to Ang II in isolated basilar arteries from both control and MeHg-treated groups. At an Ang II concentration varying from 10^{-9} to 10^{-7} M, the response in the control artery and the enhanced response in the MeHg-treated group was completely suppressed by pretreatment with Y27632 (Fig. 9). However, even in the presence of Y27632, a slightly higher vasoconstriction at higher Ang II concentrations (10^{-6} and 10^{-5} M) was observed in MeHg groups, although the difference was not significant.

To confirm the augmented activation of Rho-kinase in arteries from MeHg-treated mice, we observed the effect of Y27632 on the vascular resting tension. Application of Y27632 (0.1–3 μM) caused a concentration-dependent relaxation of the basilar artery. These responses were enhanced in arteries from MeHg-treated mice compared with that from control mice (Fig. 10A). The enhanced relaxation effect of Y27632 was more clearly observed in the precontracted condition using U-46619 (Fig. 10B). We confirmed that fasudil, another Rho-kinase inhibitor, showed results similar to those of Y27632 (data not shown). Concomitant treatment with tempol significantly prevented the increased vasodilatory response to Y27632 in precontracted arteries from MeHg-treated mice (Fig. 10C). Tempol treatment alone had no significant effect on the Y27632-induced response compared with that in the control group (Fig. 10C).

4.4. Effect of MeHg on ACh-induced relaxation

We also observed the effect of MeHg on the ACh-induced vasodilatory response. Although ACh induced relaxation of the isolated basilar artery in all groups in a dose-dependent manner, reduced vascular response to ACh was observed in the MeHg-treated group (Fig. 11A). However, relaxation induced by SNP, which causes vasodilation as a NO donor, was not significantly different between control and MeHg-treated groups (Fig. 11B). Concomitant treatment with tempol prevented the decreased relaxation response induced by ACh in the MeHg-treated groups (Fig. 11C). Conversely, significant differences were not observed in ACh-induced relaxation of thoracic aorta in both the

MeHg-treated and control groups (Fig. 11D).

5. Discussion

In the present study, we showed that *in vivo* MeHg exposure affects blood pressure and cerebral vascular reactivity in mice. The major findings of this study are that MeHg increased SBP and MBP, and selectively enhanced Ang II-induced contraction and reduced ACh-induced relaxation in the isolated mouse basilar artery (MBA). These effects of MeHg were reversed by the ROS scavenger, tempol, suggesting that ROS generation plays a pivotal role in the cardiovascular effects of MeHg in mice.

It has been shown that mercury exposure increases the risk of hypertension, atherosclerosis, and coronary heart disease [111]. An association between mercury exposure and hypertension in humans has been suggested [41]. An increased incidence of hypertension and cerebrovascular disease among aging patients with Minamata disease also reported [105]. Our results showed experimental evidence that prolonged exposure to MeHg increases SBP and MBP in mice, which is consistent with the findings of a previous study [40], which showed that subchronic exposure to MeHg induces hypertension in rats. Because MeHg induces ROS generation [11], which is an important factor in modulating blood pressure and cerebrovascular reactivity in mice [25], we studied the effects of a ROS scavenger, tempol, on MeHg-induced elevated blood pressure. Concomitant treatment with tempol attenuates the development of hypertension in MeHg-treated mice. This finding suggests that the MeHg-induced elevation in blood pressure is, at least in part, associated with increased ROS production. Our results are similar to previous findings that oral tempol prevented the increase in blood pressure in response to a slow pressure infusion of Ang II [24].

We showed an increase in vascular reactivity in response to Ang II in the basilar artery, but not in the thoracic aorta, isolated from MeHg-exposed mice. However, there was no significant difference in KCl-induced contraction, suggesting that specific mechanisms involved in Ang-II-induced vasoconstriction were impaired by MeHg exposure. Vascular tone is determined by the balance of constriction and dilation mechanisms, which depends on the vascular smooth muscle and endothelium activity, respectively. It has been reported that constriction of the MBA in response to Ang II was eliminated by the Rho-kinase inhibitor Y27632, suggesting that activation of this signaling

pathway is the main mechanism by which Ang II increases cerebral vascular tone in mice [31]. Rho-kinase has been suggested to play an important role in regulation of vascular tone [25]. Therefore, we investigated the participation of Rho-kinase in the enhanced Ang II-induced contraction in the MeHg-treated MBA. We found that Y27632 treatment reduced vasoconstrictor responses to Ang II in both MeHg-treated and control mice, which is in agreement with a previous report [31]. At Ang II concentrations of 10^{-7} M to 10^{-5} M, vasoconstriction increased in the MeHg-treated group in the absence of Y27632, but the difference in contraction between MeHg-treated and control mice was not observed in the presence of Y27632. This suggests that the hyperactivity of Rho-kinase is involved in the enhanced response to Ang II in MeHg-exposed mice. In addition, we found that Y27632 produced dilation in the basilar artery from control mice in both the resting tension and pre-contracted conditions. This result further suggests that Rho-kinase is present and functionally important in contributing to normal resting basilar artery tone. Importantly, Y27632 produced significantly greater basilar artery dilatation in MeHg-treated mice compared with control mice in both the resting and pre-contracted conditions. We also confirmed that fasudil, another Rho-kinase inhibitor, caused similar results to those with Y27632. These results also support our idea that hyperactivity of Rho-kinase is the cause of enhanced Ang II-induced contraction in MeHg-exposed MBA. Enhanced Ang II-induced contraction supports our findings of elevated blood pressure *in vivo* MeHg exposed mice.

We then focused on the participation of ROS in the modulation of the Rho-kinase activity in the MeHg-treated MBA, because ROS increases Rho-kinase activity [54] and the ROS-induced increase in Rho-kinase activity causes vascular smooth muscle contraction [97]. In this study, we showed that tempol also improved the altered contractility caused by Ang II and enhanced Rho-kinase activity in the basilar artery from MeHg-treated mice. These results suggest that the effect was due to the ROS generation by MeHg exposure. It has been reported that ROS itself enhances Rho-kinase activity [54], and the enhanced Rho-kinase activation by MeHg-induced ROS generation might be responsible for the enhanced vascular contractility caused by Ang II. Similar findings are observed in mouse cerebral arteries in type II diabetes [25] and in carotid arteries from type I diabetic rats [89], where an augmented role of Rho-kinase and enhanced Ang II-induced contraction that result from increased ROS generation has been suggested.

However, MeHg-induced modification of proteins, such as the Ang II receptor or proteins in the intracellular signaling pathways, cannot be ruled out. The effect of MeHg on receptor and signaling molecule expression needs to be clarified in future studies.

The endothelium-dependent vasodilator response induced by ACh was decreased in MeHg-treated mice, in accordance with previous studies using an *in vivo* mercury chloride treatment [121]. In a MBA, ACh-induced relaxation is reported to be largely dependent on the effect of NO released from endothelial cells [25, 50]. Because treatment with SNP, a NO donor, elicited a similar degree of vasodilatory effect on the basilar arteries from both MeHg-treated and control mice, the effect of MeHg on ACh-induced relaxation was specific to the NO signaling pathway in the vascular endothelium. ROS-induced impairment of endothelium-dependent responses in cerebral blood vessels has been reported [25, 26]. In addition, the superoxide anion interacts with NO to form peroxynitrite, thereby decreasing the NO bioavailability for smooth muscle relaxation [8]. In the hypertensive model rat, improvement of NO availability and amelioration of hypertension by tempol or other antioxidant treatments has been reported [93, 109]. Blood pressure reduction produced by chronic tempol treatment in hypertensive rats is also accompanied by increased endothelium-dependent vasodilation [2]. Previous studies demonstrated that tempol reverses endothelial NO dysfunction in animals or humans with elevated levels of oxidative stress [44]. We observed that tempol treatment restored the impaired ACh-induced vasodilatation in vessels from MeHg-treated mice, suggesting that ROS generation by MeHg plays an important role in basilar artery endothelial dysfunction. It is likely that the reduced relaxation induced by ACh in the basilar artery from MeHg-treated mice occurred in response to the decreased NO bioavailability that resulted from increased ROS levels.

Recently, it has been suggested that NO may influence vascular contractility through inhibition of Rho-kinase [20, 92]. In addition, increasing levels of superoxide are involved in increasing Rho-kinase activity and calcium sensitivity by reducing Rho-kinase inhibition caused by NO [20, 55, 92]. Therefore, tempol may improve MeHg-induced vascular hypercontractility in response to Ang II primarily by neutralizing the direct effect of ROS on Rho-kinase, or secondarily by reversing the Rho-kinase inhibition via NO. Our findings provide a potential mechanistic link between studies related to ROS-induced

impairment of vascular responses and increased Rho-kinase activity, which plays an important role in the physiological regulation of cerebral vascular tone.

It is not clear why MeHg selectively affects Ang II and ACh responses in the basilar artery but not in the thoracic aorta, but several possibilities may explain this phenomenon. NADPH oxidases are the major physiological source of ROS within the cerebral and systemic vasculature, and their activity is upregulated in pathophysiological states such as hypertension [69]. Recently, significant differences in vascular NADPH oxidase function under normal physiological conditions have been noted between cerebral and non-cerebral arteries. It has been reported that [68] NADPH oxidase activity is higher in the basilar artery than in the thoracic aorta of mice, and NADPH-mediated O_2^- production was 15-fold higher in basilar artery than in the thoracic aorta. Consequently, in a MeHg-exposed MBA, ROS generation via NADPH oxidase might be higher than that in the thoracic aorta. It is likely that higher ROS generation in a MeHg-exposed basilar artery results in an apparent alteration in Rho-kinase and NO signaling pathways. In addition, the contraction induced by Ang II in the isolated thoracic aorta was smaller than that in the basilar artery, suggesting that there is lower activity or involvement of Rho-kinase in the Ang II signaling pathway between the basilar artery and thoracic aorta in mice. It is also known that the basilar artery produces larger amounts of NO than other arteries. For example, spontaneous NO production from porcine basilar arterial endothelial cells is 4-fold higher than that from abdominal aortic endothelial cells [72]. High NO productivity in the basilar artery may result in a higher sensitivity to MeHg compared with the thoracic aorta.

