DOCTORAL THESIS

Study on the pharmacological characteristics of the avian basilar arteries

（トリ脳底動脈の薬理学的特徴に関する研究）

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Contents

ABSTRACT iii
General introduction 1
Chapter 1
Study 1
Involvement of beta$_3$-adrenergic receptors in relaxation mediated by nitric oxide in chicken basilar artery 6
1. ABSTRACT 7
2. INTRODUCTION 8
3. MATERIALS AND METHODS 9
3.1. Tissue Preparation 9
3.2. Reagents 9
3.3. Functional Study 10
3.4. Nitric Oxide (NO) Quantification Using Fluorescence 11
3.5. Statistical Analysis 11
4. RESULTS 12
4.1. Responsiveness to Noradrenaline and Effects of α- and β-AR Antagonists 12
4.2. Responsiveness to β-AR Agonists 12
4.3. Effect of β-AR Antagonists on Isoproterenol-Induced Relaxation 12
4.4. Effect of L-NNA on Isoproterenol-Induced Relaxation 13
4.5. Effect of β-AR Antagonists and L-NNA on SR 58611A-Induced Relaxation 13
4.6. Measurement of NO Production 13
5. DISCUSSION 14
Chapter 2
Study 2
Reduced nitric oxide synthase involvement in Aigamo duck basilar arterial relaxation 27
1. Simple Summary 28
2. Abstract 28
3. Introduction 30
4. Materials and Methods 31
4.1. Tissue Preparation 31
4.2. Reagents 32
4.3. Functional Study 32
4.4. Statistical Analysis 33
5. Results 33
5.1. Spontaneous Nitric Oxide and Prostaglandin Release 33

5.2. Responsiveness to Vasoactive Substances 34
5.3. Involvement of 5-Hydroxytryptamine Receptor Subtype 34
5.4. Involvement of Histamine Receptor Subtypes 34
5.5. Responsiveness to β-Adrenergic Receptor Agonists 35
5.6. Involvement of β-Adrenergic Receptor Subtype 35
5.7. Involvement of Muscarinic Receptor Subtype 35
5.8. Effects of Nitric Oxides Synthase and Cyclooxygenase Inhibitors on Ornithokinin-Induced Response 36
6. Discussion 36
7. Conclusions 40
General discussion 51
General conclusion 53
Reference 54
ACKNOWLEDGEMENT 63

ABSTRACT

The present thesis was designed to study the responsiveness of chicken basilar artery (CBA) and duck basilar artery (DBA) to intrinsic vasoactive substances in physiological condition.

In CBA, the adrenergic receptor subtypes were characterized. Functional studies of the isolated arteries to vasoactive substances were performed by micro organ bath system. The response of these arteries to noradrenaline (NA) was evaluated, induced contraction of arteries in resting tension, but induced relaxation of arteries in 5-hydroxytryptamine (5-HT) pre-contraction. Furthermore, the use of propranolol (a β-AR antagonist) and phentolamine (a α-AR antagonist) respectively enhanced those contraction and relaxation, to reveal the presence of both alpha and beta (α and β) receptors. Concentration-dependent relaxations induced by a range of β-AR agonists provided insights into their relative potency. Notably, isoproterenol demonstrated the highest potency, followed by noradrenaline, adrenaline, and procaterol.

To further explore the β-AR subtypes, several β-AR antagonists were employed, including propranolol for $\beta_{1,2,3}$-ARs, atenolol for β_1-ARs, butoxamine for β_2-ARs, and SR 59230A for β_3-ARs. In the Schild regression analysis, Propranolol was the only antagonist to yield a slope diverging from unity, suggested the presence of multiple β-AR subtypes. Our Schild regression analysis for atenolol and butoxamine also indicated that neither β_1-AR nor β_2-AR as the dominant subtype with their low pA_2 value of 5.95 and 5.14, respectively. On the contrary, SR 59230A exhibited a pA2 value (7.52) close to that reported for the relevant receptor subtype. Moreover, the study explored the influence of SR 58611A, a β_3-AR agonist, on arterial relaxation. Initially our studies showed that SR 58611A-induced relaxation was inhibited by SR 59230A, but not atenolol and butoxamine. This result suggested that SR 58611A exhibits high specificity for β_3-ARs, without measurable β_1 and β_2 activity. As a result, vasodilation mediated by β_3-ARs can be observed through SR 58611A-induced relaxation. The treatment of *Nω-nitro-L-arginine* (L-NNA, a nitric oxide synthase (NOS) inhibitor) was found to inhibit SR 58611A-induced relaxation, suggested the involvement of endothelial NOS in β_3-ARs mediated vasodilation. Additionally, experiments involving basilar arterial strips containing endothelium demonstrated that SR 58611A treatment induced nitric oxide production, which was decreased by L-

NNA, further suggested β_3-ARs mediated vasodilation in endothelial cell via NOS pathway.

These findings collectively suggest that α- and β-ARs participate in the regulation of both contraction and relaxation in chicken basilar arteries. Furthermore, β_3-ARs, particularly those localized on the endothelium, appear to play a pivotal role in vasodilation through the release of nitric oxide (NO).

In DBA, our second study investigated the responsiveness of those arteries to various vasoactive substances, encompassing 5-HT, histamine (His), angiotensin (Ang) II, NA, acetylcholine (ACh), and avian bradykinin ornithokinin (OK). This study aimed to characterize the receptor subtypes involved in arterial contraction and relaxation, as well as to examine the role of endothelial NO in vitro.

We demonstrated the involvement of spontaneously released endothelial NO based on the contraction induced with L-NNA followed by the relaxation induced with indomethacin, which inhibits cyclooxygenase, under resting tension. As a result, L-NNA-induced contraction under resting tension and indomethacin-induced relaxation under contraction induced with L-NNA, suggesting the spontaneous NO and spontaneous thromboxane A_2 are released from endothelial cells of DBA. However, a key difference was that the L-NNA-induced contraction in ducks (30.5%) was markedly weaker than that in chickens (122.1%), suggesting that ducks experience less involvement of spontaneous endothelial NO than chickens.

Furthermore, our results indicated that arterial contraction in duck basilar arteries was primarily mediated by 5-HT_1 and H_1 receptors, whereas relaxation was elicited by β_3-adrenergic and M_3 receptors. Notably, OK induced a biphasic response in duck basilar arteries, while Ang II had no discernible effect. The involvement of endothelial NO was assessed, revealing its critical role in relaxation mediated by M_3 and OK receptors. However, in contrast to chicken basilar arteries, β_3-ARs and His receptors in duck basilar arteries did not appear to contribute significantly to endothelial NO-mediated relaxation.

These differential findings between chicken and duck basilar arteries underscore distinct receptor subtypes and endothelial NO involvement, which may account for variations in vascular responses and disease outcomes, particularly in the context of avian influenza infection. Ducks exhibit greater resistance to the virus compared to

chickens, and the reduced endothelial NO contribution in duck basilar arteries may contribute to this differential susceptibility.

In conclusion, these two studies provide valuable insights into the receptor subtypes and endothelial function in avian basilar arteries. We suggest that CBA exhibit a significant contribution of β_3-ARs, especially on the endothelium, in vasodilation through NO release. In contrast, DBA is characterized by a smaller endothelial release of NO and a smaller degree of endothelial involvement in its reactivity than the BA of the chicken. NO plays a role with M_3 but not β_3-adrenergic receptors. These physiological differences may help explain varying susceptibility to diseases such as highly pathogenic avian influenza between poultry species. Further research is warranted to explore the underlying mechanisms and therapeutic implications of these findings.

General introduction

Maintaining a healthy brain condition depends on ensuring optimal brain perfusion and cerebral blood flows (CBF), which are responsible for delivering the necessary oxygen required for neuronal oxidative metabolism of energy substrates. Due to the limited capacity of neurons for anaerobic metabolism, CBF plays a crucial role in brain function and viability, ensuring the proper delivery of oxygen and energy substrates, as well as the removal of waste products of metabolism (Fantini et al., 2016). This regulation of CBF relies on vascular resistance, notably influenced by autoregulation, which maintains relative independence from perfusion pressure. Furthermore, large cerebral arteries exhibit higher resistance in the cerebral circulation, significantly contributing to total cerebral vascular resistance and microvascular pressure regulation. Various physiological stimuli, including changes in systemic blood pressure, cerebral metabolism, sympathetic activity, and humoral agents like vasopressin and angiotensin, impact the resistance of these arteries (Faraci and Heistad, 1990). It is important to note that large cerebral arteries consist of two primary pairs: the vertebral arteries and the internal carotid arteries. The vertebral arteries originate from the subclavian arteries and combine to form the basilar artery.

The basilar artery courses along the ventral side of the medulla oblongata and ultimately splits into the posterior cerebral arteries. These arteries establish connections to the internal carotid arteries via posterior communicating arteries and provide oxygenated blood to the cerebellum, brain stem, and occipital lobes. Investigating the basilar artery is significant as it constitutes one of the primary resistance vessels in the brain (Mattle et al., 2011).

The physiological responses of the basilar artery appear to influence not only CBF but also 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. 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. The

process is intricate, involving various factors, including local-chemical and endothelial factors, autacoids, and innervation systems, working together to safeguard optimal blood flow to the brain under diverse physiological and pathological conditions.

Local-chemical factors like H^+, K^+, Ca^{2+} ions, adenosine, and osmolarity affect cerebrovascular resistance during different conditions. Endothelial factors include thromboxane A_2, endothelin (ET), endothelium-derived constrictor and relaxing factors (nitric oxide, NO), and prostacyclin (PGI_2). These factors can be released due to physical stimuli, autacoids, neurotransmitters, and cytokines. Some, like NO, PGI_2, and ET, can also originate from neurons and astrocytes, connecting parenchymal function with flow. Autacoids such as histamine, bradykinin, eicosanoids, and free radicals influence cerebrovascular resistance and blood-brain barrier permeability, often due to trauma, ischemia, seizures, or inflammation. Cerebral arteries receive innervation from different systems, including the sympathetic-noradrenergic fibers from the superior cervical ganglion, the parasympathetic cholinergic system, intracerebral noradrenergic and serotonergic perivascular innervation, and a trigeminal innervation system with potential involvement in vascular issues like headaches or vasospasms (Wahl and Schilling, 1993).

Our research focuses on the responsiveness of basilar artery induced by those receptor-mediated stimuli, such as 5-Hydroxytryptamine (5-HT), noradrenaline (NA), acetylcholine (ACh), histamine (His), angiotensin (Ang) II, and bradykinin (BK). And such these researches could be investigated by organ bath system, allowing for a comprehensive examination of vascular reactions under controlled experimental conditions.

The organ bath serves as our experimental apparatus crucial for unraveling the intricate details of vascular responses in pharmacological studies. It provides a controlled and isolated environment for investigating the dynamic behavior of blood vessels, offering researchers a unique opportunity to delve into the complexities of vascular physiology. One of the distinctive advantages of the organ bath is its capacity to maintain isolated vascular tissues under optimal conditions. This controlled environment enables precise manipulations of experimental variables, including temperature, oxygenation, and exposure to pharmacological agents. Pharmacological studies within the organ bath involve the application of vasoactive substances to

observe their effects on vascular tone and reactivity. In addition to its role in observing responses qualitatively, the organ bath is instrumental in quantitative pharmacological investigations. We can employ various pharmacological calculation methods within this setup to assess parameters such as EC_{50} (half-maximal effective concentration) and E_{max} (maximal response) of vasoactive compounds. These calculations provide valuable insights into the potency and efficacy of drugs, contributing to a deeper understanding of their pharmacological profiles. Moreover, the organ bath allows for the simulation of diverse physiological and pathological conditions, enabling researchers to mimic scenarios relevant to cardiovascular health and disease. This versatility is especially beneficial when studying the effects of drugs under conditions such as hypoxia, ischemia, or inflammation.

The organ bath stands as a cornerstone in vascular research, facilitating both qualitative and quantitative investigations into the pharmacological aspects of vascular responses. Its controlled experimental conditions and versatility make it an indispensable tool for advancing our understanding of vascular physiology and developing targeted pharmacotherapies. Investigation by organ bath system, species differences in mammalian basilar arterial responsiveness to intrinsic vasoactive substances have been reported, even some of the species-specific responses are very unique and characteristic.

For example, 5-HT was discovered as a substance that caused contraction of smooth muscle in the mid-1900s. Approximately 95% of total 5-HT in the human body is produced by enterochromaffin cells in the intestinal mucosa. While blood platelets don't synthesize 5-HT, they acquire it from the intestine, circulating it throughout the vascular system. Local vascular injury triggers a release of 5-HT from activated platelets, impacting the functions of vascular smooth muscle cells and endothelial cells, influencing processes like contraction, proliferation, migration, and release of vasoactive mediators (Machida et al., 2013). The cerebral artery also receives a supply of 5-HT from perivascular nerves, where it functions as a neurotransmitter, influencing vascular tone modification (Lincoln, 1995). Furthermore, species differences are evident in 5-HT-mediated responsiveness in mammalian basilar arteries, where the specific receptors involved vary among different species. Specifically, 5-HT-induced contractions of basilar arteries are

mediated via stimulation of 5-HT_1 receptors in guinea-pigs, rabbits, and humans; of 5-HT_2 receptors in rats, horse and monkeys; and of both types of receptors in dogs, sheep, monkeys (Macaca fasciculuris), and pigs (Miyamoto et al., 1996).

In other case, NA also influence cerebral artery as a vascular neurotransmitter. Recent advancements reveal expanded knowledge of adrenoceptor subtypes (α_1, α_2, β_1, β_2, β_3). The α_1 adrenoceptor subtype is more implicated in the maintenance of vascular basal tone and of arterial blood pressure in conscious animals, while the α_2 adrenoceptor subtype in particular control arterial contraction, and is responsible above all for venous vasoconstriction (Civantos Calzada and Aleixandre de Artiñano, 2001). The β_1, β_2, and β_3 adrenoceptor subtypes classically identified in cardiac, airway smooth muscle, and adipose tissue, respectively (Johnson, 1998). In basilar arteries, previous reports on their reactivity to NA in mammals include our findings that NA induced contractions in horses (Miyamoto et al., 1995) and bats (Islam et al., 2021), relaxation in pigs (Miyamoto et al., 1993) and dolphins (Islam et al., 2020), and no response in mice (Islam et al., 2014). Other researchers have reported that noradrenaline induced contractions in dogs (Sakakibara et al., 1982), guinea pigs (Chang et al., 1988), sheep (Gaw and Wadsworth, 1989), no response under resting tension (Chang et al., 1988), and relaxation under precontraction by K^+ in rats (Hempelmann and Ziegler, 1993). Additionally, β_1 adrenoceptor subtypes typically play a dominant role in mediating vasodilation in these arteries, with a minor population of β_2 adrenoceptor subtypes. To date, there have been no reports demonstrating the presence of β_3 adrenoceptor subtypes in the basilar artery.

Avian, as vertebrates, share common anatomical and physiological features with mammals, despite distinct evolutionary paths. Both avian and mammalian species belong to the phylum Chordata and share a vertebral column, indicating a common ancestry and fundamental structural similarities. In the context of neurological research, the study of avian brain vasculature holds value due to the shared characteristics with mammalian counterparts. Both bird and mammal brains exhibit complexity in structure and function, with comparable regions responsible for

cognition, sensory processing, and motor control. Understanding the vascular systems in birds can offer insights into the evolutionarily conserved principles governing cerebral blood supply and its role in supporting complex neural functions. Additionally, birds, like mammals, are endothermic or warm-blooded, requiring efficient oxygen delivery to sustain their high metabolic rates. The study of avian brain vasculature provides an opportunity to explore adaptations that enable birds to meet the energetic demands associated with flight and other complex behaviors. This comparative approach allows researchers to uncover universal principles of vascular organization and function across vertebrates.

For poultry species, the assumed role of basilar arteries parallels that in mammals, despite limited research, primarily focused on the chicken basilar artery (CBA). In the CBA, research indicates that the 5-HT_1 receptor is implicated in arterial contraction, while relaxation involves the $H_{1\&2}$ and M_3 receptors. Notably, nitric oxide (NO) plays a role in conjunction with M_3 and H_1 receptors. A distinctive feature of chicken basilar arteries, when compared to mammalian counterparts, is the unique mediation of relaxations by $H_{1\&2}$ receptors in both smooth muscle and endothelial cells. This is noteworthy since the H_1 receptor has been established as the predominant histamine receptor in cerebral smooth muscle cells of reported mammals such as cattle, pigs, and horses (Miyamoto and Nishio, 1993; 1994). The divergence in the mediation of relaxations in chicken basilar arteries, particularly through $H_{1\&2}$ receptors, underscores the species-specific differences in vascular regulation between poultry and mammals.

Chapter 1

Study 1

Involvement of beta$_3$-adrenergic receptors in relaxation mediated by nitric oxide in chicken basilar artery

1. ABSTRACT

The response of basilar arteries to noradrenaline varies among many animal species, but remains little studied in poultry. Accordingly, we aimed to characterize the adrenergic receptor (AR) subtypes that modulate vascular response in basilar arteries in the chicken, with isometric recording of arterial ring tension using an organ bath. We demonstrated the presence of both alpha and beta (α and β) receptor subtypes through evaluating the response to noradrenaline, with and without a range of β-AR and α-AR antagonists. The concentration-dependent relaxations then induced by a range of β-AR agonists indicated a potency ranking of isoproterenol > noradrenaline > adrenaline > procaterol. We then investigated the effects of β-AR antagonists that attenuate the effect of isoproterenol (propranolol for $\beta_{1,2,3}$-ARs, atenolol for β_1-ARs, butoxamine for β_2-ARs, and SR 59230A for β_3-ARs), with Schild regression analysis, ascertaining multiple β-AR subtypes, with neither the β_1-AR nor the β_2-AR as the dominant subtype. SR 59230A was the only antagonist to yield a pA_2 value (7.52) close to the reported equivalent for the relevant receptor subtype. Furthermore, treatment with SR 58611 (a β_3-AR agonist) induced relaxation, which was inhibited ($P < 0.01$) by L-NNA and SR 59230A. Additionally, treating basilar arterial strips (containing endothelium) with SR 58611 induced nitric oxide (NO) production, which was inhibited ($P < 0.01$) by L-NNA and SR 59230A. Based on this first characterization of AR subtypes in chicken basilar arteries (to our knowledge), we suggest that α- and β-ARs are involved in contraction and relaxation, and that β_3-ARs, especially those on the endothelium, may play an important role in vasodilation via NO release.

Key words: β-adrenoceptor, chicken, basilar artery, vasorelaxation, nitric oxide.

2. INTRODUCTION

The basilar artery performs vital functions across mammalian and avian species. It constantly supplies blood to the brainstem, is involved in the regulation of heart rate and respiratory rate, and contributes to the maintenance of the cerebral circulatory volume (Rahamt and Gilland, 2014). The physiological response of the basilar artery to stimuli is thus of crucial importance; however, this vascular reactivity is much better investigated in mammals than poultry.

Previous reports on basilar artery reactivity to noradrenaline in mammals include our findings that noradrenaline induced contractions in horses (Miyamoto et al., 1995) and bats (Islam et al., 2021), relaxation in pigs (Miyamoto et al., 1993) and dolphins (Islam et al., 2020), and no response in mice (Islam et al., 2014). Other researchers have reported that noradrenaline induced contractions in dogs (Sakakibara et al., 1982), guinea pigs (Chang et al., 1988), sheep (Gaw and Wadsworth, 1989), no response under resting tension (Chang et al., 1988), and relaxation under precontraction by K^+ in rats (Hempelmann and Ziegler, 1993).

For poultry species, basilar arteries are widely assumed to play a similar role as in mammals, although the only relevant reports so far are of contraction as a vascular response to noradrenaline (Okuno et al., 2008; Matsumoto et al., 2012). In both poultry and mammalian species, the basilar artery is lined by the endothelium, controls the degree of vascular relaxation and contraction, and regulates regional blood flow (Krüger-Genge et al., 2019). In chickens, virulent avian influenza A viruses produce lethal disease, with apoptosis of vascular endothelial cells in the liver, kidney, and brain (Ito et al., 2002; Vreman et al., 2022). Thus, it is biologically plausible that viral injury to endothelial cells in the basilar artery contributes to the high mortality rates. Accordingly, investigations of the actions of vasoactive substances on endothelial cells and the location of the relevant receptors in the chicken basilar artery have the potential to benefit poultry health.

Adrenergic receptors are crucial to vasoconstriction and vasodilation. To date, the α-adrenergic receptor (AR) has been classified into α_1- and α_2-AR subtypes,

whereas the β-AR has been classified into β_1-, β_2-, and β_3-AR subtypes, which have been primarily identified in cardiac smooth muscle, airway smooth muscle, and adipose tissue, respectively (Bylund, 1992; Johnson, 1998). The β-AR is a widely distributed class of G protein-coupled receptor usually mediating vascular relaxation, occurring not only in vascular smooth muscle cells, but in endothelial cells as well (Vanhoutte, 2001). In several previous studies of mammalian cerebral arteries, β_1-ARs have been found to play a dominant role, while β_2-ARs are less prevalent in cats (Edvinsson and Owman, 1974), humans (Tsukahara et al., 1986), cows (Ayajiki and Toda, 1992), and pigs (Miyamoto et al., 1993). Similar studies are lacking in avian species. There have been no reports showing the presence of β_3-ARs in basilar arteries.

Against this background, this study aimed to characterize the AR subtypes that modulate vascular responses in chicken basilar arteries, by exposing sections of basilar arteries from slaughtered chickens to noradrenaline as well as a range of agonists and antagonists.

3. MATERIALS AND METHODS

3.1. Tissue Preparation

Chicken basilar arteries were obtained from freshly slaughtered broiler chickens (Gallus gallus domesticus; n = 100, both sexes, 48-days old) at a local poultry processing plant. The tissues were transferred to our laboratory in ice-cold physiological saline (119 mM NaCl, 4.7 mM KCl, 1.6 mM CaCl2, 1.2 mM MgCl2, 25 mM NaHCO3, 1.2 mM KH2PO4, and 10 mM glucose, pH 7.4) aerated with carbogen [95% (vol/vol) O2, 5% (vol/vol) CO2]. Each artery was immediately dissected free of adherent tissues under a stereomicroscope. All experiments were performed in accordance with the Guidelines for Animal Experiments of Kagoshima University.

3.2. Reagents

The following reagents were used at the described concentrations: butoxamine hydrochloride (10^{-5}–10^{-4} M, Sigma-Aldrich, St. Louis, MO), isoproterenol

hydrochloride (10^{-9}–10^{-5} M, Sigma-Aldrich), SR 59230A (10^{-7}–10^{-6} M, Sigma-Aldrich), SR 58611A (10^{-5} M, Sigma-Aldrich), N^{ω}-nitro-L-arginine (L-NNA; 10^{-4} M, Sigma-Aldrich), noradrenaline (10^{-9}–10^{-5} M, Tokyo Chemical Industry, Tokyo, Japan), phentolamine mesylate (10^{-6}–10^{-5} M, Tokyo Chemical Industry), 5-hydroxytryptamine (serotonin)-creatinine sulfate (5-HT; 10^{-6} M; Merck, Darmstadt, Germany), adrenaline (10^{-9}–10^{-5} M, Daiichi Sankyo, Tokyo, Japan), procaterol (10^{-9}–10^{-4} M, Fujifilm-Wako, Tokyo, Japan), propranolol hydrochloride (10^{-9}–10^{-8} M, ICI, London, UK), atenolol (10^{-6}–10^{-5} M, LKT Laboratories, Tokyo, Japan), and sodium nitroprusside (10^{-4} M; Nacalai Tesque, Kyoto, Japan). The NO2/NO3 Assay Kit-FX (Dojindo, Kumamoto, Japan) was used following the manufacturer's instructions.

3.3. Functional Study

Three rings, approximately 2 mm wide, were cut from each chicken basilar artery. Each ring was mounted horizontally between 2 L-shaped stainless-steel holders (outer diameter 0.03 mm), with 1 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 physiological saline at 41°C (pH 7.4). Each suspended ring was left to equilibrate for at least 30 min under a resting tension of 0.5 mN. This tension was chosen because it allowed us to induce maximum contractions in the artery without deflection of the steel. KCl (60 mM) was applied every 30 min until the amplitude of the contraction reached a constant value.

Changes in the KCl concentration of 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. In a preliminary experiment, 5-HT induced sufficient contraction with retention of the tonic phase, whereas prostaglandin $F_{2\alpha}$ and uridine-5-triphosphate and other vasoconstrictors failed to induce sufficient contractions with a tonic phase [maximal response (E_{max}) <25% relative to contraction induced by 60 mM KCl]. Therefore, we selected 5-HT as

the agent to induce precontraction before applying the β-AR agonist. Cumulative concentration-response curves were obtained for agonists by adding a solution of each agonist directly to the fluid in the bath. Antagonists or inhibitors were added to the bathing media 30 min before each agonist. The antagonists had no effect on the resting vascular tone. The log concentration ratio of EC_{50} values (i.e., the concentration producing a half-Emax in the absence or presence of an antagonist) was calculated and plotted against the logarithm of the antagonist concentration to obtain the relevant pA_2 value as a measure of potency (Arunlakshana and Schild, 1959).

At the end of the relaxant response, sodium nitroprusside (10^{-4} M) was applied to produce maximal relaxation, which was taken as 100%.

3.4. Nitric Oxide (NO) Quantification Using Fluorescence

Arteries were prepared for NO quantification using the method described by De Caterina et al. (1985), with some modifications. Briefly, 4 chicken basilar arteries (total: 1.24 ± 0.06 mg wet tissue) were cut into 2-mm segments and immersed in a 500-μL tube containing oxygenated physiological saline at 41°C (pH 7.4). For each experimental condition, the segments were treated with SR 58611A as a positive control and pretreated with L-NNA or SR 59230A for 1 h of incubation. The incubation solution was centrifuged at 2,000 × *g* for 20 min and the supernatant was used to measure the nitrate and nitrite (products of NO).

We used a fluorescence method based on 2,3-diaminonaphthalene, which is a newer NO assay with higher sensitivity than the Griess method. All the nitrate in the solution was converted into nitrite using nitrate reductase, and the total nitrite was measured by its fluorescence intensity (λex = 360 nm, λem = 450 nm) with a microplate reader (Tecan, Mannedorf, Switzerland). The combined amount of nitrate and nitrite per mg of wet tissue was calculated as the NO production.

3.5. Statistical Analysis

Results are expressed as the mean ± SEM, and statistical analyses were performed using Student t test or the Bonferroni test after one-way ANOVA (Stat

View J-4.5, Abacus Concepts Inc., Berkeley, CA). *P* values < 0.05 were considered statistically significant.

4. RESULTS

4.1. Responsiveness to Noradrenaline and Effects of α- and β-AR Antagonists

To ascertain the presence or absence of α- and β-ARs in the chicken basilar artery, we first investigated the vascular responsiveness to noradrenaline, a nonselective α- and selective β_1-AR agonist under resting tension conditions. Noradrenaline induced contraction in a concentration-dependent manner (pEC_{50}: 4.85, Emax: 124.2 ± 5.8%, n = 5). Propranolol, a nonselective β-AR antagonist (10^{-5} M), shifted the concentration-response curve for noradrenaline 6-fold to the left with a 56.3 ± 14.4% upshift (Figure 1A). We also investigated the vascular responsiveness to noradrenaline under precontraction by 5-HT (10^{-6} M). Under this condition, noradrenaline induced relaxation (Emax: 28.9 ± 3.1%, n = 5) followed by contraction. Phentolamine, a nonselective α-AR antagonist shifted the concentration-response curve for noradrenaline downward and abolished contraction at 10^{-5} M (Figure 1B).