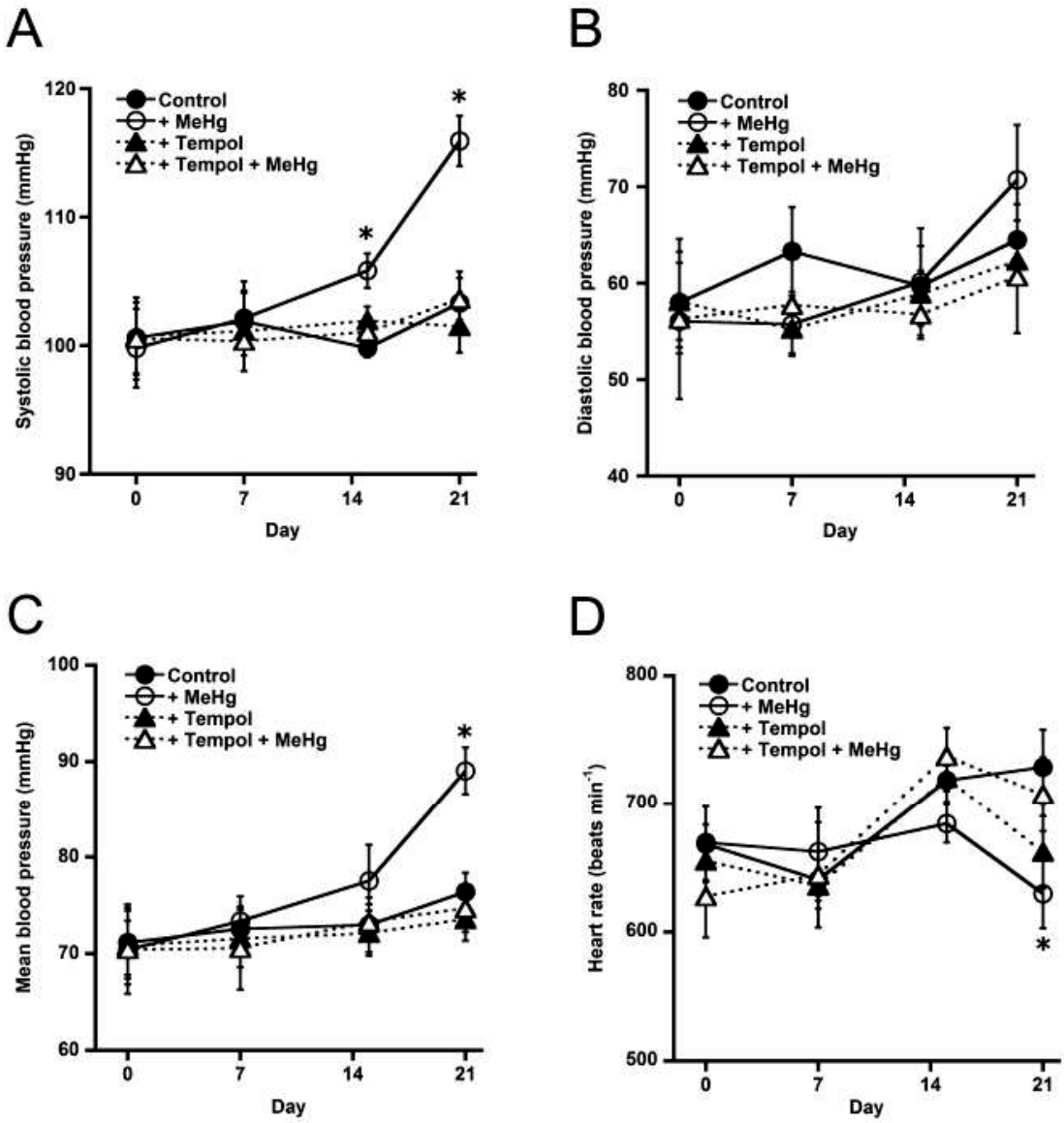

Fig. 7. Effect of *in vivo* MeHg treatment on blood pressure and heart rate. MeHg (40 ppm) or tempol (2 mM) were administered in the drinking water for 21 days. The systolic [A], diastolic [B], and mean [C] blood pressure and the heart rate [D] were measured each week in conscious mice using an indirect tail-cuff method. Each point represents the mean ± SEM of five mice. *P < 0.05 vs. control.

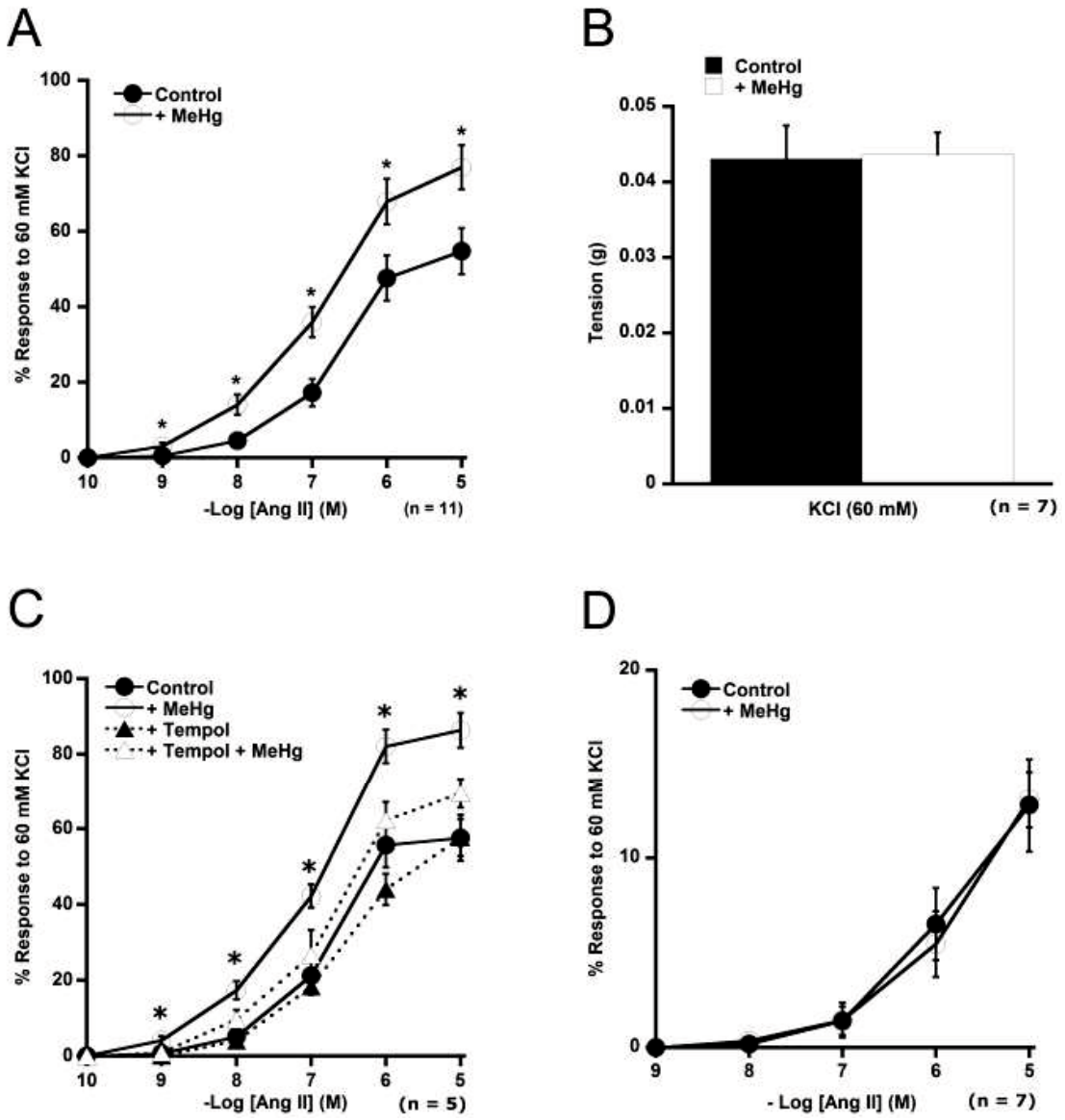

Fig. 8. Effect of *in vivo* MeHg treatment on Ang II- and KCl-induced contraction in the basilar artery and thoracic aorta. After 21 days' treatment with MeHg, the effect of MeHg on the Ang II- and KCl-induced contraction in the isolated mouse basilar artery ([A] and [B], respectively), and Ang II-induced contraction in the thoracic aorta [D] was observed. Ang II-induced contraction was also observed in the basilar artery from mice concomitantly treated with MeHg and tempol (2 mM) [C]. Each point represents the mean ± SEM. *P < 0.05 vs. control.

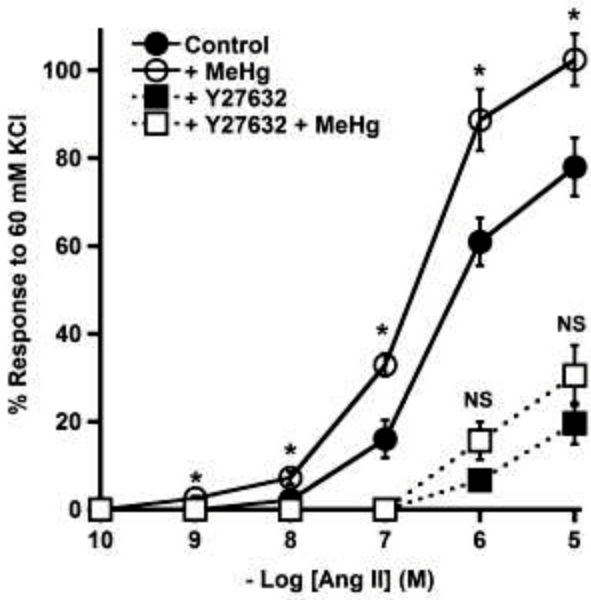

Fig. 9. Effect of Y27632 on Ang II-induced contraction in the basilar artery. Effect of Y27632, a Rho-kinase inhibitor, on Ang II-induced basilar artery contraction was observed in control and MeHg-treated mice. Each point represents the mean ± SEM of six mice. *P < 0.05 vs. control; NS: not significant vs. control+Y27632 group.