4.2. Responsiveness to β-AR Agonists

To characterize chicken basilar artery responsiveness to different β-AR agonists, we generated the relaxation effects of isoproterenol (a nonselective β-AR agonist), noradrenaline (a β_1-AR agonist, in the presence of 10^{-5} M phentolamine), adrenaline (a nonselective β-AR agonist, in the presence of 10^{-5} M phentolamine), and procaterol (a β_2-AR agonist) (Figure 2). Each of these β-AR agonists produced concentration-dependent relaxation of the chicken basilar arteries. The rank order of their potency as inducers of relaxation was isoproterenol > noradrenaline > adrenaline > procaterol (Table 1).

4.3. Effect of β-AR Antagonists on Isoproterenol-Induced Relaxation

To evaluate which of the receptor subtypes is predominant in chicken basilar arteries, we investigated the effect of a range of antagonists on isoproterenol-induced

relaxation. Figure 3 shows the effects of propranolol (10^{-9} M, 10^{-8} M), atenolol (a β_1-AR antagonist, 10^{-6} M, 10^{-5} M), butoxamine (a β_2-AR antagonist, 10^{-5} M, 10^{-4} M), and SR 59230A (a β_3-AR antagonist, 10^{-7} M, 10^{-6} M) on the isoproterenol-induced relaxation under precontraction. The Schild plots are shown in Figure 4, with slopes for propranolol, atenolol, butoxamine, and SR 59230A against isoproterenol of 0.71 ± 0.08, 1.05 ± 0.17, 1.06 ± 0.07, and 1.21 ± 0.09, respectively. The slope for propranolol diverged ($P < 0.05$) from unity, while the other 3 slopes did not. The calculated pA_2 values of atenolol, butoxamine, and SR 59230A were 5.95 ± 0.18, 5.14 ± 0.11, and 7.52 ± 0.11, respectively.

4.4. Effect of L-NNA on Isoproterenol-Induced Relaxation

To investigate the role of endothelial NO in isoproterenol-induced relaxation, we applied L-NNA [an NO synthase (NOS) inhibitor, 10^{-4} M] to the tested chicken basilar artery samples. As shown in Figure 5, pretreatment with L-NNA shifted the concentration-response curve for isoproterenol to the right, but had no significant effect on the Emax elicited by isoproterenol.

4.5. Effect of β-AR Antagonists and L-NNA on SR 58611A-Induced Relaxation

To further evaluate the role of β_3-ARs, we investigated the relaxation effect of 10^{-5} M SR 58611A (a β_3-AR agonist) under precontraction. As shown in Figure 6, SR 59230A (10^{-5} M) inhibited SR 58611A (10^{-5} M)-induced relaxation by approximately 90%, while atenolol (10^{-5} M) and butoxamine (10^{-5} M) had no significant effect on this response. Additionally, L-NNA (10^{-4} M) inhibited SR 58611A (10^{-5} M)-induced relaxation by approximately 65%.

4.6. Measurement of NO Production

To investigate the role of NO in β_3-AR mediated relaxation, we quantified NO production under a range of conditions. As shown in Figure 7, in the absence of SR 58611A (10^{-5} M, negative control), the total NO production per mg of wet tissue was 0.54 ± 0.04 nmol/mg, lower ($P < 0.01$) than that (2.09 ± 0.12) in the presence of SR 58611A (positive control). As pretreatment agents, SR 59230A (10^{-5} M) and L-NNA

(10^{-4} M) shifted the total NO production per mg of wet tissue to 0.57 ± 0.08 and 0.66 ± 0.06, respectively, and these results differed ($P < 0.01$) from those obtained with the positive control, but not the negative control.

5. DISCUSSION

To our knowledge, the present study is the first to characterize the AR in the basilar artery of a poultry species, specifically the chicken. As the key finding of this study, we found that the β_3-AR subtype is the predominant endothelial subtype in this artery and plays an important role in AR-mediated vasodilation via NO.

Considering previous reports on the coexistence of α- and β-ARs in bovine and porcine cerebral arteries from in vitro studies with noradrenaline (Ayajiki and Toda, 1992; Miyamoto et al., 1993), we started our investigation by ascertaining whether similar patterns are seen in chicken basilar arteries. Our findings on the response elicited by noradrenaline were similar to those in the previous mammalian studies, demonstrating concentration-dependent contraction under resting tension and relaxation under 5-HT-induced contraction. These results indicate the coexistence of α- and β-ARs in the basilar arteries of chickens. These α- and β-ARs were further suggested to interact by our findings of shifts in the concentration-response curve; specifically an approximately 55% upshift by propranolol (10^{-5} M), a nonselective β-AR (Figure 1A), and an approximately 50% downshift by phentolamine (10^{-5} M), a nonselective α-AR antagonist, under a contracted condition (Figure 1B). In our experiments with noradrenaline, 10^{-5} M propranolol and phentolamine were confirmed to exhibit the maximum inhibitory effect, and the similarity of their maximal inhibitory effects (in percentage terms) indicated a 1:1 ratio for α- to β-ARs.

We then evaluated the relaxation effect of other β-AR agonists on chicken basilar arteries in vitro. Experiments were conducted in the presence of 10^{-5} M phentolamine (which had exhibited the maximum inhibitory effect on the α-AR) so that we could evaluate β-AR independently. The rank order of potency for β-AR agonists in the relaxation of chicken basilar arteries was isoproterenol > noradrenaline (with phentolamine) > adrenaline (with phentolamine) > procaterol, with respective pEC_{50}

values of 7.83, 7.06, 5.97, and 5.64 (Table 1). These data were similar to those observed in monkey detrusors (Takeda et al., 2002), which suggested that the β_3-AR was the predominant subtype. Furthermore, procaterol yielded a lower pEC50 (5.64) in the present study in chicken basilar arteries than it had in previous research (O'Donnell and Wanstall, 1985) on its role as a β_2-AR agonist (7.65–8.38), suggesting that the β_2-AR may not play a predominant role in the chicken basilar artery.

We then further investigated the presence of β-ARs, by applying Schild regression analysis in a comparison with a range of β-AR antagonists in the relaxation induced by isoproterenol, a nonselective β-AR agonist. In this analysis, propranolol was the only antagonist to yield a slope diverging ($P < 0.05$) from unity in the Schild plot (0.71, as shown in Figure 4), suggesting that multiple β-AR subtypes are involved in isoproterenol-induced relaxation of chicken basilar arteries. This finding is consistent with that of Oriowo (1994) in the rat carotid artery. The Schild plot for propranolol in that study also showed a slope less ($P < 0.05$) than unity, indicating interactions of isoproterenol with sites of high (classical β) and low (atypical β) affinity.

Our Schild regression analysis also indicated that neither the β_1-AR nor β_2-AR is the dominant subtype in chicken basilar arteries. The pA_2 yielded by the β_1-AR antagonist atenolol in this study (5.95, Figure 4) was much smaller than those reported in guinea pig colonic, guinea pig atrial, rat atrial, and feline tracheal tissues (6.49, 6.78–7.37), where β_1-ARs predominate (O'Donnell and Wanstall, 1983; Chino et al., 2018) and was similar to that (5.88) observed in guinea pig tracheas (O'Donnell and Wanstall, 1979), which reportedly possess 15% β_1-ARs and 85% β_2-ARs, based on ligand-binding experiments (Carswell and Nahorski, 1983). The pA_2 yielded by the a β_2-AR antagonist butoxamine in this study (5.14, Figure 4) was smaller than reported in guinea pig tracheal tissue (6.51), where β_2-ARs predominate (Tanaka et al., 2004) and similar to that (5.1) observed in guinea pig atria (O'Donnell and Wanstall, 1979), which reportedly possess 25% β_2-ARs and 75% β_1-ARs, based on ligand-binding experiments (Molenaar and Summers, 1987).

In contrast to the Schild regression results for β_1-AR and β_2-AR antagonists, our results for β_3-AR antagonists provided evidence that the β_3-AR is predominant in the chicken basilar artery. SR 59230A effectively antagonized the isoproterenol-induced relaxation of chicken basilar arteries, and its pA_2 value (7.52, Figure 4) was similar to those reported in porcine detrusor muscle (7.7) and rat urinary bladder tissues (7.27), where β_3-ARs predominate (Yamanishi et al., 2002; Obara et al., 2019), and the guinea pig gastric fundus (7.35), where the β_3-AR mediates isoproterenol-induced relaxation (Horinouchi et al., 2001). Notably, the later study involved investigating the pA_2 value for SR 59230A against isoproterenol in the presence of atenolol (10^{-4} M) and butoxamine (10^{-4} M) to block the β_1- and β_2-ARs. Taking our results together with the above-stated findings in mammalian studies, we strongly suggest that the β_3-AR is predominantly involved in isoproterenol-induced relaxation in the chicken basilar artery.

Our findings extend scientific knowledge on the distribution of the β_3-AR. This receptor was first cloned and identified in 1989 (Emorine et al., 1989). Since then, studies have focused on its metabolic effect on the adipose tissue (Lowell and Flier, 1997; Haddish and Yun, 2022) and its relaxation regulation in the gastrointestinal tract (Bianchetti and Manara, 1990; De Ponti et al., 1996) and bladder (Seguchi et al., 1998; Fujimura et al., 1999; Mitidieri et al., 2022). In the cardiovascular system, the β_3-AR was found to attenuate cardiac myocyte contractility via eNOS/cGMP signaling in the human myocardium (Tavernier et al., 2003) and increase myocardial perfusion by producing NO and endothelium-derived hyperpolarizing factor in the coronary microvascular endothelium (Dessy et al., 2004). Additionally, the mechanism of β_3-AR-mediating relaxation is suggested to involve NO released from the endothelium, and the location of β_3-ARs is indicated to be mainly endothelial cells based on immunohistochemistry in rat aorta (Trochu et al., 1999; Rautureau et al., 2002). To our knowledge, this is the first report of the presence of β_3-ARs in basilar arteries in any species.

To obtain further evidence on the mechanism of vascular reactivity in chicken

basilar arteries, we conducted experiments with pretreatment by the NOS inhibitor, L-NNA. This pretreatment shifted ($P < 0.01$) the concentration-response curve for isoproterenol to the right and did not change the maximal relaxant response (Figure 5). This result differs from the findings in rat basilar arteries, where treatment with L-NNA did not affect isoproterenol-induced relaxation (Moore et al., 2015). Endothelial involvement in isoproterenol-induced relaxation may be species dependent. For the chicken basilar artery, our results suggest that isoproterenol-induced relaxation may involve NO partially and may be mediated by multiple β-AR subtypes in both of endothelium and smooth muscle.

To clarify the relationship between the β_3-AR and NO, we further evaluated chicken basilar artery responses to the β_3-AR agonist, SR 58611A in the presence and absence of L-NNA. We selected a high concentration (10^{-5} M) of SR 58611A to investigate the effect of L-NNA on β_3-AR-mediated relaxation because SR 58611A did not elicit a concentration-related response under 5-HT-induced contraction, due to the slow development of relaxation delay in reaching a steady-state (>30 min), which was not within the approximately 20-min window for constant contraction with 5-HT. We also took into account the reportedly greatly reduced potency of SR 58611A in thoracic and carotid arteries vs. the gastrointestinal tract in rats (Trochu et al., 1999).

As the first step, we checked the affinity of SR 58611A as the β_3-AR agonist. SR 58611A-induced relaxation was confirmed to be inhibited by SR 59230A (10^{-5} M), but not atenolol (10^{-5} M) or butoxamine (10^{-5} M) (Figure 6), suggesting that the β_3-AR is the only subtype to mediate this relaxation. Similar results had previously been obtained in the thoracic aorta of the rat, where SR 58611A was inhibited by SR 59230A, but not nadolol, a β_1- and β_2-AR antagonist (Trochu et al., 1999). Wang et al. (2021) have also shown that the enhancement of AMPA receptor-mediated excitatory postsynaptic currents via β_3-AR induced by SR 58611A is inhibited by SR 59230A, but not betaxolol (a β_1-AR antagonist) or ICI118551 (a β_2-AR antagonist) in prefrontal cortex cells. Overall, the results of this and previous studies indicate that SR 58611A shows high specificity to the β_3-AR without measurable β_1 and β_2 activity.

L-NNA inhibited the relaxation response induced by SR 58611A (Figure 6), suggesting that NO is involved in the β_3-AR mediated relaxation.

We then quantified NO for further evaluation. An increased NO production level was observed when artery segments were treated with SR 58611A, while pretreatment with SR 59230A and L-NNA almost totally abolished this change (Figure 7). These results strongly suggest that the β_3-AR induces relaxation in endothelial cells via the NOS pathway. In the chicken basilar artery, histamine H_2 receptors and muscarinic M_3 receptors have been reported to be located on endothelial cells (Okuno et al., 2008; Matsumoto et al., 2012). Any virulent avian influenza A virus infection may thus injure cerebral endothelial cells, and render β_3-ARs, as well as H_2- and M_3-receptors, dysfunctional. Such effects may explain the high mortality rate of this viral infection in chickens.

In conclusion, noradrenaline-induced contraction and relaxation are involved in α- and β-ARs in chicken basilar arteries. In particular, the relaxation response was predominantly mediated by β_3-ARs on endothelial cells.

Table 1. The pEC_{50} values and maximal response (E_{max}) for agonists

Agonists	pEC_{50}	E_{max} (%)
Resting tension		
5-Hydroxytryptamine	5.84 ± 0.06	143.2 ± 9.3[a]
Histamine	4.37 ± 0.09	104.3 ± 14.0[a]
Noradrenaline	-	No response
Noradrenaline + Propranolol	-	No response
Precontracted condition		
Acetylcholine	6.18 ± 0.17	-81.0 ± 5.4[b]
Noradrenaline	6.28 ± 0.05	-69.7 ± 3.7[b]
Noradrenaline + Phentolamine	6.30 ± 0.11	-68.8 ± 6.3[b]
Ornithokinin	6.20 ± 0.25	-85.4 ± 2.7[b]
Isoproterenol	7.22 ± 0.11	-82.8 ± 9.2[b]
Adrenaline	5.89 ± 0.10	-60.9 ± 9.5[b]
Procaterol	-	No response

[a]The contraction induced by 60 mM KCl was taken as 100%.

[b]The relaxation induced by 10^{-4} M sodium nitroprusside was taken as 100%.

Each value represents the mean ± SEM of 4–6 ducks.
The concentration of propranolol and phentolamine is 10^{-5} M.

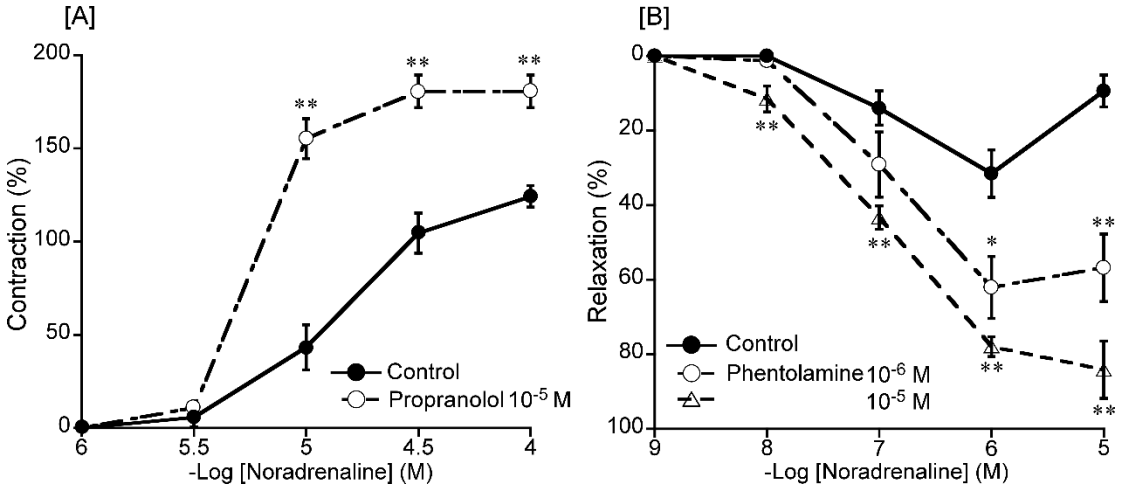

Figure 1. (A) Effect of propranolol (○: 10^{-5} M) on noradrenaline-induced contraction (●) in chicken basilar arteries under resting tension. (B) Effect of phentolamine (○: 10^{-6} M, △: 10^{-5} M) on noradrenaline-induced relaxation (●) in chicken basilar arteries precontracted with 10^{-6} M 5-hydroxytryptamine (5-HT). The contraction induced by 60 mM KCl (A) and the relaxation induced by 10−4 M sodium nitroprusside (B) were taken as 100%, respectively. Each point represents the mean ± SEM of 5 chickens (*P < 0.05, **P < 0.01 vs. control).

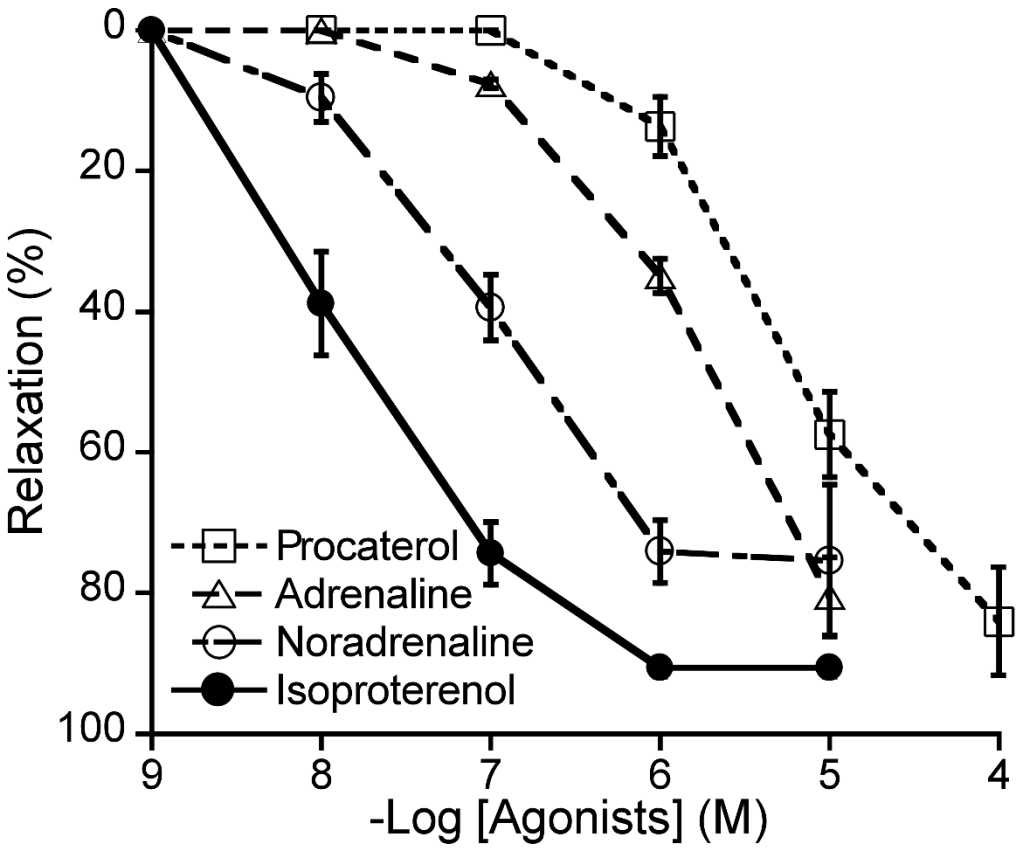

Figure 2. Responsiveness to β-adrenergic receptor agonists (●: isoproterenol, ○: noradrenaline in the presence of 10^{-5} M phentolamine, △: adrenaline in the presence of 10^{-5} M phentolamine, □: procaterol) in chicken basilar arteries precontracted with 10^{-6} M 5-HT. The relaxation induced by 10^{-4} M sodium nitroprusside was taken as 100%. Each point represents the mean ± SEM of 5 chickens.

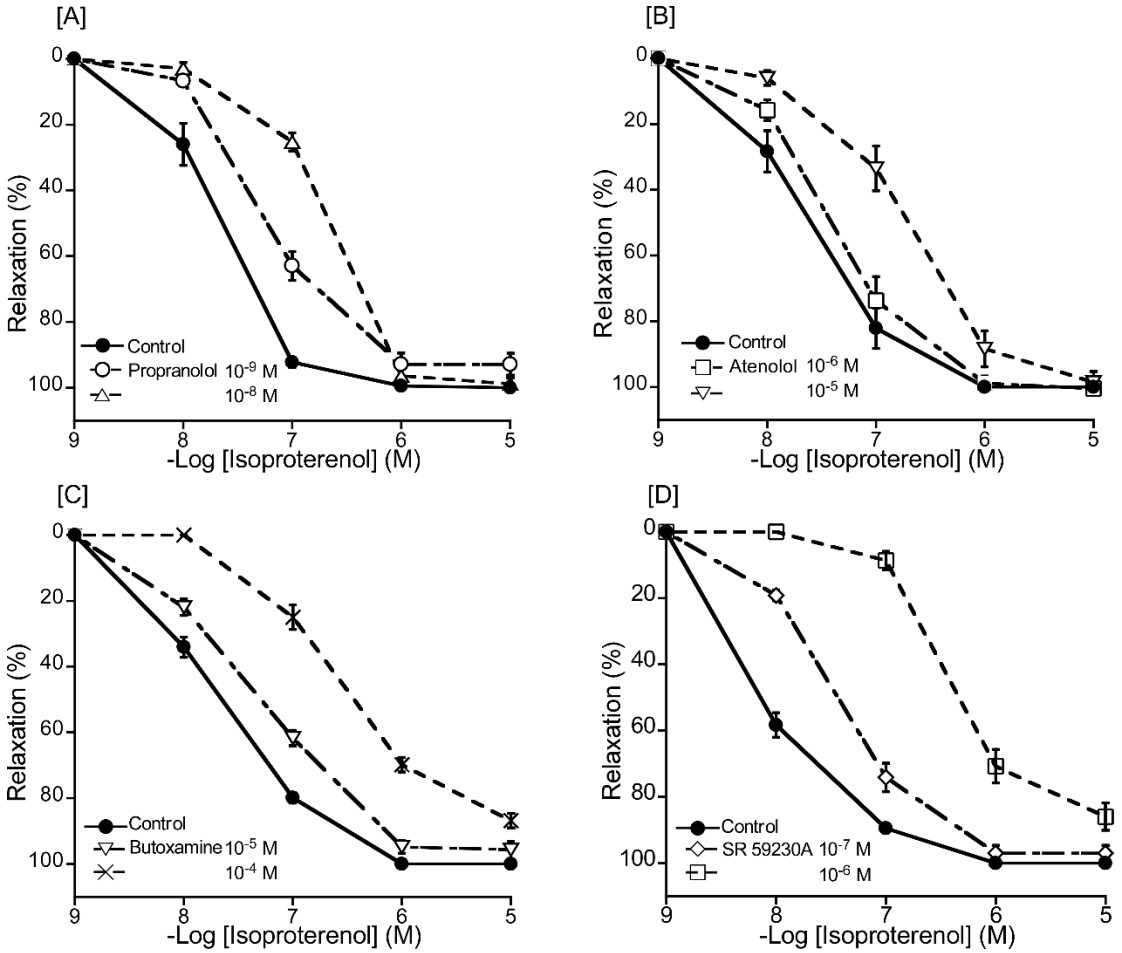

Figure 3. Effect of propranolol (○: 10^{-9} M, △: 10^{-8} M) (A), atenolol (□: 10^{-6} M, ▽: 10^{-5} M) (B), butoxamine (▽: 10^{-5} M, ×: 10^{-4} M) (C), and SR 59230A (◇: 10^{-7} M, □: 10^{-6} M) (D) on isoproterenol-induced relaxation (●) in chicken basilar arteries precontracted with 10^{-6} M 5-HT. The maximal relaxation induced by isoproterenol was taken as 100%. Each point represents the mean ± SEM of 5 or 6 chickens.

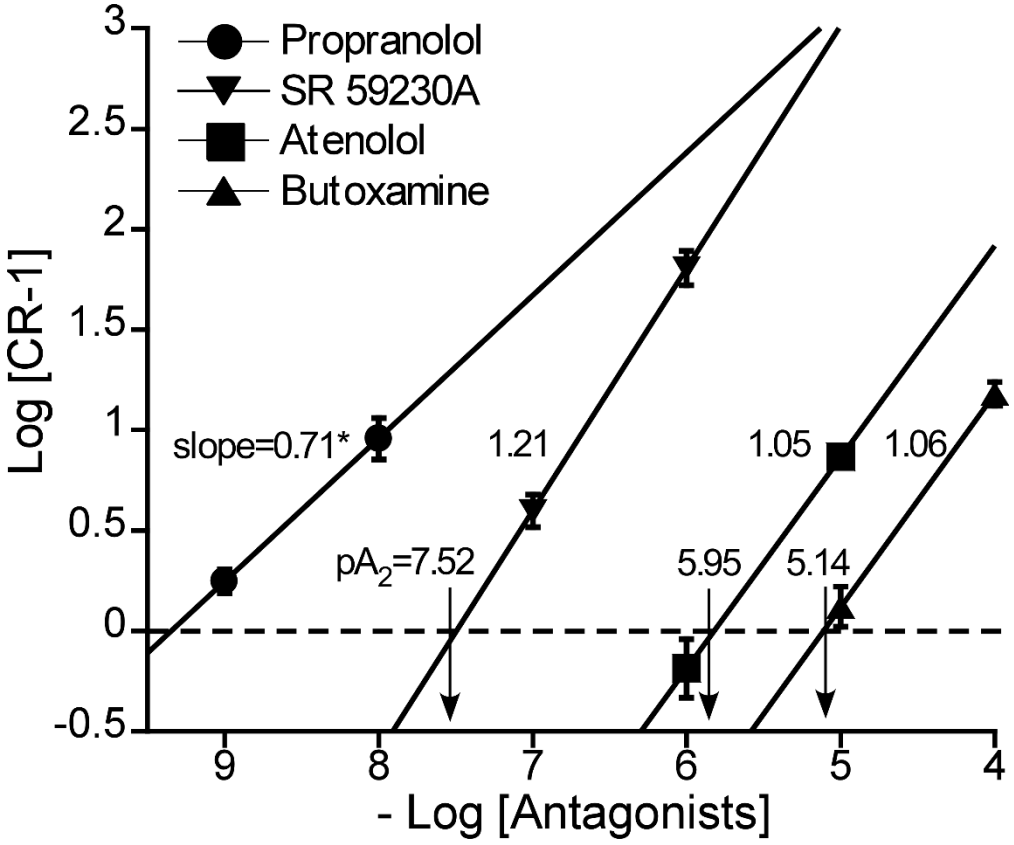

Figure 4. Schild plots of propranolol (●), atenolol (■), butoxamine (▲), and SR 59230A (▼) for isoproterenol-induced relaxation in chicken basilar arteries. The relaxation induced by 10^{-4} M sodium nitroprusside was taken as 100%. Each point represents the mean ± SEM of 5 or 6 chickens. CR: Equieffective isoproterenol concentration ratio [concentration producing 50% maximal (EC_{50})] in the presence of antagonists/EC_{50} in the absence of antagonists (*P < 0.05 vs. unity [1.00]).