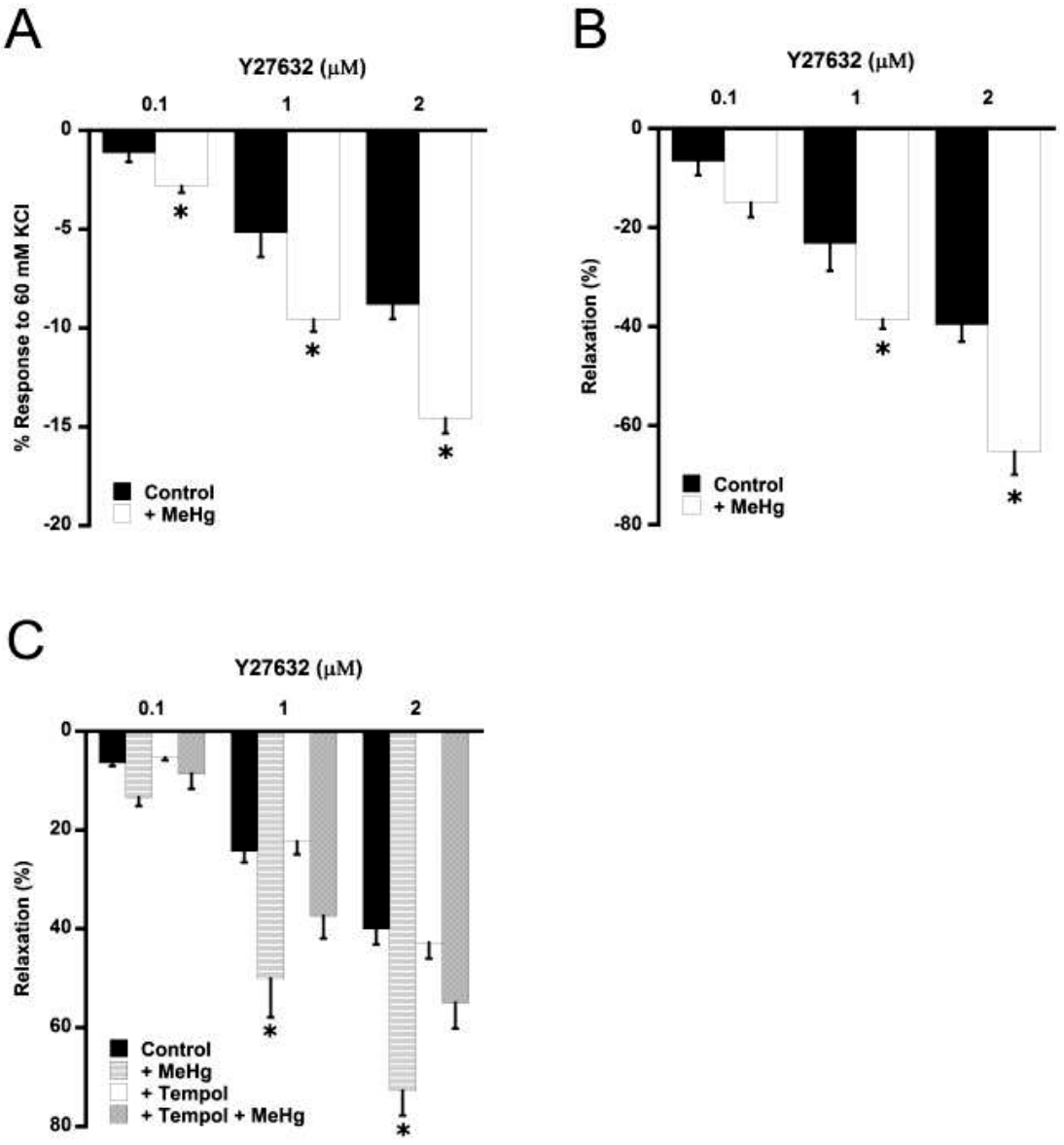

Fig. 10. Effect of Y27632 on myogenic tone in the isolated basilar artery. The direct effect of Y27632 on the isolated basilar artery was measured at resting tension [A] and in the pre-contracted condition [B]. Y27632-induced relaxation was also observed in the basilar artery from mice concomitantly treated with MeHg and tempol (2 mM) [C]. Each point represents the mean ± SEM of five mice. *P < 0.05 vs. control.

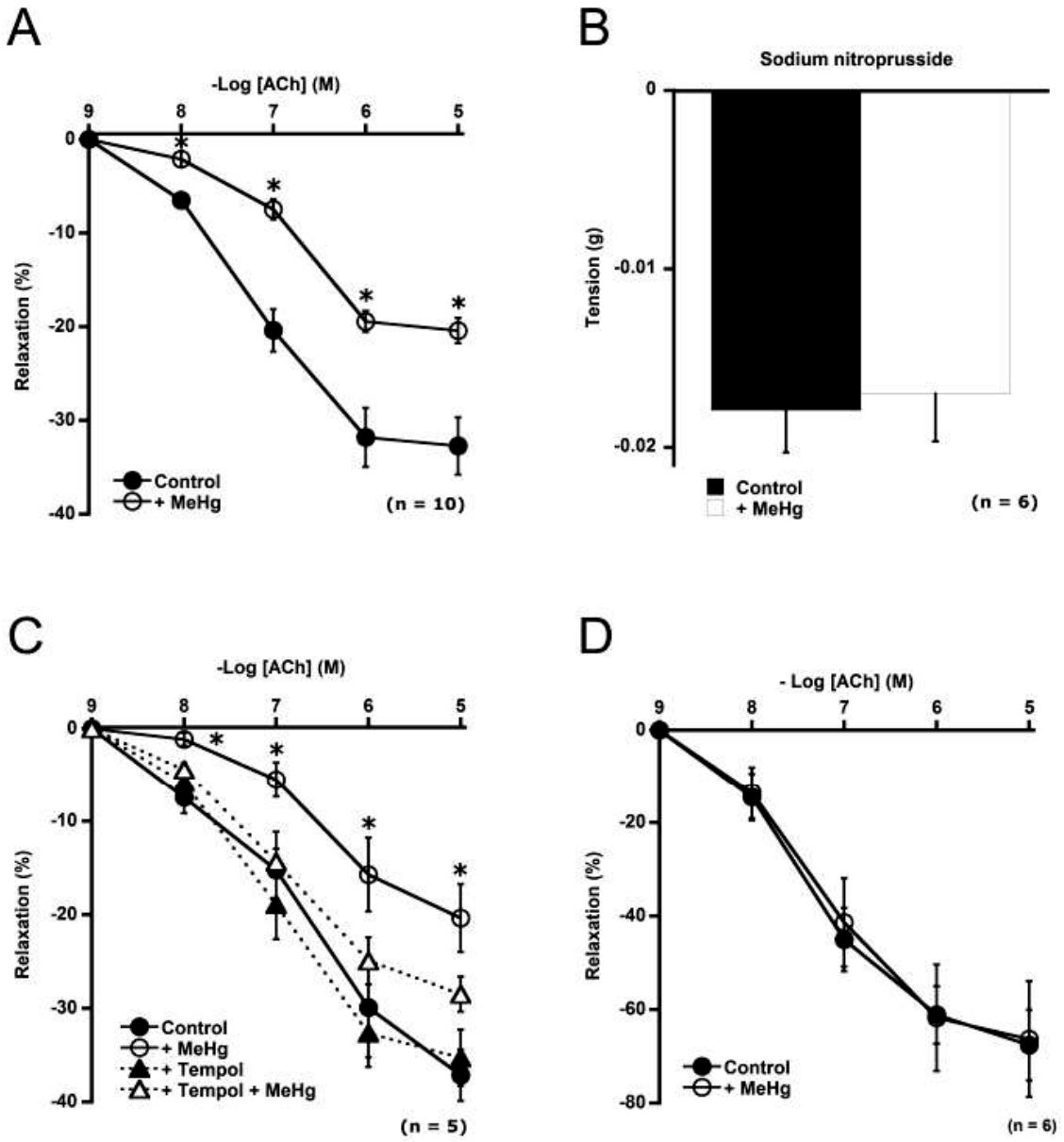

Fig. 11. Effect of *in vivo* MeHg treatment on ACh-induced relaxation in the basilar artery and thoracic aorta. The effect of MeHg on ACh- and SNP-induced relaxation in the isolated mouse basilar artery ([A] and [B], respectively), and on ACh-induced relaxation in the thoracic aorta [D] was observed. ACh-induced relaxation was also observed in the basilar artery from mice concomitantly treated with MeHg and tempol (2 mM) [C]. Each point represents the mean ± SEM. *P < 0.05 vs. control.

Chapter 2

Study 3

Bradykinin induces NO and $PGF_{2\alpha}$ production via B_2 receptor activation from cultured porcine basilar arterial endothelial cells

1. ABSTRACT

Our previous *in vitro* study demonstrated that bradykinin (BK) induced relaxation and contraction of porcine basilar artery (PBA) mediated via activation of endothelial B_2 receptors. The main relaxing and contracting factors appeared to be nitric oxide (NO) and prostaglandin (PG)H_2, respectively, but not thromboxane A_2. After obtaining these findings, we succeeded in cultivating endothelial cells isolated from the PBA. Although PGH_2 has different functionally active isoforms, including PGD_2, PGE_2 and $PGF_{2\alpha}$, we have not yet clarified which of them is responsible for BK-induced contraction. Therefore, we attempted to quantify NO and PG production from cultured porcine basilar arterial endothelial cells (PBAECs) and to identify which of the PGs was involved in this contraction. The cultured PBAECs produced NO spontaneously, and BK enhanced this production in a concentration-dependent manner. The NO synthase inhibitor Nω-nitro-L-arginine (L-NNA) and the B_2 receptor antagonist HOE140, but not the B_1 receptor antagonist des-Arg^9-[Leu^8]-BK, completely abolished it. In a functional study, PGD_2, PGE_2 and $PGF_{2\alpha}$ induced concentration-dependent contractions in isolated porcine basilar arterial rings, the order of maximum contraction being $PGF_{2\alpha} > PGE_2 > PGD_2$. The cultured PBAECs produced PGD_2, PGE_2 and $PGF_{2\alpha}$ spontaneously, and BK significantly enhanced the production of $PGF_{2\alpha}$, but not that of PGD_2 and PGE_2. The B_2, but not B_1, antagonist completely abolished the BK-enhanced production of $PGF_{2\alpha}$. These results suggest that BK induces production of NO and $PGF_{2\alpha}$ simultaneously from PBAECs via B_2 receptor activation.

2. INTRODUCTION

Through autoregulation, the brain is able to maintain a relatively constant cerebral blood flow, despite changes in perfusion pressure [3]. It is known that the basilar artery is able to produce larger amounts of nitric oxide (NO) and prostaglandins (PGs) than other arteries. For example, it has been reported that spontaneous NO production from porcine basilar arterial endothelial cells (PBAECs) is 4 times higher than that from abdominal aortic endothelial cells [72]. Spontaneous production of thromboxane (TX) B_2 (a stable metabolite TXA_2) from the porcine basilar artery (PBA) is also 4 times higher than that from the mesenteric artery [76]. Abundant production of NO and TXA_2 might allow maintenance of vascular homeostasis through changes in their relative amounts. In the PBA, bradykinin (BK) may also potentially contribute to autoregulation, as BK induces relaxation followed by contraction via B_2 receptors on endothelial cells [74, 75]. BK is a proinflammatory peptide that mediates a variety of physiological and pathophysiological responses [38, 63]. BK increases during inflammation, because of augmented release from inflammatory cells, including macrophages, neutrophils, and monocytes [62] and might disrupt vascular homeostasis. We have previously reported that the main endothelium-derived contracting factor (EDCF) in the PBA might be PGH_2, and not endothelin, leukotrienes or TXA_2, and that the endothelium-derived relaxing factor (EDRF) may be NO [75]. In the basilar arteries of other animals, it has been reported that the main EDRF produced in response to BK may be NO in rat [98], dog [114] and human [43], and that the main EDCF produced in response to BK may be TXA_2 in dog [104]. However, there is no direct or quantitative evidence of BK-induced NO production from cultured basilar arterial endothelial cells from animals of any species. Moreover, PGH_2 is converted to biologically active vasoconstrictive PGE_2, PGD_2, $PGF_{2\alpha}$ and TXA_2, and there is no information to indicate which of these specific PGs evokes contraction in response to BK. Therefore, the primary goal of the present study was to confirm quantitatively that BK enhances NO production as an EDRF, and then using cultured PBAECs our second goal was to quantify and specify the PG isoform responsible for BK-induced contraction. It was anticipated that quantitative and specific analysis of NO and PGs might provide a useful index of pharmacological, physiological, and pathological mechanisms operating in the cerebral circulation.

3. MATERIALS AND METHODS

3.1. Tissue preparation

Basilar arteries were obtained from freshly slaughtered pigs (both sexes, about 6 or 7 months old, Landrace-Large White-Duroc crossbreed) at a local abbatoir and transferred to our laboratory in ice-cold physiological saline (119 mM NaCl, 4.7 mM KCl, 1.6 mM CaCl2, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, and 10 mM glucose, pH 7.4) aerated with carbogen (95% (v/v) O_2, 5% (v/v) CO_2). Each artery was dissected free of adherent tissues. All experiments were performed in accordance with the Guideline for Animal Experiments of Kagoshima University.