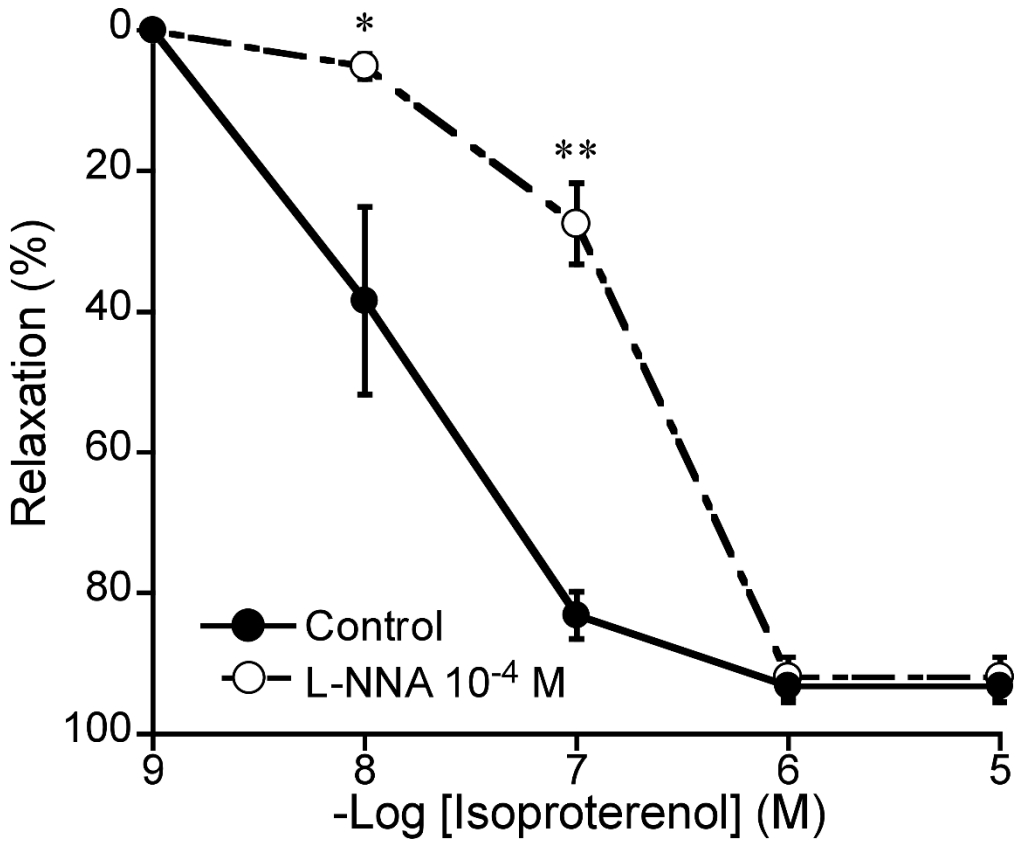

Figure 5. Effect of Nω-nitro-L-arginine (L-NNA, ○) on isoproterenol-induced relaxation (●) in chicken basilar arteries precontracted with 10^{-6} M 5-HT. The relaxation induced by 10^{-4} M sodium nitroprusside was taken as 100%. Each point represents the mean ± SEM of 5 chickens (*P < 0.05, **P < 0.01 vs. control).

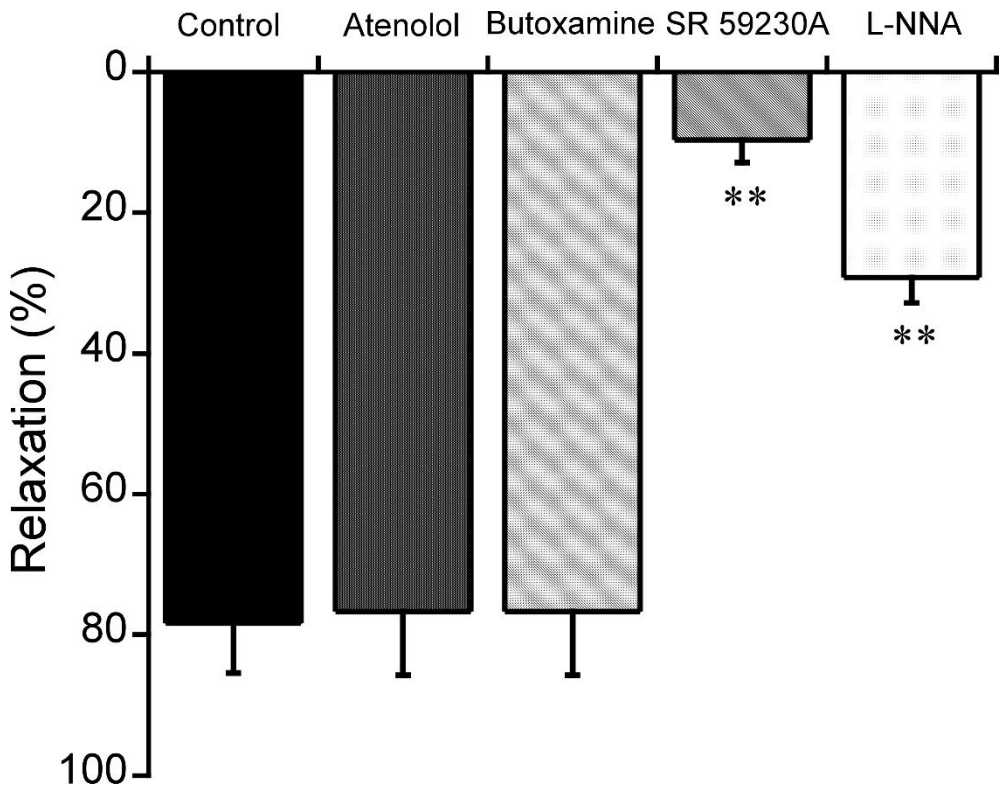

Figure 6. Effects of atenolol (10^{-5} M), butoxamine (10^{-5} M), SR 59230A (10^{-5} M), and Nω-nitro-L-arginine (L-NNA, 10^{-4} M) on SR 58611A (10^{-5} M)-induced relaxation. The relaxation induced by 10^{-4} M sodium nitroprusside was taken as 100%. Each point represents the mean ± SEM of 5 chickens (**$P < 0.01$ vs. control).

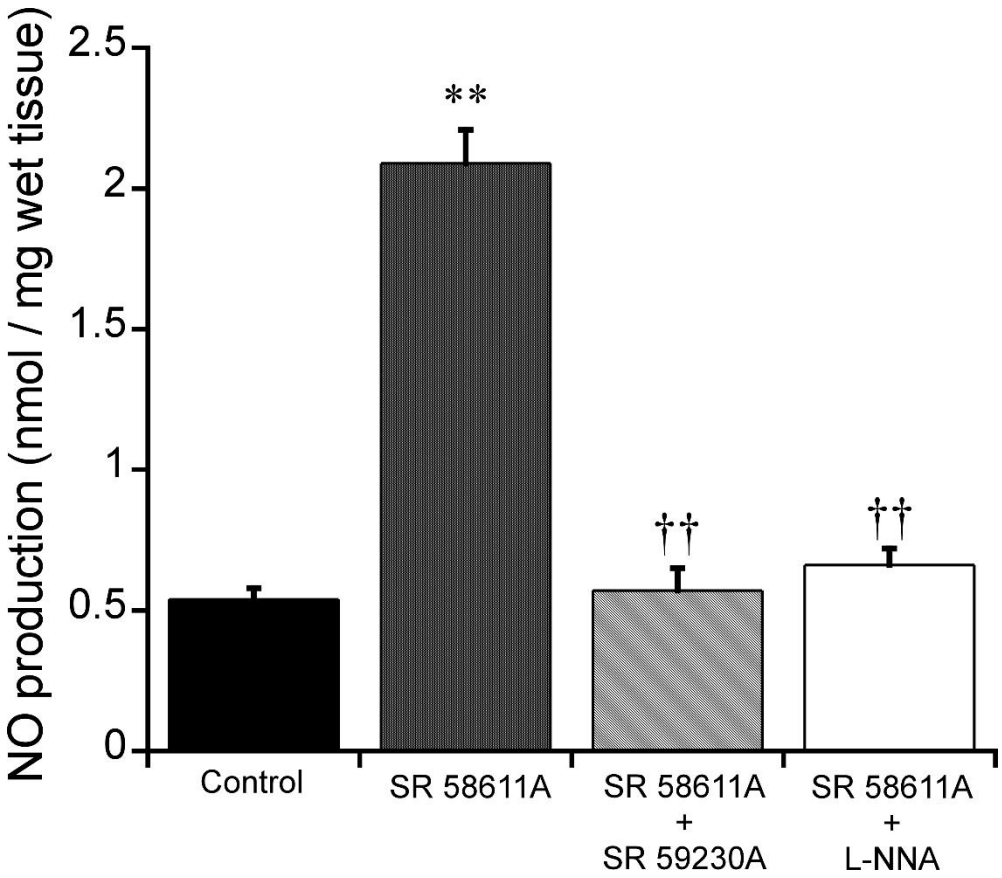

Figure 7. Nitric oxide (NO) production ($[NO^{2-}] + [NO^{3-}]$) treated with SR 58611A (10^{-5} M) from 4 isolated chicken basilar arterial strips with intact endothelium for 1 h, and the effects of SR 59230A (10^{-5} M) and N^{ω}-nitro-L-arginine (L-NNA, 10−4 M) on the NO production. Control: spontaneous NO production. Each column represents the mean ± SEM of arteries from 12 chickens. (**$P < 0.01$ vs. control and ††$P < 0.01$ vs. SR 58611A).

Chapter 2

Study 2

Reduced nitric oxide synthase involvement in Aigamo duck basilar arterial relaxation

1. Simple Summary

The basilar artery is a vital cerebral blood vessel common in most vertebrates and constantly supplies blood to the hindbrain where many vital functions are coordinated. Avian basilar arterial responsiveness to vasoactive substances has been characterized only in chickens. In this artery, the endothelium plays an important role in relaxation, and endothelial dependence may explain the lethality of the highly pathogenic avian influenza virus, which reportedly induces apoptosis in the cerebrovascular endothelium. Our present results in ducks suggest a contrast to the previously reported results in chickens with regard to basilar arterial relaxation: The involvement of endothelial nitric oxide as a relaxing factor appears to be reduced in duck basilar arteries. Our research may help scientists to better understand the resistance to the highly pathogenic avian influenza virus that may be conferred by the cerebrovascular endothelium in ducks.

2. Abstract

The basilar arterial endothelium mediates blood vessel relaxation partly through the release of nitric oxide (NO). Apoptosis of cerebrovascular endothelial cells is linked to a high mortality rate in chickens infected with the highly pathogenic avian influenza virus, but interestingly, ducks exhibit a greater resistance to this virus. In this study, we examined the responsiveness of duck basilar arteries (BAs) to various vasoactive substances, including 5-hydroxytryptamine (5-HT), histamine (His), angiotensin (Ang) II, noradrenaline (NA), acetylcholine (ACh), and avian bradykinin ornithokinin (OK), aiming to characterize the receptor subtypes involved and the role of endothelial NO in vitro. Our findings suggest that arterial contraction is mediated with 5-HT1 and H1 receptors, while relaxation is induced with β_3-adrenergic and M_3 receptors. Additionally, OK elicited a biphasic response in duck BAs, and Ang II had no effect. Endothelial NO appears to be crucial in relaxation mediated with M_3 and OK receptors but not β_3-adrenergic receptors in the duck BA. The reduced endothelial NO involvement in the receptor-mediated relaxation response in duck BAs represents

a clear difference from the corresponding response reported in chicken BAs. This physiological difference may explain the differences in lethality between ducks and chickens when vascular endothelial cells are infected with the virus.

Keywords: cerebral artery; duck; endothelium; highly pathogenic avian influenza; nitric oxide

3. Introduction

Ducks appear to be markedly more resilient to highly pathogenic avian influenza virus (HPAIV) than chickens (Davis et al., 2018; de Bruin et al., 2022). For example, in one study, all wild ducks (from the genus Anas) experimentally infected with HPAIV remained free of symptoms and survived (Keawcharoen et al., 2008). In chickens, by contrast, HPAIV infection often leads to fatal outcomes in which the vascular endothelium may be implicated. Vascular endothelial cells in the liver, kidney, and brain in chickens with HPAIV are reportedly prone to apoptosis, and this phenomenon has been linked to the deaths of infected birds (Ito et al., 2002; Vreman et al., 2022). Acute apoptosis is reportedly associated with high levels of endothelial nitric oxide (NO) release during HPAIV infection, and the released NO may act with other damaging oxidants to promote excessive inflammation (Burggraaf et al., 2011). Reports from human physiology suggest that elevated NO levels can promote proinflammatory effects, including increased vascular permeability, cytotoxicity, and inflammatory cell infiltration (Burggraaf et al., 2011; Cirino et al., 2003; Redington, 2006), and any related loss of endothelial function in vital arteries could quickly prove fatal. Thus, it is plausible that ducks' resilience to HPAIV may stem from distinctive characteristics of their vascular endothelium, but vasoreactivity and endothelial NO release remain largely uncharacterized in duck species.