3.2. Reagents

We used the following reagents: BK, des-Arg^9-[Leu^8]-BK, Nω-nitro-L-arginine (L-NNA), Dulbecco's Modified Eagle Medium (DMEM), nutrient mixture F-12 HAM, penicillin, streptomycin and amphotericin B (Sigma-Aldrich Co, Saint Louis, MO, USA), heat-inactivated horse serum (Invitrogen Corp., NY, USA), a NO_2/NO_3 assay kit (Wako, Osaka, Japan), fluorescent acetylated low-density lipoprotein (Harbor Bio-Product, Norwood, MA, USA), PGD_2, PGE_2 and $PGF_{2\alpha}$ EIA kits (Cayman Chemical Company, Ann Arbor, MI), and HOE140 (Peptide Institute, Inc. Osaka, Japan). All drugs were dissolved in distilled water.

3.3. Functional study

Several (3 or 4) rings approximately 4 mm long were cut from each basilar artery. The rings were mounted vertically between two L-shaped stainless steel holders, with the upper part fixed to an isometric force transducer (TB-611T, Nihon Kohden Kogyo, Tokyo, Japan), and immersed in a 5-ml water-jacketed organ bath containing oxygenated salt solution at 37 ˚C (pH 7.4). Each suspended ring was left to equilibrate for at least 120 min under a resting tension of 0.75 g. This tension was chosen because it allowed us to induce maximum contractions in the artery. KCl (60 mM) was applied every 30 min until the amplitude of the contraction reached a constant value. Changes in the KCl concentration of the physiological saline were compensated for by equimolar adjustment of the NaCl concentration. The isometric tension was recorded with an amplifier (AP-621G, Nihon Kohden Kogyo, Tokyo, Japan), digitized with an analog-digital converter (PowerLab/8SP,

AD Instruments Co., Castle Hill, NSW, Australia), and stored on the hard disk of a personal computer. The cumulative concentration-response curves of the PGs were obtained by adding a solution of each PG directly to the fluid in the bath. The contraction responses were calculated as percentages of the response produced by 60 mM KCl, which was taken as 100%. The presence of endothelial cells was confirmed pharmacologically by testing the relaxant response to sodium fluoride (10^{-2}–3×10^{-2} M), which is abolished by endothelial denudation [75].

3.4. Cell culture

Primary PBAECs were isolated by infusing 0.05% trypsin-EDTA solution into the vessel through a polyethylene tube (SP10, I.D. 0.28 mm, O.D. 0.61 mm, Natsume, Tokyo, Japan) and cultured in a growth medium containing 45% DMEM, 45% nutrient mixture F-12 HAM, 10% horse serum and an antibiotic mixture comprising 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B. The method employed has already been reported by our group [72]. Endothelial cells were characterized by their morphology using phase-contrast microscopy (Olympus, IX70, Tokyo, Japan) and by staining for fluorescent acetylated low-density lipoprotein [72]. Endothelial cells that had been cultured for less than 6 passages were used.

3.5. Quantification of NO and PGs

When the nitrite (NO_2^-) and nitrate (NO_3^-) level was measured as an indicator of NO production, and PG isoforms was measured, a mixed medium of 50% DMEM and 50% F-12 HAM without phenol red was used to avoid disturbance of the fluorometric assay [72]. Confluent endothelial cells ($3\text{-}5\times10^6$) were treated with experimental drugs. Sampling times for quantifying spontaneously produced NO were set at 0, 6, 12 and 24 h after changing the medium. With this assay kit, the minimum detectable dose of NO_2/NO_3 is <1 μM, and the coefficient of variation is 3.5 ± 0.5%. The concentrations of the different PG isoforms in the PBAEC culture medium were measured using the supernatant employing a specific ELISA, in accordance with the instructions provided by the manufacturer. The data were analyzed using a computer spreadsheet provided on the manufacturer's website. The sampling times for quantifying the PGs were set at 0 and 60 min after changing the medium.

3.6. Statistical analysis

Results are expressed as the mean ± SEM. Statistical analyses were performed by Student's *t* test or the Bonferroni test after one-way analysis of variance (Stat View J-4.5, Abacus Concepts Inc., USA). Significance was established when the probability level was equal to or less than 5%.

4. RESULTS

4.1. Spontaneous NO production from cultured PBAECs and the effect of BK upon it

As shown in Fig. 12A, the cultured PBAECs produced NO spontaneously in a time-dependent manner, and BK significantly enhanced this NO production in a concentration-dependent manner.

4.2. Effect of B_1 and B_2 antagonists and L-NNA on BK-enhanced NO production

A B_2 receptor antagonist, HOE140, and a NO synthase inhibitor, L-NNA, completely abolished BK (10^{-7} M)-enhanced NO production, whereas a B_1 receptor antagonist, des-Arg^9-[Leu^8]-BK, had no significant effect on it (Fig. 12B).

4.3. Responsiveness to PGD_2, PGE_2 and $PGF_{2\alpha}$

Fig. 13A shows the concentration-response curves for PGD_2, PGE_2 and $PGF_{2\alpha}$ in isolated porcine basilar arterial rings. All three PGs induced concentration-dependent contractions. The maximum contraction induced by $PGF_{2\alpha}$ was significantly stronger than those induced by PGD_2 and PGE_2. The pEC_{50} values for PGE_2 and $PGF_{2\alpha}$ were significantly higher than that for PGD_2 (Table 2).

4.4. Effect of BK on production of PGD_2, PGE_2 and $PGF_{2\alpha}$

The cultured PBAECs spontaneously produced PGD_2 (1.90 ± 0.25, n=3), PGE_2 (4.10 ± 0.29, n=3) and $PGF_{2\alpha}$ (3.89 ± 0.66, n=3) for 1 h. BK significantly enhanced the production of $PGF_{2\alpha}$ (7.50 ± 0.59, n=3), but not that of PGD_2 (2.30 ± 0.35, n=3) or PGE_2 (4.35 ± 0.37, n=3) (Fig. 13B).

4.5. Effects of B_1 and B_2 antagonists on $PGF_{2\alpha}$ production from cultured PBAECs

The B_2 receptor antagonist, HOE140, but not the B_1 receptor antagonist, des-Arg^9-[Leu^8]-BK, completely abolished BK-enhanced $PGF_{2\alpha}$ production from PBAECs (Fig. 14). Neither the B_1 nor the B_2 antagonist had any significant effect on the spontaneous production of PGD_2 and PGE_2 (data not shown).

5. DISCUSSION

To our knowledge, this is the first study to have demonstrated quantitatively that BK induces production of NO and $PGF_{2\alpha}$ simultaneously via endothelial B_2 receptors. One kind of receptor, B_2, appears to mediate the opposing vasoactive activities. It has been reported that stimulation of B_2 receptors activates the NO synthase pathway [36] and the cyclooxygenase pathway of arachidonic acid [12] via heterotrimeric G-proteins of the Gi and Gq family [77]. The stimulation of Gi protein production by BK can potentially decrease the production of cAMP via inhibition of adenylyl cyclase activity, leading to production of arachidonic acids, whereas Gq protein can activate phosphoinositide-specific phospholipase C, which mobilizes intracellular calcium via hydrolysis of phosphatidylinositol 4,5-bisphosphate. Intracellular calcium is necessary for NO production [77]. In PBAECs, BK might activate these two signaling pathways, leading to production of NO and $PGF_{2\alpha}$.

Available reports describing the mechanisms involved in BK-induced relaxation have been based mostly on *in vitro* functional experiments using basilar arteries from rat [98] and dog [60]. Our present results based on cell culture experiments directly indicate that BK stimulates production of NO via activation of endothelial B_2 receptors.

PGD_2 is known to be a strong vasodilator of the human middle cerebral artery [120]. PGE_2 has been reported to induce both contraction and relaxation of basilar arteries isolated from human [86]. In other cerebral vascular beds, PGE_2 is known to act as a vasodilator [4] or a vasoconstrictor in porcine circle of Willis arteries [52]. $PGF_{2\alpha}$ is a potent constrictor of basilar arteries isolated from dog [45], rhesus monkey [96] and human infant (gestational age 30-40 weeks) [9], but low concentrations of $PGF_{2\alpha}$ produce endothelium-dependent relaxation in monkey cerebral artery [59]. In our present study, however, all of the three PGs induced contractions of the PBA, as shown in Fig. 13A. The maximal contractions induced by the PGs, and their pEC_{50} values, suggested that $PGF_{2\alpha}$

was the most potent in the PBA. $PGF_{2\alpha}$ is one of the most abundant PGs in the brain [124].

As we were unable to identify which of the PGs was involved in PBA contraction purely on the basis of our *in vitro* experiment, we attempted to quantify PG production from cultured PBAECs. BK had no significant effect on production of PGE_2 and PGD_2, but significantly increased production of $PGF_{2\alpha}$, as shown in Fig. 13B. These findings suggested that $PGF_{2\alpha}$ is the main BK-induced contracting factor in the PBA. Although there is no information regarding the specific PGs released in response to BK in basilar or cerebral arterial endothelial cells, our result is partially consistent with that of a previous study [85], which showed that BK stimulated the production of PGE_2 and $PGF_{2\alpha}$, but not PGD_2, in bovine coronary arterial endothelial cells. Another study [67] also reported human endothelial cells synthetize 7 times higher amount of $PGF_{2\alpha}$ than PGD_2 and PGE_2 in normal conditions. Endothelial cells vary in the metabolites of arachidonic acid that they produce depending upon the species of animal and vascular origin of the cells. Also, endothelial cells from different vascular beds respond differently to specific vasoactive agents. We also investigated the effects of both B_1 and B_2 antagonists on the basal production of PGE_2 and PGD_2. Neither of these antagonists had significant effect on PGD_2 or PGE_2 production. BK enhanced the production of $PGF_{2\alpha}$ from cultured PBAECs about two-fold. Pretreatment with the B_2 antagonist HOE140 abolished BK-enhanced $PGF_{2\alpha}$ production, whereas the B_1 antagonist des-Arg^9-[Leu^8]-BK had no such effect. Our present results directly indicate that BK stimulates production of $PGF_{2\alpha}$ via activation of endothelial B_2 receptors in cell culture. This result is consistent with our previous *in vitro* study, which showed that BK-induced contraction was inhibited by [$Thi^{5,8}$, D-Phe^7]-bradykinin (B_2-receptor antagonist) [75].

In conclusion, our present study indicates that BK stimulates the production of NO and $PGF_{2\alpha}$ simultaneously as relaxation and contraction factors, respectively, via B_2 receptor activation from PBAECs under physiological conditions. Further studies conducted under pathological conditions or using animal models of different diseases will be needed.

Table 2. pEC_{50} values and maximal responses (Max) to PGs.

Prostaglandins	**pEC_{50}**	**Max (%)**
PGD_2	5.79 ± 0.03*	55.0 ± 4.7**
PGE_2	7.22 ± 0.02	88.8 ± 5.4*
$PGF_{2\alpha}$	7.25 ± 0.02	119.9 ± 5.0

Contraction induced by 60 mM KCl was taken as 100%.

Each point represents the mean ± SEM of 5 animals.