The basilar artery (BA) may provide a useful location for investigating vasoreactivity and endothelial release in ducks. It is a vital artery that constantly supplies blood to the hindbrain and a common cerebrovascular feature across vertebrates that contributes to the maintenance of cerebral circulatory volume (Rahamt and Gilland, 2014). Its role in birds appears to be the same as that in mammals, although chickens are the only avian species in which BA reactivity has been studied (Matsumoto et al., 2012; Okuno et al., 2008; Wu et al., 2023). In chickens, the BA endothelium has been identified as a powerful source of spontaneous NO release, which was based on strong contractions noted after the application of N^{ω}-nitro-L-arginine (L-NNA), a NO synthase (NOS) inhibitor (Okuno

et al., 2008). Furthermore, previous investigations of endothelial cell receptors have indicated that beta-3 adrenergic receptors (β_3-ARs), histamine (His) H_1 receptors, and muscarinic acetylcholine (ACh) M_3 receptors are involved in the relaxation of the chicken BA via the NO pathway, suggesting this arterial relaxation is strongly dependent on the endothelial release of NO (Matsumoto et al., 2012; Okuno et al., 2008; Wu et al., 2023).

Evolutionarily speaking, ducks and chickens are separated by 90 million years, and avian genomes tend to be more strongly conserved than mammalian genomes (Ramirez et al., 1993; Skinner et al., 2009). We thus considered it plausible that ducks may have a less complex vascular endothelium than chickens, who were later to emerge as a species. Interestingly, HPAIV reportedly rarely shows endothelial tropism in either wild or domestic ducks (Short et al., 2014; Tong et al., 2021).

Against this background, we aimed to investigate the vascular endothelium of the BA in ducks by characterizing its capacity for endothelial NO release and its response to a range of vasoactive substances previously evaluated in our studies of this artery in chickens.

4. Materials and Methods

4.1. Tissue Preparation

BAs were obtained from freshly slaughtered Aigamo ducks (Anas platyrhynchos/Anas platyrhynchos var. domesticus hybrid; n = 71, both sexes, weight: 2.97 ± 0.04 kg). The ducks had been raised for meat and allowed to forage on insects in rice paddies on farms in Kagoshima Prefecture, Japan, and were slaughtered in accordance with the relevant Japanese law on agricultural animals. The sampled arteries were transferred to our laboratory in 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% (vol/vol) O_2, 5% (vol/vol) CO_2]. Each artery was immediately dissected free of adherent tissues under a stereomicroscope. All experiments were performed in accordance with the Guidelines

for Animal Experiments of Kagoshima University. Our data were obtained from Aigamo ducks, not from species-pure ducks, and this represents a limitation of this study.

4.2. Reagents

The following reagents were used at the described concentrations: His hydrochloride (10^{-6}–10^{-3} M, Sigma-Aldrich, St. Louis, MO, USA), isoproterenol (10^{-9}–10^{-5} M, Sigma-Aldrich), ketanserin tartrate (10^{-6} M, Sigma-Aldrich), methiothepin maleate (10^{-8}–10^{-6} M, Sigma-Aldrich), diphenhydramine hydrochloride (10^{-7}–10^{-5} M, Sigma-Aldrich), cimetidine (10^{-5} M, Sigma-Aldrich), angiotensin (Ang) II (human) acetate salt (10^{-8}–10^{-4} M, Sigma-Aldrich), butoxamine hydrochloride (10^{-6} M, Sigma-Aldrich), SR 59230A (10^{-7}–10^{-6} M, Sigma-Aldrich), L-NNA (10^{-4} M, Sigma-Aldrich), noradrenaline (NA, 10^{-9}–10^{-4} M, Tokyo Chemical Industry, Tokyo, Japan), phentolamine mesylate (10^{-5} M, Tokyo Chemical Industry), 5-hydroxytryptamine (5-HT)-creatinine sulfate (10^{-8}–10^{-4} M; Merck, Darmstadt, Germany), adrenaline (10^{-9}–10^{-5} M, Daiichi Sankyo, Tokyo, Japan), ACh chloride (10^{-9}–10^{-5} M, Daiichi Sankyo), procaterol (10^{-9}–10^{-5} M, Fujifilm-Wako, Tokyo, Japan), atenolol (10^{-6}–10^{-5} M, LKT Laboratories, Tokyo, Japan), hexahydro-sila-difenidol hydrochloride, p-fluoroanalog (pFHHSiD, 10^{-7}, 10^{-6} M; Research Biochemical, Natick, MA, USA), avian bradykinin ornithokinin (OK, 10^{-9}–10^{-5} M, BEX Co. Ltd., Tokyo, Japan), and sodium nitroprusside (SNP, 10^{-4} M; Nacalai Tesque, Kyoto, Japan). All reagents were used in accordance with the relevant manufacturer's instructions. Each of the selected agonists had previously been validated in vertebrate species (including avian species) (Fujita et al., 2023; Miki et al., 1992; Perry and Capaldo, 2011; Wächtler, 1980). Their reported effects on cerebral arteries in a range of species as demonstrated in vitro studies are shown in Table 1.

4.3. Functional Study

The methods applied in the functional study were as described in our previous study in chickens (Wu et al., 2023). Briefly, three or four rings, approximately 2 mm wide, were cut from each duck BA. Each ring was mounted horizontally between two L-shaped, stainless-steel holders (outer diameter 0.03 mm) with one part fixed to an isometric force transducer. The mounted ring was then immersed in a 4 mL water-jacketed micro-tissue organ bath (UMTB-1, Unique Medical Co. Ltd., Tokyo, Japan) containing oxygenated physiological saline at 41 °C (pH 7.4). For experiments with precontraction, 5-HT was selected as the inducing agent. Isometric tension was measured with an amplifier.

The log concentration ratio of EC_{50} values (i.e., the concentration producing a half-maximal response) in the absence or presence of an antagonist was calculated and plotted against the logarithm of the antagonist concentration to obtain the pA_2 values (Arunlakshana and Schild, 1959).

At the end of the relaxant response, SNP (10^{-4} M) was applied to produce maximal relaxation, which was taken as 100%.

4.4. Statistical Analysis

Results are expressed as the mean ± SEM, and statistical analyses were performed using Student's t-test or the Bonferroni test after one-way ANOVA (Stat View J-4.5, Abacus Concepts Inc., Berkeley, CA, USA). *p*-values < 0.05 were considered statistically significant.

5. Results

5.1. Spontaneous Nitric Oxide and Prostaglandin Release

The typical responses to L-NNA (a NOS inhibitor, 10^{-4} M) with a subsequent application of indomethacin (a cyclooxygenase inhibitor, 10^{-5} M) under the resting tone are illustrated in Figure 1: L-NNA-induced contraction (30.5 ± 5.4% to 60 mM KCl, n = 4 ducks) under resting tension and indomethacin-induced relaxation (−36.1 ± 2.1% to 10^{-4} M SNP, n = 4 ducks) under contraction induced with L-NNA. The

contraction induced with 60 mM KCl was 1.84 ± 0.04 mN.

5.2. Responsiveness to Vasoactive Substances

To ascertain the dominant receptor subtypes in duck BAs, we investigated the effect of a range of substances with a known vasoactive effect in chickens and other species. Specifically, the agents used to generate effects on the vascular endothelium were 5-HT, His, Ang II, ACh, NA [a beta-1 (β_1) and non-selective alpha (α) AR agonist], and OK (an avian bradykinin receptor agonist). The contractile response was measured under a resting tension condition. Under this condition, 5-HT and His induced contraction in concentration-dependent manners with respective half-maximal effective concentrations (logarithmically adjusted, hereafter, pEC_{50} values) of 5.84 ± 0.06 and 4.37 ± 0.09, whereas no effect was noted for NA in the absence or presence of propranolol (a non-selective β-AR antagonist, 10^{-5} M) or Ang II (Figure 2A and Table 2). The relaxation response was measured under precontraction with 5-HT. Following precontraction, ACh, NA, and OK induced relaxation in a concentration-dependent manner, yielding respective pEC_{50} values of 6.18 ± 0.17, 6.28 ± 0.05, and 6.20 ± 0.25. Phentolamine (10^{-5} M), a non-selective α-AR antagonist, had no significant effect on the response to NA (Figure 2B and Table 2).

5.3. Involvement of 5-Hydroxytryptamine Receptor Subtype

To ascertain the predominant 5-HT receptor subtype in the duck BA, we investigated the effects of methiothepin (a non-selective 5-HT receptor antagonist) and ketanserin (a 5-HT_2 receptor selective antagonist) on 5-HT-induced contraction. The Schild plot for methiothepin showed a slope of 1.16 ± 0.19 (Figure 3C), which did not significantly diverge from unity. Its calculated pA_2 value was 8.52 ± 0.17, whereas ketanserin (10^{-6} M) had no significant effect on the 5-HT-induced contraction in the duck BAs (Figure 3B).

5.4. Involvement of Histamine Receptor Subtypes

To ascertain the predominate His receptor subtype in the duck BA, we investigated the effects of diphenhydramine (an H_1 receptor antagonist) and cimetidine (an H_2 receptor selective antagonist) on His-induced contraction. The Schild plot for diphenhydramine (10^{-7}–10^{-5} M) showed a slope of 1.09 ± 0.15 (Figure 4B), which did not significantly diverge from unity. Its calculated pA_2 value was 6.89 ± 0.13, whereas cimetidine (10^{-5} M) had no significant effect (Figure 4).

5.5. *Responsiveness to β-Adrenergic Receptor Agonists*

To elucidate the roles of β-ARs in the duck BA, we applied different β-AR agonists that may elicit relaxation effects and monitored the vascular response. The agents applied were isoproterenol (a non-selective β-AR agonist), NA (a β_1-AR and non-selective α-AR agonist), adrenaline (a non-selective β-AR and non-selective α-AR agonist), and procaterol (a β_2-AR agonist). Three of the four β-AR agonists induced concentration-dependent relaxation of the duck BAs; the exception was procaterol (Figure 5). Their rank order of potency as inducers of relaxation was isoproterenol > NA > adrenaline (Table 2). None of these agents induced contraction.

5.6. *Involvement of β-Adrenergic Receptor Subtype*

To ascertain the predominant β-AR subtype in the duck BA, we investigated the effects of atenolol (a β_1-AR antagonist), butoxamine (a β_2-AR antagonist), and SR 59230A (a β_3-AR antagonist) on isoproterenol-induced relaxation. In this experiment, atenolol (a β_1-AR antagonist, 10^{-6} M) and butoxamine (a β_2-AR antagonist, 10^{-6} M) had no significant effect on isoproterenol-induced relaxation (Figure 6A). In contrast, only SR 59230A effectively antagonized the isoproterenol-induced relaxation, and its slope of the Schild plot was 1.18 ± 0.11, which did not significantly diverge from unity (Figure 6C). Its calculated pA_2 value was 7.03 ± 0.08. Furthermore, L-NNA (10^{-4} M) did not affect the isoproterenol-induced relaxation in duck BAs (Figure 6A).

5.7. *Involvement of Muscarinic Receptor Subtype*

To ascertain the predominant muscarinic ACh receptor subtype in the duck BA, we investigated the effects of atropine (a non-selective muscarinic ACh receptor antagonist), pirenzepine (a muscarinic ACh M_1 receptor selective antagonist), methoctramine (a muscarinic ACh M_2 receptor selective antagonist), and pFHHSiD (a muscarinic ACh M_3 receptor selective antagonist) on ACh-induced contraction. Atropine shifted the concentration-response curve for ACh to the right at 10^{-8} M and largely abolished ACh-induced relaxation at 10^{-7} M (Figure 7A). For selective muscarinic antagonists, the respective slopes of Schild plots for pirenzepine and pFHHSiD were 1.06 ± 0.10 and 0.97 ± 0.08, neither of which significantly diverged from unity. The respective pA_2 values yielded with pirenzepine and pFHHSiD were 6.52 ± 0.12 and 8.06 ± 0.13 (Figure 7E, F), whereas methoctramine did not affect the ACh-induced relaxation in duck BAs. L-NNA mostly abolished the ACh-induced relaxation of duck BAs (Figure 7D).

5.8. *Effects of Nitric Oxides Synthase and Cyclooxygenase Inhibitors on Ornithokinin-Induced Response*

To investigate OK-induced relaxation in the duck BA, we applied L-NNA and L-NNA plus indomethacin. In the presence of L-NNA (10^{-4} M), OK-induced relaxation was attenuated significantly ($p < 0.01$) and even transferred to contraction at 10^{-5} M, whereas indomethacin (10^{-5} M) abolished this contraction (Figure 8).

6. Discussion

The present study is the first to characterize basilar arterial responsiveness to a range of vasoactive substances in ducks. We showed that NO is a diminished endothelial mediator in ducks relative to its reported role in chickens. This information will prove especially useful for comparisons between ducks, who are not prone to disease and death after HPAIV infection, and chickens, who are.

We demonstrated the involvement of spontaneously released endothelial NO based on the contraction induced with the NOS inhibitor L-NNA followed by the

relaxation induced with indomethacin, which inhibits cyclooxygenase, under resting tension (Figure 1). Our findings in ducks are similar to the results obtained in some mammalian species, including dogs and bats (Islam et al., 2021; Shirahase et al., 1987). In porcine BAs, further studies demonstrated that spontaneous NO is released from endothelial cells and spontaneous thromboxane A_2, which is formed from PGH_2 and inhibited with indomethacin, is also released from endothelial cells (Miyamoto et al., 1999; 2007). The mechanism for maintaining cerebrovascular tone in ducks partially resembles that in chickens. However, a key difference was that the L-NNA-induced contraction in ducks (30.5%) was markedly weaker than that in chickens (122.1%) (Okuno et al., 2008), suggesting that ducks experience less involvement of spontaneous endothelial NO than chickens.

We also investigated the receptor subtypes involved in contraction and relaxation and their location (smooth muscle or endothelial cells) in the duck BA.