*p<0.05, **p<0.01 vs $PGF_{2\alpha}$

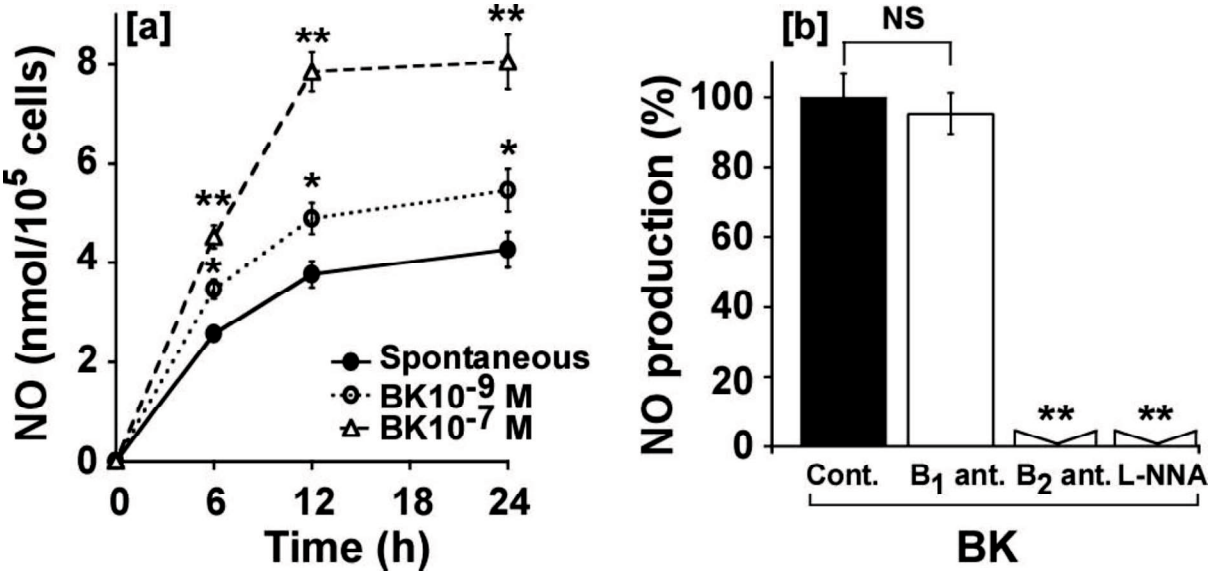

Fig. 12. Spontaneous production of NO (●) and the effect of BK (○, 10^{-9} M and △, 10^{-7} M) upon it [A] and effects of B_1 antagonist (des-Arg^9-[Leu^8]-BK, 10^{-6} M), B_2 antagonist (HOE140, 10^{-7} M), and L-NNA (10^{-4} M) on BK (10^{-7} M)-enhanced NO production from cultured porcine basilar arterial endothelial cells. BK-enhanced NO production during 12 h was taken as 100% [B]. Each point represents the mean ± SEM for 3 independent experiments in triplicate. *p<0.05, **p<0.01 vs control, NS: not significant.

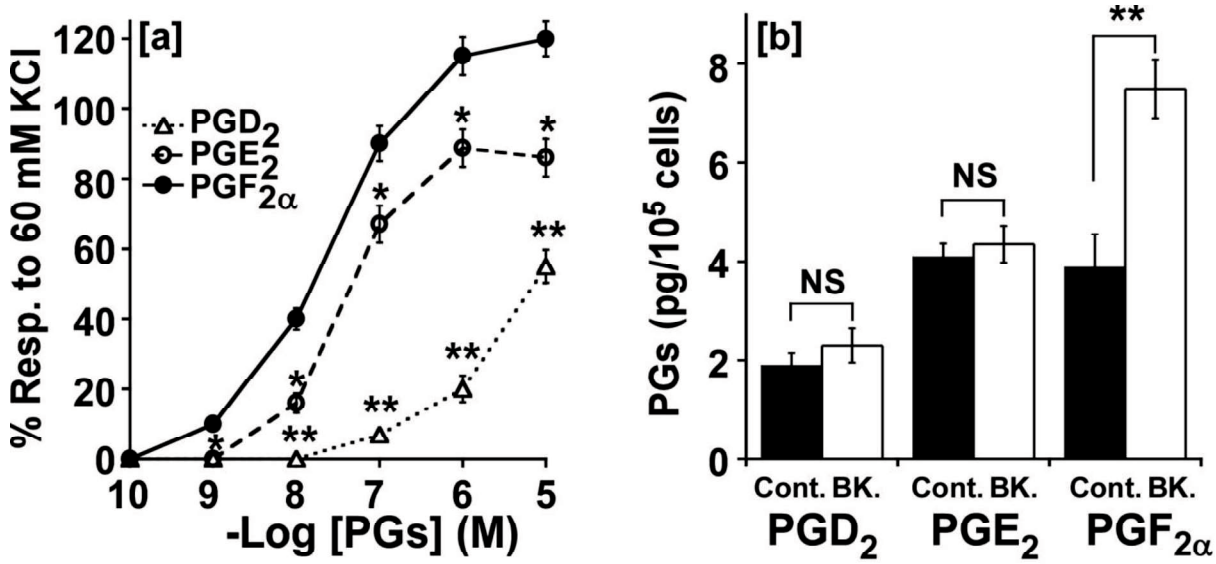

Fig. 13. Concentration–response curves for PGD_2 (△), PGE_2 (○) and $PGF_{2\alpha}$ (●) in isolated porcine basilar arterial rings [A] and effect of BK (10^{-7} M, □) on spontaneous PGD_2, PGE_2 and $PGF_{2\alpha}$ production (■) from cultured porcine basilar arterial endothelial cells after 1 h of treatment. Each point represents the mean ± SEM for 5 animals [A] and 3 independent experiments in triplicate [B]. *$p<0.05$, **$p<0.01$ vs control, NS: not significant.

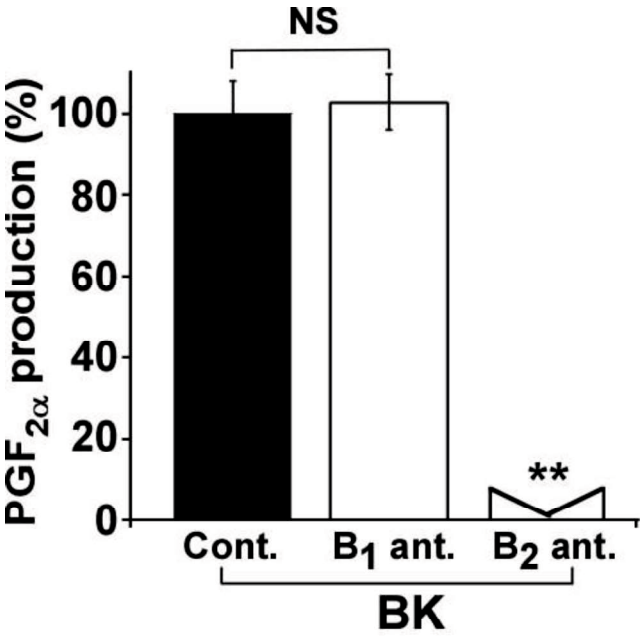

Fig. 14. Effects of B_1 antagonist (des-Arg^9-[Leu^8]-BK, 10^{-6} M) and B_2 antagonist (HOE140, 10^{-7} M) on BK (10^{-7} M)-enhanced $PGF_{2\alpha}$ production from cultured porcine basilar arterial endothelial cells after 1 h of treatment. BK-enhanced $PGF_{2\alpha}$ production was taken as 100%. Each point represents the mean ± SEM. for 3 independent experiments in triplicate. **$p<0.01$ vs control, NS: not significant.

Chapter 2

Study 4

Angiotensin II alter bradykinin-induced biphasic response by decreasing endothelial NO and increasing $PGF_{2\alpha}$ release from the Microminipig basilar artery

1. Abstract:

Angiotensin II (Ang II) is known to promote vascular disease and hypertension in part by its effect on vascular endothelium. Endothelial dysfunction might affect blood pressure and vascular activity to endothelial dependent vasoactive substances. In this study, we evaluated the effect of intravenous infusion of Ang II on basilar arterial responsiveness to bradykinin (BK) in microminipig (MMPig). Ang II (200 ng. kg^{-1} min^{-1}) or vehicle was infused for two weeks by using osmotic mini-pump and the blood pressure was monitored regularly. Both systolic and diastolic blood pressure were significantly increased in Ang II-infused MMPig compared to vehicle-infused group. The resting vascular tone of the isolated basilar artery had not changed between the groups. However, BK-induced dilation were abolished and the contraction was significantly enhanced in isolated basilar artery from Ang II-infused MMPig. Later, endothelial cells from the MMPig basilar artery were cultured and stimulated with Ang II (10^{-7} M) or vehicle for 48 hours. BK-induced NO production was significantly decreased but $PGF_{2\alpha}$ production was increased in Ang II stimulated cells than the vehicle treated cells. These results suggest that the decreased NO and increased $PGF_{2\alpha}$ production from the endothelial cells might be the causes of altered BK responses in Ang II-induced hypertension.

Key words: Hypertension, Cerebral artery, Bradykinin, NO, $PGF_{2\alpha}$, MMPig.

2. Introduction

The brain is critically dependent on a continuous and well-regulated blood supply to support its dynamic needs for oxygen and glucose and to remove metabolic by-products of brain activity [83]. Complex regulatory mechanisms ensure that the brain receives sufficient cerebral blood flow to maintain the homeostasis of the cerebral microenvironment. Hypertension disrupts these control mechanisms and increases the susceptibility of the brain to vascular insufficiency [49].

The renin-angiotensin system, and particularly angiotensin (Ang) II underlie many of the changes in vascular structure and function that occur in animal's models of hypertension as well as patients with hypertension. Ang II promotes atherosclerosis and vascular disease in the presence of other cardiovascular risk factors including diabetes and aging [23]. It produces cerebrovascular inflammation and remodeling, and impairs cerebral blood flow regulation [27]. Intravenous infusion of Ang II acutely increases blood pressure, levels of reactive oxygen species (ROS) [13, 14], and impairs vascular function including endothelium dependent responses in brain [13, 14] or enhancement of vasoconstrictor response to different agonists [110].

The endothelium, recognized as a fundamental homeostatic organ for the regulation of the vascular tone and structure. Under physiologic conditions, endothelial stimulation induces the production and release of NO, which exerts its cardiovascular protective role [99]. In disease conditions, the endothelium undergoes functional and structural alterations and loses its protective role, becoming a pro-atherosclerotic structure. The loss of the normal endothelial function is characterized by impaired NO bioavailability. This can follow either a reduced production of NO by eNOS [99]. When NO availability is significantly reduced, the endothelium activates various compensatory physiological pathways. Of importance, a dysfunctioning endothelium also becomes a source of other substances and mediators which are detrimental to the arterial wall, including endothelin-1, thromboxane A_2 (TXA_2), prostaglandin (PG) H_2 and ROS [99], with various pro-atherosclerotic features, including a vasoconstricting action.

It is generally accepted that prostanoids play a key role in the vascular alterations associated with hypertension [35]. The specific prostanoid involved in these alterations depends on the blood vessel studied. PGH_2, $PGF_{2\alpha}$ and TXA_2 are mainly responsible for the endothelial dysfunction in hypertension [35].

Bradykinin (BK) is a proinflammatory peptide that mediates a variety of physiological and pathophysiological responses [38, 63]. In PBA, BK is a unique endothelium dependent agonist which induce relaxation followed by contraction via NO and $PGF_{2\alpha}$ respectively [51, 75]. BK increases during inflammation, because of augmented release from inflammatory cells, including macrophages, neutrophils, and monocytes [62] and might disrupt vascular homeostasis. In this study, we hypothesized that Ang II dependent hypertension might affect endothelium dependent biphasic response of BK by altering NO and $PGF_{2\alpha}$ production.