It appears that 5-HT receptors play a similar role in ducks and chickens, as the receptor agonist induced a concentration-dependent contraction in isolated duck BAs in both the avian species we evaluated, and we found that the 5-HT_1 receptor may be the dominant receptor of this subtype (Figure 3). Based on an evaluation of the half-maximal effect concentrations, we found that the 5-HT receptor antagonist methiothepin produced a similar effect on the duck BA to that observed on the chicken BA and the rabbit saphenous vein where the vascular response (vasocontraction) is mediated via activation of the 5-HT_1 receptor (Matsumoto et al., 2012; van Heuven-Nolsen et al., 1990). Furthermore, the effects of methiothepin and the 5-HT_2 antagonist ketanserin in the duck BA (respective rightward shifts in the concentration–response curve of 35.5-fold and 2.7-fold) were similar to the corresponding shifts (30-fold and 3-fold, respectively) we previously observed in the chicken BA (Matsumoto et al., 2012), suggesting ducks and chickens share a similar characterization for this receptor type.

We also profiled the involvement of His receptors. The His-induced, concentration-dependent contraction in isolated duck BAs in this study resembled

those observed in porcine, bovine, and equine BAs, with a similar half-maximal effect to that in the bovine report but a smaller half-maximal effect than those in the porcine and equine (Miyamoto and Nishio, 1993; 1994) reports. The H_1-receptor antagonist diphenhydramine (10^{-7}–10^{-5} M) had an effect on ducks, but this effect was of a smaller magnitude than those reported in cattle, pigs, and horses (Miyamoto and Nishio, 1993; 1994). The H_2-receptor antagonist cimetidine had no effect, and we thus consider that H_1 receptor activation induces contraction of the duck BA. Our findings related to His receptors in ducks present a contrast to the corresponding findings previously reported in chickens. Okuno et al. (2008) (Okuno et al., 2008) reported that both H_1 and H_2 receptors, which are located on respective endothelial cells and smooth muscle cells, are involved in the relaxation of the chicken BA. However, in ducks, H_1 receptors play a dominant role in His-induced contraction, while H_2 receptors were not involved.

For adrenergic receptors, noradrenaline had no effect on the BA at resting tension, and phentolamine (a non-selective α-ARs antagonist) had no effect on noradrenaline-induced relaxation under precontraction (Figure 2), suggesting that α-ARs are not involved in duck BA reactivity. This presents a contrast with the chicken BA where α-ARs are present and involved in contraction, and phentolamine inhibits this contraction (Wu et al., 2023). For β-ARs, the duck BA appears to possess β_1-ARs but with a smaller population for this receptor subtype than in chickens based on the similar potency ranking but smaller effect size for isoproterenol and NA (Wu et al., 2023). β_2-ARs appear not to play a major role in the duck BA, although they are present as a non-dominant subtype in chickens based on findings for the effect of procaterol (Wu et al., 2023). Interestingly, the effect of the β-AR agonist isoproterenol-induced relaxation was not inhibited with the NOS inhibitor L-NNA (Figure 6A); therefore, we suggest that NO plays no role in β-AR mediation in ducks, which represents a physiological difference from chickens.

We consider that β_3-AR is the predominant β-AR subtype in the duck BA based on the results obtained with a range of β-AR antagonists (Figure 6). Most pertinently,

only the β_3-AR antagonist SR 59230A demonstrated an effective antagonistic effect (pA2 value: 7.03) in duck BAs comparable to that in rat urinary bladder tissue (7.27) and the guinea pig gastric fundus (7.35) where β_3-ARs are known to predominate (Horinouchi et al., 2001; Obara et al., 2019). Accordingly, we suggest that β_3-AR is predominantly involved in isoproterenol-induced relaxation in the duck BA, a phenomenon similar to that reported in chickens. However, this relaxation is endothelium-dependent in chickens but not in ducks.

Our findings for muscarinic ACh receptors (Figure 7) resembled those observed in bat, chicken, and mouse BAs (Matsumoto et al., 2012; Islam et al., 2014; 2021), suggesting their activation is involved in the response to ACh. We further suggest that the M_3 receptor is the predominant subtype involved in ACh-induced relaxation, and M_1 and M_2 receptors may not be involved in duck BAs. We found no effect for the M_2 antagonist methoctramine and only a weak effect for the M_1 antagonist pirenzepine. The latter effect was smaller than that reported in cat cerebral arteries (8.08) where muscarinic M_1 receptors predominate (Dauphin et al., 1991). However, the M_3 antagonist pFHHSiD yielded a calculated pA_2 value (8.06) similar to those reported in bovine coronary arteries (7.64), human uterine arteries (8.17), and chicken BAs (7.55) where the muscarinic M_3 receptor predominates (Matsumoto et al., 2012; Friedrich et al., 1991; Jovanović et al., 1994). In contrast to our findings on β-ARs, we consider that NO is involved in muscarinic-M_3-receptor-mediated relaxation in ducks, as it is in other species, including mice, bats, and chickens (Matsumoto et al., 2012; Islam et al., 2014b; 2021), based on the abolition of the ACh-induced relaxation with the NO inhibitor L-NNA seen in this study (Figure 7D).

OK, which has been purified from duck plasma, is reported to be an avian bradykinin (Kimura et al., 1989). We revealed that OK induced relaxation and contraction in duck BAs via regulation of the respective NO and cyclooxygenase pathways. In porcine BAs, bradykinin produced NO and prostaglandin $F_{2\alpha}$ then induced relaxation followed by contraction (Miyamoto et al., 2007; Islam et al., 2014a]. OK receptors are the avian homolog to mammalian bradykinin receptors and

are known to be involved in blood vessel dilatation, smooth muscle contraction, increased vascular permeability, and inflammation (Schmaier, 2016). OK receptors reportedly potentiate proinflammatory responses in chicken macrophages (Guabiaba et al., 2017). Pharmacological research on these receptors is hampered with a lack of established antagonists; accordingly, it was not possible to investigate the relevant receptor subtypes in this study. However, we anticipate this line of research will be enabled with advances in cell cultures in the future.

BA responsiveness to vasoactive substances is known to differ widely between mammalian species according to differences in receptor subtypes and their distribution on smooth muscle or endothelial cells. To our knowledge, BA responses have never shown fully identical characteristics in any two mammalian species, and we speculate that similar diversity will be observable across avian species based on the present study, which is the second characterization of BA response in an avian species. However, one feature common to ducks in this study and to chickens in our previous report was β_3-AR-mediated vasodilation, which has never been reported in mammalian cerebrovascular systems (Wu et al., 2023; Miyamoto et al., 1993). We speculate that β_3-ARs may play an important role in the cardiovascular system of birds, and this represents an interesting line of future research. These physiological differences could be explained with the earlier evolutionary emergence of ducks than chickens, which means that ducks may exhibit simpler endothelial physiology. In contrast, chickens have undergone significant adaptations to suit terrestrial life, leading to more complex functionalities mediated by the endothelium.

7. Conclusions

In conclusion, we suggest that the BA of the duck is characterized by a smaller endothelial release of NO and a smaller degree of endothelial involvement in its reactivity than the BA of the chicken. The 5-HT_1 and H_1 receptors may be involved in arterial contraction, and the β_3 and M_3 receptors may be involved in relaxation. NO plays a role with M_3 but not β_3-adrenergic receptors. These physiological differences

may help to explain why severe effects (including death) of HPAIV infection are seen in chickens but not ducks.

Table 1. Effects of agonists on cerebral arteries in different animal species.

Agonists	Effect (receptor subtype)	Species
Noradrenaline	Contraction (α) Relaxation (β_1, β_2)	Chicken (Wu et al., 2023) Pig (Miyamoto et al., 1993)
Acetylcholine	Relaxation (M_3)	Chicken (Matsumoto et al.,2012), Cat (Dauphin and Hamel, 1990)
5-Hydroxytryptamine	contraction (5-HT_1, 5-HT_2)	Chicken (Matsumoto et al.,2012), Rat (Kovács et al., 2012)
Histamine	contraction (H_1)	Pig (Miyamoto and Nishio, 1993), Cattle (Miyamoto and Nishio, 1994)
Angiotensin II	contraction (AT_1)	Bat (Islam et al., 2021), Mouse (Walker et al., 2019)
Bradykinin	contraction and relaxation (B_2) relaxation (B_2)	Pig (Miyamoto et al., 1999) Mouse (Islam et al., 2014)

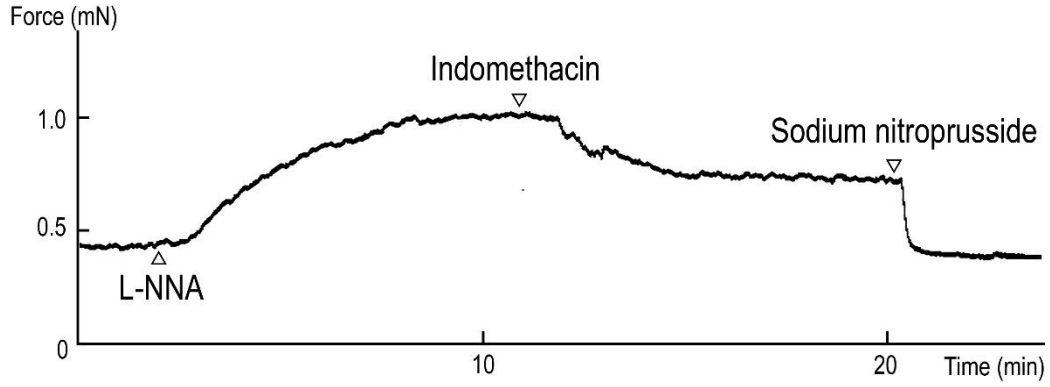

Figure 1. Typical contraction induced with N^{ω}-nitro-L-arginine (L-NNA, 10^{-4} M) and relaxation induced with indomethacin (10^{-5} M) under precontracted conditions induced with L-NNA.

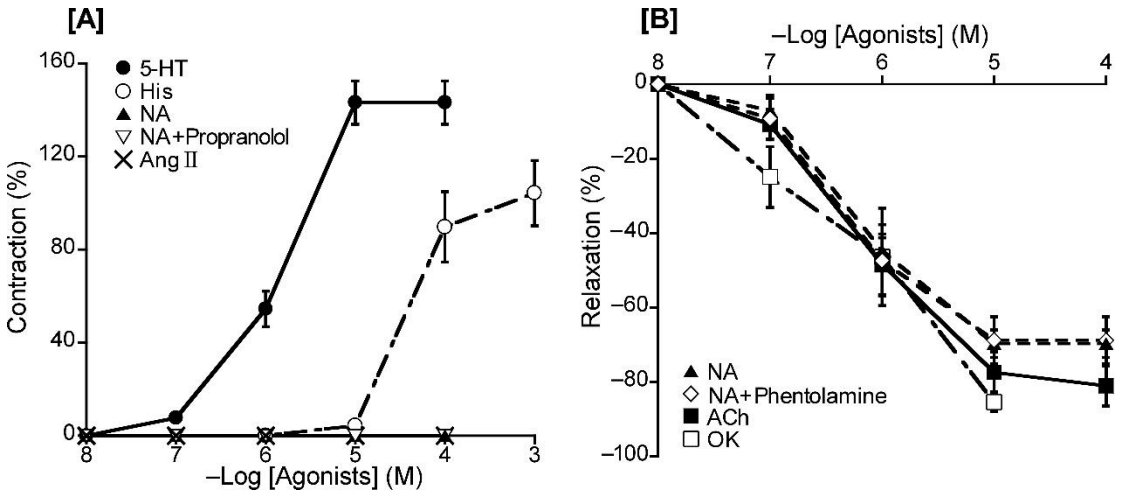

Figure 2. Responsiveness to 5-hydroxytriptamine (5-HT), histamine (His), noradrenaline (NA), and angiotensin (Ang) II under resting tension (**A**) and to NA, acetylcholine (ACh), and ornithokinin (OK) under a precontracted condition induced with 5-HT (**B**). The contraction induced with 60 mM KCl (**A**) and the relaxation induced with 10^{-4} M sodium nitroprusside (**B**) was taken as 100% contraction and relaxation, respectively. Each point represents the mean ± SEM of 4–6 ducks. The percentage of reacting vessels is 100%.

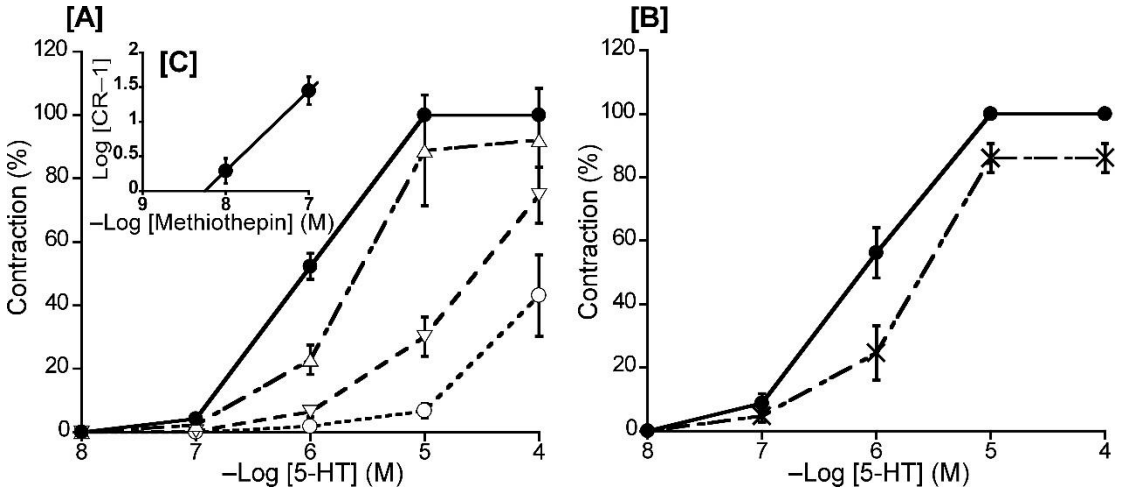

Figure 3. Effects of 5-hydroxytriptamine (5-HT) antagonists on 5-HT-induced contraction in the duck basilar artery. The antagonists were methiothepin (Δ: 10^{-8} M, ▽: 10^{-7} M, ○: 10^{-6} M) (**A**) and ketanserin (×: 10^{-6} M) (**B**). The Schild plot for methiothepin is shown in (**C**). The maximal contraction induced with 5-HT was taken as 100%. Each point represents the mean ± SEM of 6 ducks.