An appropriate animal model that reproduces human physiology and pathology would be ideal for investigating hypertension. Swine represent a potentially useful non-rodent animal model because, their anatomy, physiology and habits of feeding and sleep are very similar to those of human. It is well known that porcine models often relate well to human conditions, [71] and of the major cerebral arteries in non-primates, the basilar artery is perhaps most like that in humans [37]. It is reported that pig develop chronic pulmonary hypertension similar to that found in human patients [88]. The MMPig has recently been established as an experimental animal. This is the world's smallest pig, which has very close similarities to human with respect to lipid metabolism and atherogenesis [58]. Therefore, in the present study, we use this newly develop MMPig to study Ang II dependent hypertension, its effect and mechanisms on cerebral artery.

3. Materials and Methods

3.1. Animal preparation

Adult male MMPigs were used. The sinus venarum cavarum catheterization was performed. Two groups were designed: Saline and Ang II groups. MMPigs were infused with saline or Ang II at a dose rate of 200 ng. kg^{-1} min^{-1} for 14 days by using osmotic minipump. Blood pressures were regularly monitored at the designated time of the day. All experiments were performed in accordance with the Kagoshima University Guidelines for Animal Experimentation.

3.2. Tissue preparation

MMPigs were sacrificed under anesthesia. The brain was removed from the cranial cavity. The basilar arteries were then gently isolated from the brain and transferred to ice-

cold physiological saline (119 mM NaCl, 4.7 mM KCl, 1.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, and 10 mM glucose, pH 7.4) aerated with carbogen (95% (v/v) O_2, 5% (v/v) CO_2). Each artery was immediately dissected free of adherent tissues under a stereomicroscope.

3.3. Reagents

We used the following reagents: Angiotensin (Ang) II acetate salt, bradykinin (BK) acetate salt, Nω-nitro-L-arginine (L-NNA, a NO synthase inhibitor), indomethacin, sodium nitro prusside (SNP), Dulbecco's modified Eagle's medium (DMEM), nutrient mixture F-12 HAM and trypsin–EDTA (Sigma-Aldrich, Saint Louis, MO, USA), Clonetics® EMB®-2 cell culture medium (Lonza, Walkersville, MD USA), NO_2/NO_3 assay kit (Wako, Osaka, Japan) and $PGF_{2\alpha}$ EIA kit (Cayman Chemical Company, Ann Arbor, MI). All drugs were dissolved in distilled water.

3.4. Functionan studies

Several rings (3-4) approximately 4 mm long were cut from each basilar artery. Each ring was mounted horizontally between two L-shaped stainless steel holders (outer diameter 0.05 mm), with one part fixed to an isometric force transducer, and immersed in a 4-ml water-jacketed micro tissue organ bath (UMTB-1, Unique Medical Co. Ltd, Tokyo, Japan) containing oxygenated salt solution at 37°C (pH 7.4). Each suspended ring was left to equilibrate for at least 120 min under a resting tension of 0.5 g. This tension was chosen because it allowed us to induce maximum contractions in the artery. KCl (60 mM) was applied every 30 min until the amplitude of the contraction reached a constant value. Changes in the KCl concentration of the physiological saline were compensated for by equimolar adjustment of the NaCl concentration. The isometric tension was recorded with an amplifier (AP-621G, Nihon Kohden Kogyo, Tokyo, Japan), digitized with an analogue-digital converter (PowerLab/8SP, ADInstruments Co., Castle Hill, NSW, Australia), and stored on the hard disk of a personal computer. The cumulative concentration-response curve of BK was obtained by adding a solution of BK directly to the fluid in the bath. Before application of BK, basilar arteries were precontracted submaximally (50-60% of the response to 60 mM KCl) with the thromboxane mimetic U-46619. BK-induced responses were calculated as the percentages of response produced by 60 mM KCl.

3.5. Cell culture studies

Primary MMPigs basilar arterial endothelial cells were isolated by infusing 0.05% trypsin–EDTA solution into the vessel lumen through a polyethylene tube (SP10, I.D. 0.28 mm, O.D. 0.61 mm, Natume, Tokyo, Japan) and cultured in Clonetics® EMB®-2 medium. When the nitrite (NO_2^-) and nitrate (NO_3^-) level was measured as an indicator of NO production, and $PGF_{2\alpha}$ was measured, a mixed medium of 50% DMEM and 50% F-12 HAM without phenol red was used to avoid disturbance of the fluorometric assay [70]. The endothelial cells were characterized by their morphology using phase-contrast microscopy (Olympus, IX70, Tokyo, Japan) and by staining for fluorescent acetylated low-density lipoprotein [112]. Only endothelial cells of less than 6 passages were used. Confluent endothelial cells ($3\text{-}5\times10^6$) were treated with experimental drugs. In this assay kit, the minimum detectable dose of NO2/NO3 is <1 μM with a coefficient of variation of 3.5±0.5%. The concentration of $PGF_{2\alpha}$ in the PBA endothelial cell culture medium were measured from the supernatant using $PGF_{2\alpha}$ measuring ELISA kit according to instructions provided by the manufacturer (Cayman Chemical Company, Ann Arbor, MI). The data were analyzed using a computer spreadsheet provided on the manufacturer's website. Sampling times for quantifying NO and $PGF_{2\alpha}$ were set at 0, 6, 12 and 24 h after changing the medium.

3.6. Statistical analysis

Results are expressed as means ± SEM. Statistical analyses were performed by Student's *t* test or the Bonferroni test after one-way analysis of variance (Stat View J-4.5, Abacus Concepts Inc., Berkeley, California, USA). Significance was established when the probability level was equal to or less than 5%.

4. Results

4.1. Effect of in vivo infusion of Ang II on blood pressure

Both systolic and diastolic blood pressure was significantly increased in Ang II infused group than the saline infused group. Blood pressure starts increasing from the first day of infusion and remains persistent until day 14 (Fig. 15).

4.2. Responsiveness to L-NNA and Indomethacin

No significant difference had been observed in L-NNA (a NO synthase inhibitor)-

induced contraction (Fig. 16A) and indomethacin (a cyclooxygenase inhibitor)-induced relaxation (Fig. 16B) between the groups. *In vivo* infusion of Ang II did not change the resting vascular tone.

4.3. Responsiveness to BK, SNP and KCl

In Ang II-infused MMPig, BK-induced relaxation was completely abolished but significantly increased its contraction (Fig. 17A). However, No significant difference had been observed in SNP and 60 mM KCl-induced responses (Fig. 17B and 17C).

4.4. Effect of Ang II on cultured endothelial cells to BK-induced NO and $PGF_{2\alpha}$ production

Endothelial cells treated with Ang II (10^{-7} M) for 48 hours significantly decreased NO (Fig. 18) but increased $PGF_{2\alpha}$ (Fig. 19) production by BK (10^{-7} M) from the cultured MMPig basilar artery endothelial cells.

5. Discussion

This study demonstrated that *in vivo* infusion of Ang II increased both systolic and diastolic blood pressure and alter basilar arterial responsiveness to BK with decreased relaxation and increased contraction in MMPig. To our knowledge, this is the first demonstration that showed a direct evidence of significantly decreased NO and increased $PGF_{2\alpha}$ production from the Ang II treated basilar artery endothelial cells by BK.

In vivo infusion of Ang II has emerged as an important experimental model to investigate vascular biology. This model has the advantages of being technically simple to perform and yielding a highly reproducible vascular response. Like others, iv infusion of Ang II for 14 days significantly increased the systolic blood pressure of MMPigs in the present study.

The resting vascular tone of PBA is maintained by spontaneous release of NO and TXA_2 from the endothelial cells [72]. In the present study, no significant difference had observed in L-NNA- and indomethacin induced responses which indicated that the resting vascular tension had not changed. Similar findings had been also observed in some studies using Ang II dependent hypertension [23], diabetes, as well as nicotine and alcohol, produced impairment of endothelial function even without changes in baseline diameter of

vessels [29, 66]. It is interesting that baseline diameter of cerebral arterioles was not altered even though endothelial impairment had found. The explanation for these findings is not clear, but this might occur if the level of NO needed to change resting vascular tone is less than the concentration needed to alter baseline vascular tone.

Ang II-induced hypertension abolished BK-induced relaxation but enhanced its contraction of the isolated basilar artery of MMPig. In contrast, vasodilatation in response to sodium nitroprusside and vasoconstriction in response to 60 mM KCl were not affected, indicating that the effects of Ang II were selective for endothelium. It also indicates NO paucity rather insensitivity of smooth muscle to NO. Others have demonstrated that Ang II impairs endothelial function and decreased BK-induced relaxation in rabbit cerebral arterioles [26], attenuated the cerebral blood flow increase induced by ACh and BK in mice [14]. Decreased relaxation and enhanced contraction response might be the causes of significantly increased blood pressure or hypertension.

Previously, we have demonstrated that BK-induced endothelium dependent relaxation and contraction mediated by NO and $PGF_{2\alpha}$ respectively in PBA [51, 75]. Therefore, it was speculated that alteration in the production or synthesis of this mediators could explain the mechanism of impaired BK-induced biphasic response in Ang II-induced hypertension. So we cultured the MMPig basilar artery endothelial cells to measure NO and $PGF_{2\alpha}$ production by BK after Ang II stimulation.

Ang II treatment of the cultured MMPig basilar artery endothelial cells had significantly decreased BK-induced NO and increased $PGF_{2\alpha}$ production. A decline in NO may be due to the decrease release from the endothelium, or by alterations in cellular signaling involved in eNOS activation or decreased bioavailability by high production of superoxide due to Ang II induced oxidative stress. It is well known that Ang II induced oxidative stress through ROS generation. Superoxide can impair endothelium-dependent responses of cerebral blood vessels [26, 29]. The superoxide anion interacts with NO and forms peroxynitrite, thereby decreasing the NO bioavailability for smooth muscle relaxation [8]. Beside this, a previouse study [129], suggested a reduction in the net production of NO may occur due to the lack of the enzyme substrate L-arginine by enhanced activity of vascular arginase in some forms of experimental hypertension. Ang II-infused rat model [81] showed that NO in the aortic segments was markedly reduced. Thus, the dysfunctional

endothelium, characterized by a decrease in NO, appeared to lead to an increase in arterial blood pressure in Ang II-infused MMPig.

Beside a decreased in NO production, the striking observation of the present study is that significantly increased $PGF_{2\alpha}$ production by BK from Ang II treated cells which may also be responsible for impaired relaxation by BK. It is also generally accepted that prostanoids play a key role in the vascular alterations associated with hypertension [35]. Thus contractile prostanoids impaired endothelium-dependent vasodilator and increased vasoconstrictor responses observed in vessels from hypertension models [35]. Similarly, previous study [35, 102], also reported significantly increased release of $PGF_{2\alpha}$ by endothelium-dependent relaxant ACh in SHR and renovascular hypertensive rats respectively. It is also noted that the pathological role of $PGF_{2\alpha}$ has been recently revealed, as this PG increases blood pressure and promotes atherogenesis in mice [127]. Our previous report [75], demonstrated that BK-induced contraction of isolated PBA was inhibited by pretreatment with indomethacin (a non-selective COX inhibitor). The cyclooxygenase metabolite that is responsible for BK-induced contraction was further identified as $PGF_{2\alpha}$ [51]. In the present study, significantly increased BK-induced $PGF_{2\alpha}$ production by Ang II treated endothelial cells could be the effect of Ang II on cyclooxygenase pathway. Participation of COX-2-derived contractile prostanoids, in the reduction of the relaxation observed in middle cerebral arteries of SHRs [46]. This research showed the functional importance of cyclooxygenase derived $PGF_{2\alpha}$ in response to Ang II-induced hypertension.

The production of endothelium-derived prostanoids is augmented in arteries with dysfunctional endothelium [47], and hypertensive arteries [100]. In brief, the chain of events leading to endothelium-dependent contractions requires an abnormal increase in intracellular calcium in the endothelial cells [100]. The rise in calcium activates phospholipase A_2 to release arachidonic acid from the cell membrane phospholipids. Then COX breaks down arachidonic acid to form prostanoids that activate prostanoid receptors located in the vascular smooth muscle, resulting in contraction [100]. So, in the perspective research it would be necessary to investigate COX and NOS expression level in endothelial cells after Ang II treatment.

It is difficult to make a direct comparison/extrapolation from our *in vivo* infusion of Ang II data (200 ng. $kg^{-1}min^{-1}$ for 14 days) to those of cell culture (10^{-7} M for 48 h). This dose of infusion significantly increased systolic blood pressure in MMPig. Similar result

also observed in several previous investigation with same dose of Ang II infusion in rats [115] and mice [14] where it increased blood pressure and cause endothelial impairment. Like *in vivo* infusion, 14 days Ang II treatment is not possible in cultured cells. However, in our cell culture study, a single dose of Ang II (10^{-7} M) and a 48 h exposure period were selected because 10^{-7} M Ang II induced maximum contraction of isolated PBA [73]. In a previous study [130], we observed that a similar concentration of Ang II exposure of human vascular endothelial cells produced maximal level of oxidative stress. Ang II induces hypertension occurs as a result of prolong exposures, which are not reproducible *in vitro* cell culture experiment. However, our results have also important to clarify the mechanism of Ang II-induced hypertension and its effect on endothelial dependent agonist responses.

In this study, we introduce a promising experimental animal, the MMPig. In September 2010, legislation by the European Parliament has broadly banned the use of great apes such as chimpanzees, bonobos, gorillas, and orangutans for scientific testing. The Commission's draft law would also have in principle restricted the use of other non-human primates such as ouistitis and macaques. Therefore, substitute animals will be required hereafter, and the MMPig will be one of the promising candidates. In addition, the pig is a recognised model for several human cardiovascular diseases like atherosclerosis [58] and myocardial infarction [94]. While rodents can often be used for the evaluation of respiratory and CNS functions, cardiovascular investigations generally use non-rodent species. Pigs are similar to humans in their cardiovascular physiology, size, anatomy and the perfusion distribution of blood flows make minipigs better subjects than other species. Its heart-to-body weight ratio, its spontaneous development of atherosclerosis and its propensity towards obesity comparable to man.

In conclusion, we present here the first observation that a decrease in NO and increase in $PGF_{2\alpha}$ production by BK from the endothelial cells of Ang II infused MMPigs caused alteration in BK response in isolated basilar artery. Moreover, the model described here seems useful as an alternative of non-rodent experimental animal for understanding some important pathophysiological characteristics of hypertension, atherosclerosis and vascular biology research.

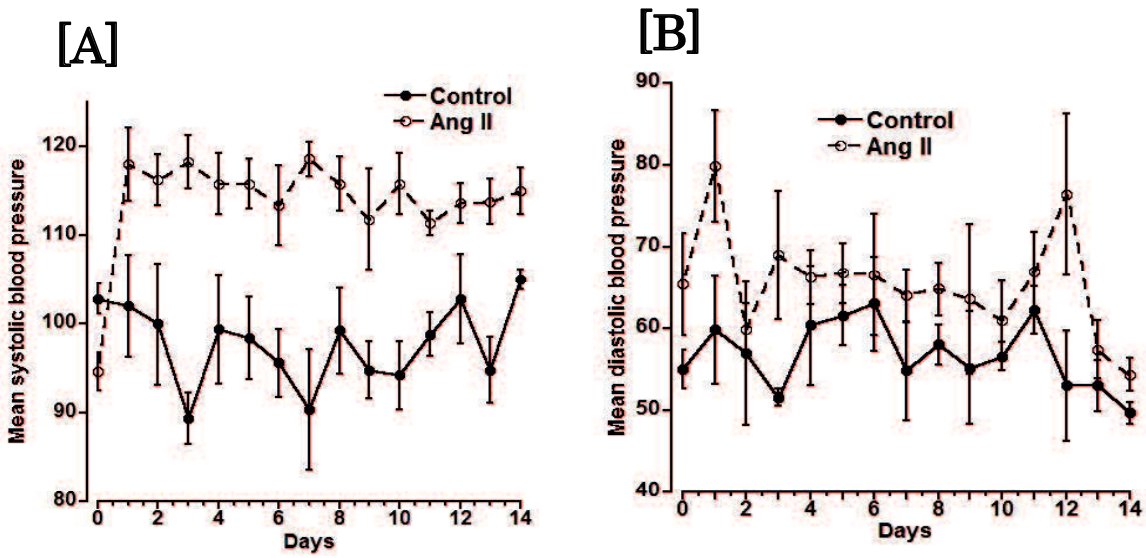

Fig. 15. Effect of *in vivo* infusion of Ang II (○) on mean systolic [A] and diastolic [B] blood pressure (●) of MMPigs. Both the systolic and diastolic blood pressure were increased by *in vivo* infusion of Ang II. Each point represents the mean ± SEM of 5 MMPigs.

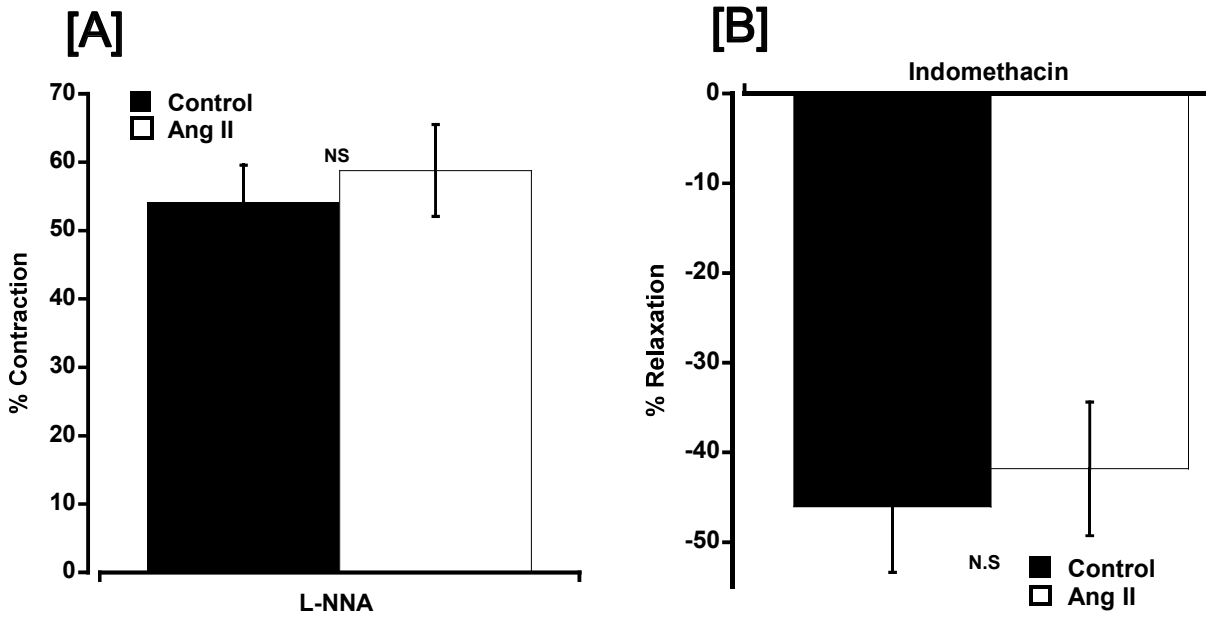

Fig. 16. Effect of *in vivo* infusion of Ang II (○) on L-NNA (a NO synthase inhibitor) [A], and indomethacin (a non-selective cyclogenase inhibitor) [B] on resting vascular tone of isolated basilar artery of MMPigs. *In vivo* infusion of Ang II did not change the resting vascular tone. Each point represents the mean ± SEM of 5 MMPigs. NS: not significant

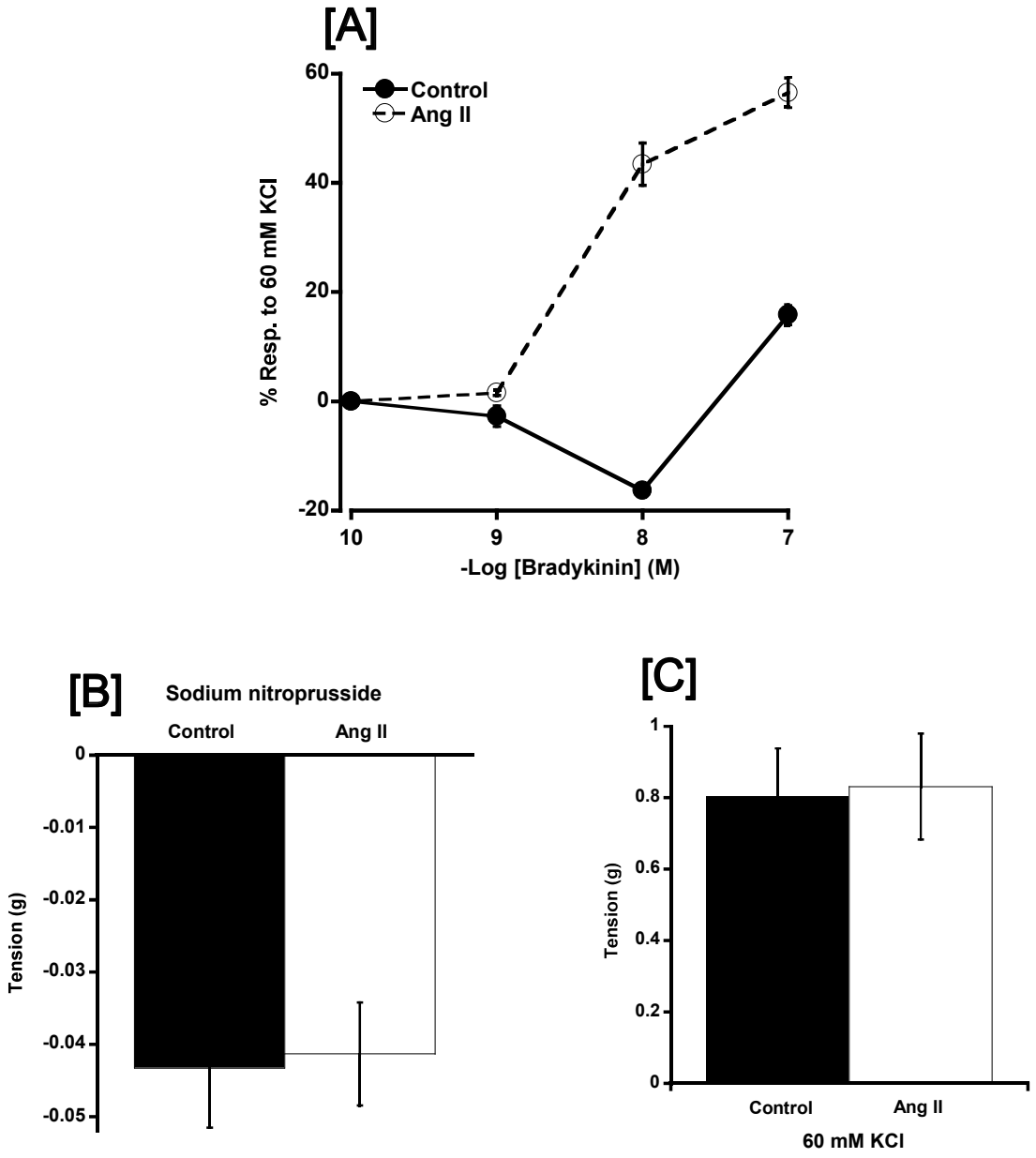

Fig. 17. Effect of *in vivo* infusion of Ang II (○) on BK-induced biphasic response (●) [A], SNP-induced relaxation [B] and 60 mM KCl-induced contraction [C]of isolated

basilar artery of MMPigs. Ang II specifically altered BK-induced biphasic response of isolated MMPig basilar artery. Each point represents the mean ± SEM of 5 MMPigs.

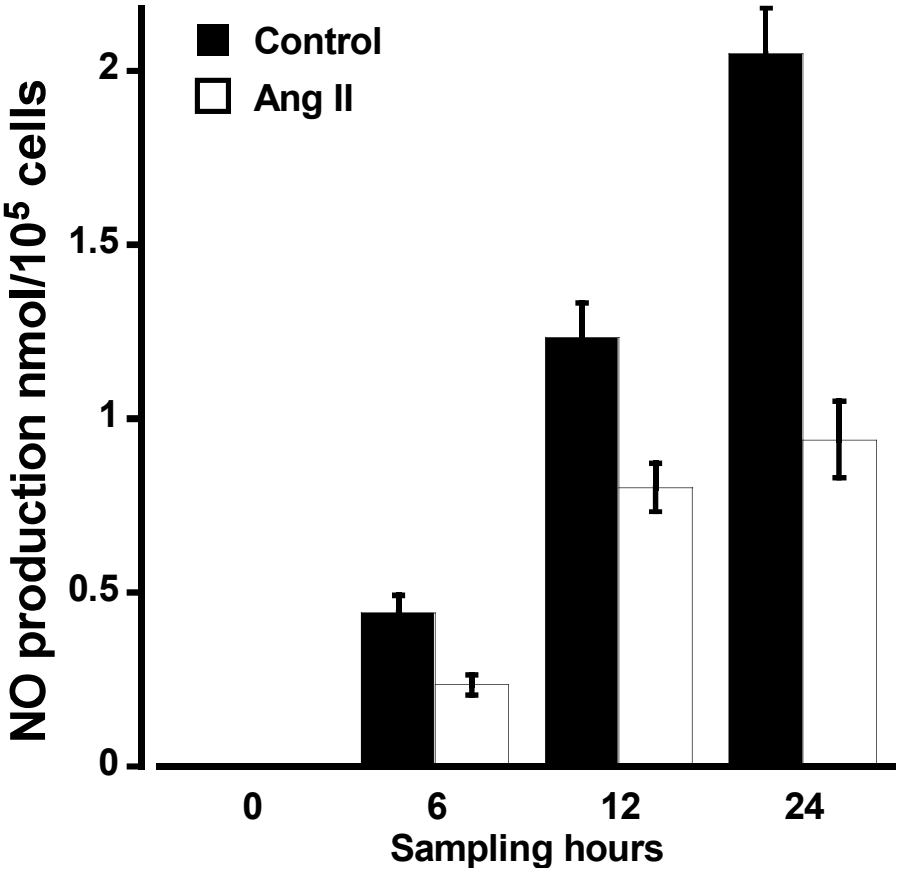

Fig. 18. Effect of Ang II treatment on BK-induced NO production from the cultured MMPigs basilar artery endothelial cells. Ang II significantly decreased BK-induced NO production from the cultured endothelial cells. Each point represents the mean±SEM of 3 independent experiments in triplicate.

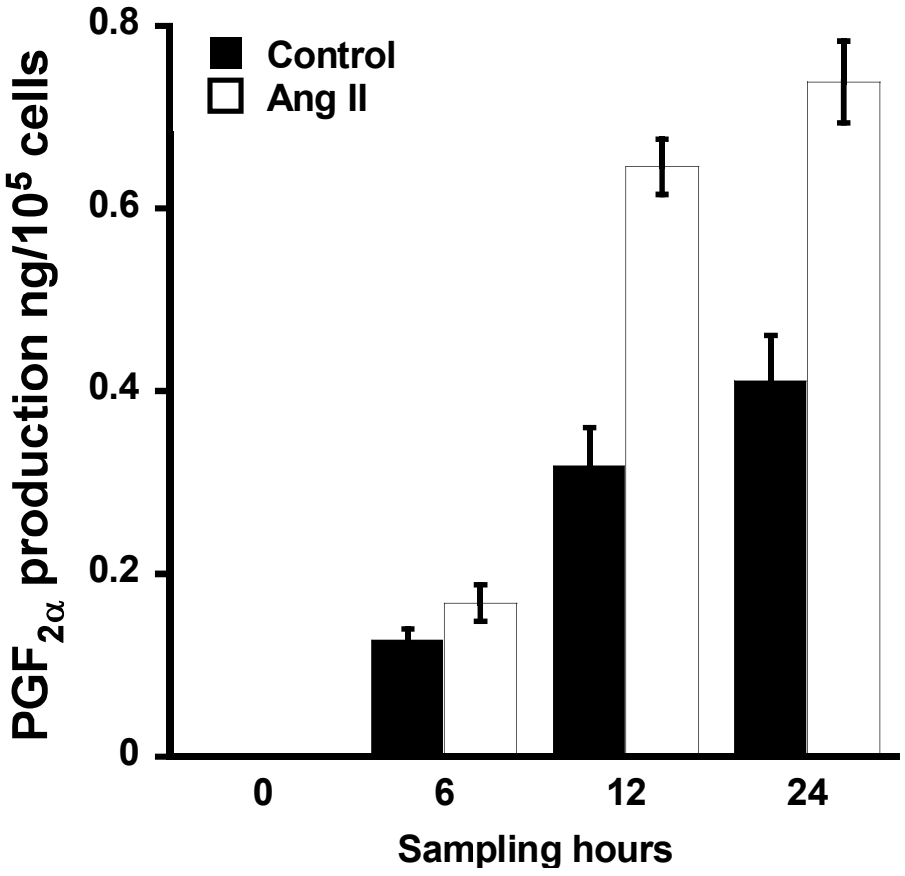

Fig. 19. Effect of Ang II treatment on BK-induced $PGF_{2\alpha}$ production from the cultured MMPigs basilar artery endothelial cells. Ang II significantly increased BK-induced $PGF_{2\alpha}$ production from the cultured endothelial cells. Each point represents the mean±SEM of 3 independent experiments in triplicate.

General discussion

The chapter 1, study 1, characterizes the responsiveness of the mouse basilar artery (MBA) to a number of pharmacological agents that are modulators of cerebrovascular circulation in both normal and pathological condition. MBA is responsive to ACh and BK with endothelium-dependent NO mediated relaxation, and to 5-HT, His and Ang II with contraction, but is unresponsive to NA. ACh-induced relaxation via activation of muscarinic receptor apart from M_1, M_2 and M_3 whereas, BK-induced relaxation via activation of B_2 receptor. His and Ang II induced contraction via H_1 and AT_1 receptor respectively in MBA. Mice are frequently used in cerebrovascular research, mice being particularly useful, because an increasing number of transgenic and knockout models are becoming available. Mice are often used as small animal models of brain ischemia, venous thrombosis or vasospasm, and Alzheimer's disease. My study provides the physiological responsiveness of the MBA to intrinsic vasoactive substances which could be altered in pathological condition. In order to reveal this point, studies on different pathological conditions which affect the cardiovascular function are required, from which a number of molecular and structural elements of cerebral disorders could be explain.

In chapter 1, study 2, MBA responsiveness was investigated in *in vivo* MeHg exposed mice. MeHg increased blood pressure, enhanced Ang II-induced contraction and impaired endothelium-dependent relaxation to ACh. Increased Ang II-induced contraction in MeHg exposed mice were due to the hyperactivation of Rho-kinase which was confirmed by observing significantly stronger dilation induced by Y27632 (a selective Rho-kinase inhibitor) in this group. ROS scavenger tempol recovered all the changes induced by MeHg in mice. This result suggested that ROS generation by MeHg plays the pivotal role in the alteration of vascular reactivity. As MeHg is an environmental pollutant, which is naturally present in the environment in a comparatively lower concentration and affect population for long period of time. So a lower dosage of MeHg with a longer duration of exposure is required in perspective study. Moreover, my study also showed that ROS and Rho-kinase play important role in vascular responsivensess. So it would be required to study the vascular responsiveness in diseases or toxicological conditions implicated with ROS generations and Rho-kinase activation.

In chapter 2, I investigated the vasoactive substances produced by BK in PBAECs. BK stimulates the production of NO and $PGF_{2\alpha}$ simultaneously as relaxation and contraction

factors, respectively, via B_2 receptor activation from PBAECs under physiological conditions. Since *in vivo* infusion of Ang II altered BK-induced biphasic response of the isolated basilar artery, I investigated the effect of Ang II on BK-induced NO and $PGF_{2\alpha}$ production from the cultured PBAECs. Cell culture study clearly indicated that Ang II decreased NO and increased $PGF_{2\alpha}$ production from the PBAECs.

Finally, the present thesis used a rodent (mice) and a non rodent animal (MMPig). Mice is frequently used laboratory animal for numerous human cerebrovascular disorders. My result would further extend the understanding of the pathophysiology of cerebrovascular diseases and therapeutic strategy. The present thesis introduces MMPig (registered with the Japanese of Agriculture, Forestry and Fisheries as a novel variety of swine) as a promising animal model for non-clinical pharmacological/toxicological use. In September 2010, legislation by the European Parliament has broadly banned the use of great apes such as chimpanzees, bonobos, gorillas, and orangutans for scientific testing. The Commission's draft law would also have in principle restricted the use of other non-human primates such as ouistitis and macaques. Therefore, substitute animals will be required hereafter, and the Microminipig will be one of the promising candidates. My study indicated that MMPig would be a useful alternative of non-rodent experimental animal for understanding consequences of hypertension and vascular biology research.

General Conclusions

In conclusions, the present thesis showed the reactive and productive characteristics of mouse and MMPig basilar artery in response to intrinsic vasoactive substances. These characteristics of basilar artery were altered in *in vivo* exposure of MeHg in mouse and *in vivo* infusion of Ang II in MMPig. I expect that future study in some toxicological or pathological state will indicate the value of the physiological characteristics of the basilar artery and the importance of mouse and MMPig in cardiovascular research.

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