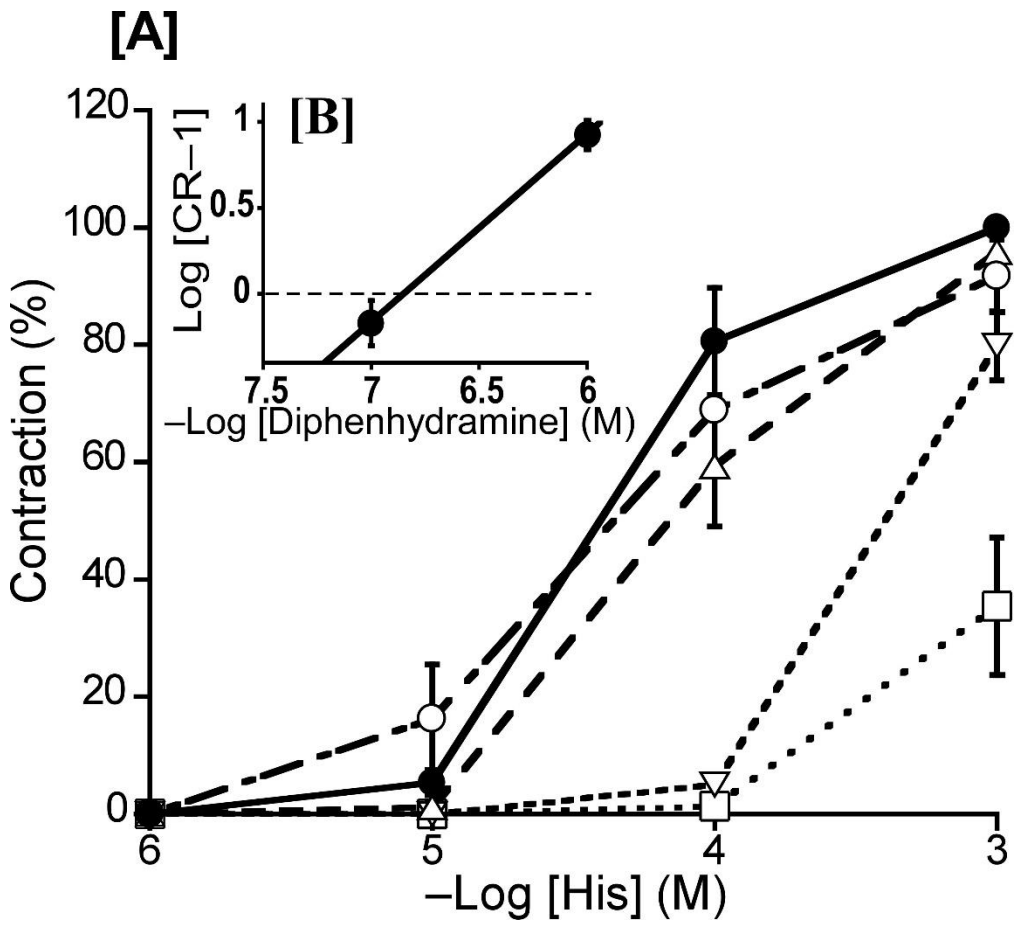

Figure 4. Effects of histamine (His) antagonists on His-induced contraction in the duck basilar artery. The antagonists were diphenhydramine (Δ: 10^{-7} M, ▽: 10^{-6} M, □: 10^{-5} M) and cimetidine (○: 10^{-5} M) (**A**). The Schild plot for diphenhydramine is shown in (**B**). The maximal contraction induced with His was taken as 100%. Each point represents the mean ± SEM of 5 ducks.

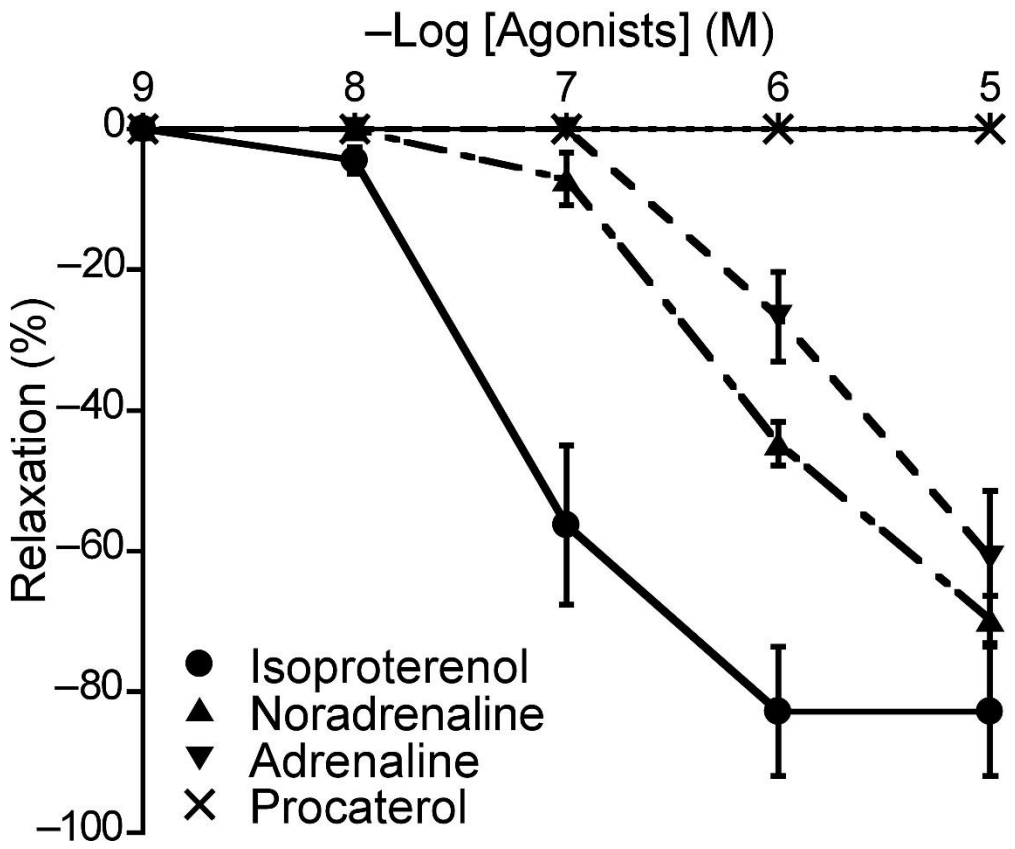

Figure 5. Responsiveness of duck basilar arteries to isoproterenol (●), noradrenaline (▲), adrenaline (▼), and procaterol (×) under precontracted conditions. The relaxation induced with 10^{-4} M sodium nitroprusside was taken as 100%. Each point represents the mean ± SEM of 4–6 ducks.

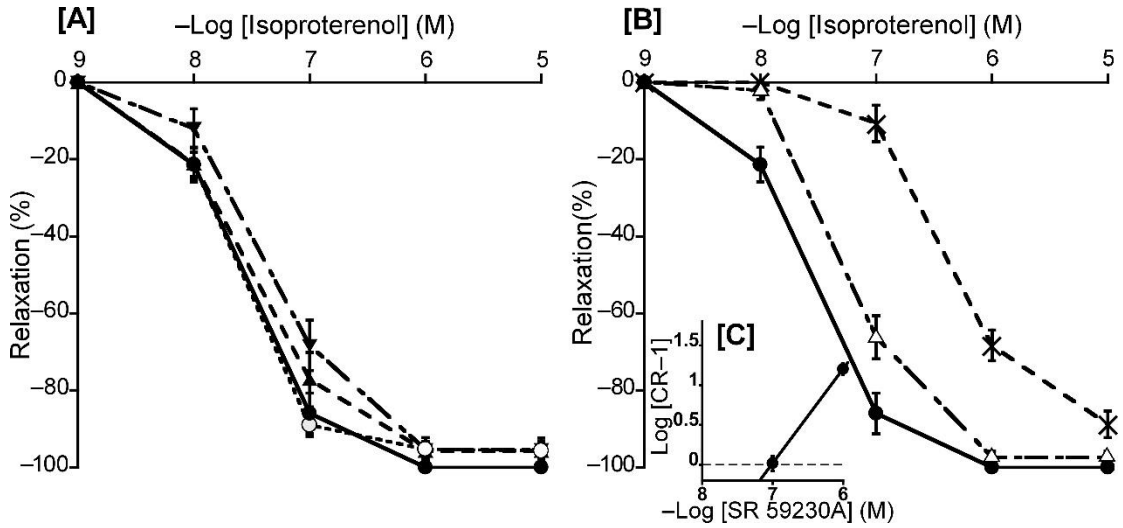

Figure 6. Effects of β-adrenergic receptor antagonists and a NOS inhibitor (L-NNA) on isoproterenol-induced relaxation in the duck basilar artery. The antagonists or inhibitor were atenolol (▲: 10^{-6} M), butoxamine (▼: 10^{-6} M), L-NNA (○: 10^{-4} M) (**A**), and SR 59230A (Δ: 10^{-7} M, ×: 10^{-6} M) (**B**). The Schild plot for SR 59230A is shown in (**C**). The maximal relaxation induced with isoproterenol was taken as 100%. Each point represents the mean ± SEM of 4 or 5 ducks.

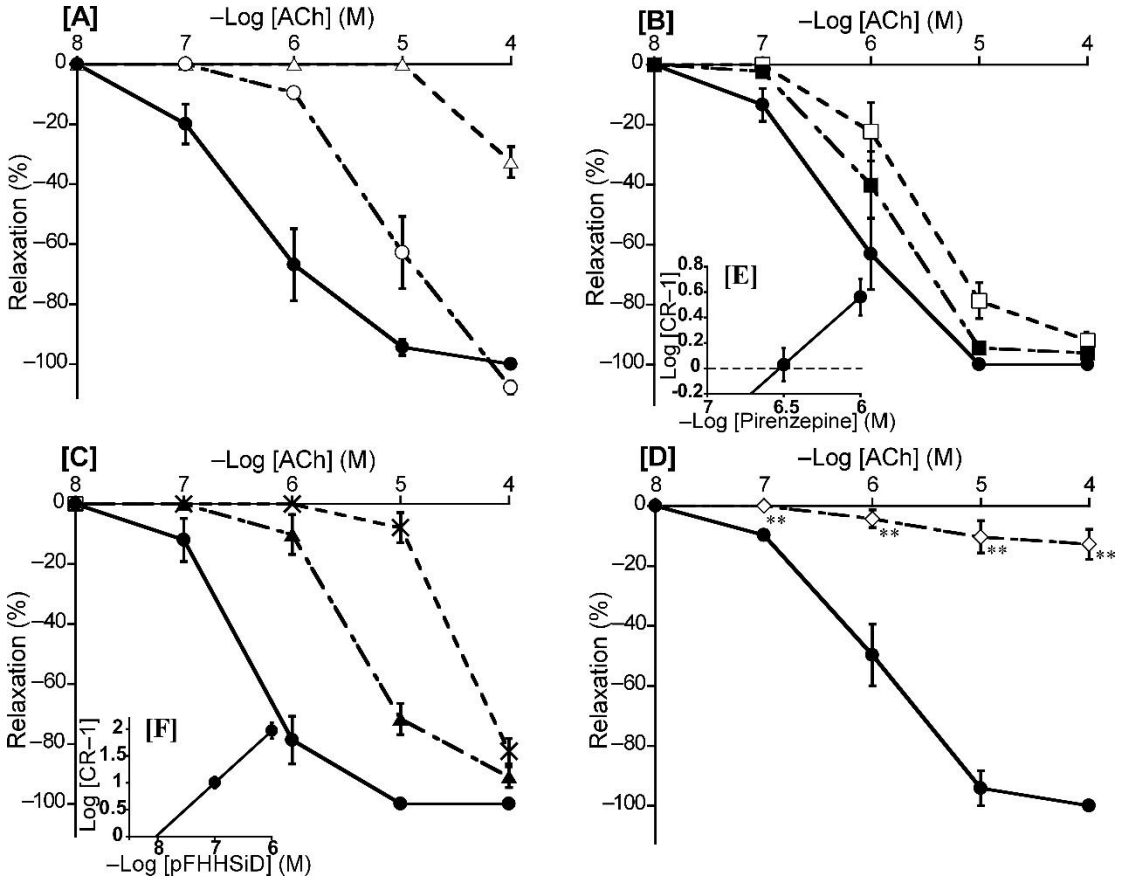

Figure 7. Effects of acetylcholine (ACh) antagonists and a NOS inhibitor (L-NNA) on ACh-induced relaxation in the duck basilar artery. The antagonists or inhibitor were atropine (○: 10^{-8} M, Δ: 10^{-7} M) (**A**), pirenzepine (■: $10^{-6.5}$ M, □: 10^{-6} M) (**B**), hexahydro-sila-difenidol hydrochloride, *p*-fluoroanalog (pFHHSiD; ▲: 10^{-7} M, ×: 10^{-6} M) (**C**), and L-NNA (◇:10^{-4} M) (**D**). The Schild plots for pirenzepine and pFHHSiD were shown in (**E**, **F**), respectively. The maximal relaxation induced with ACh was taken as 100%. Each point represents the mean ± SEM of 5 ducks. (**$p < 0.01$ vs. control).

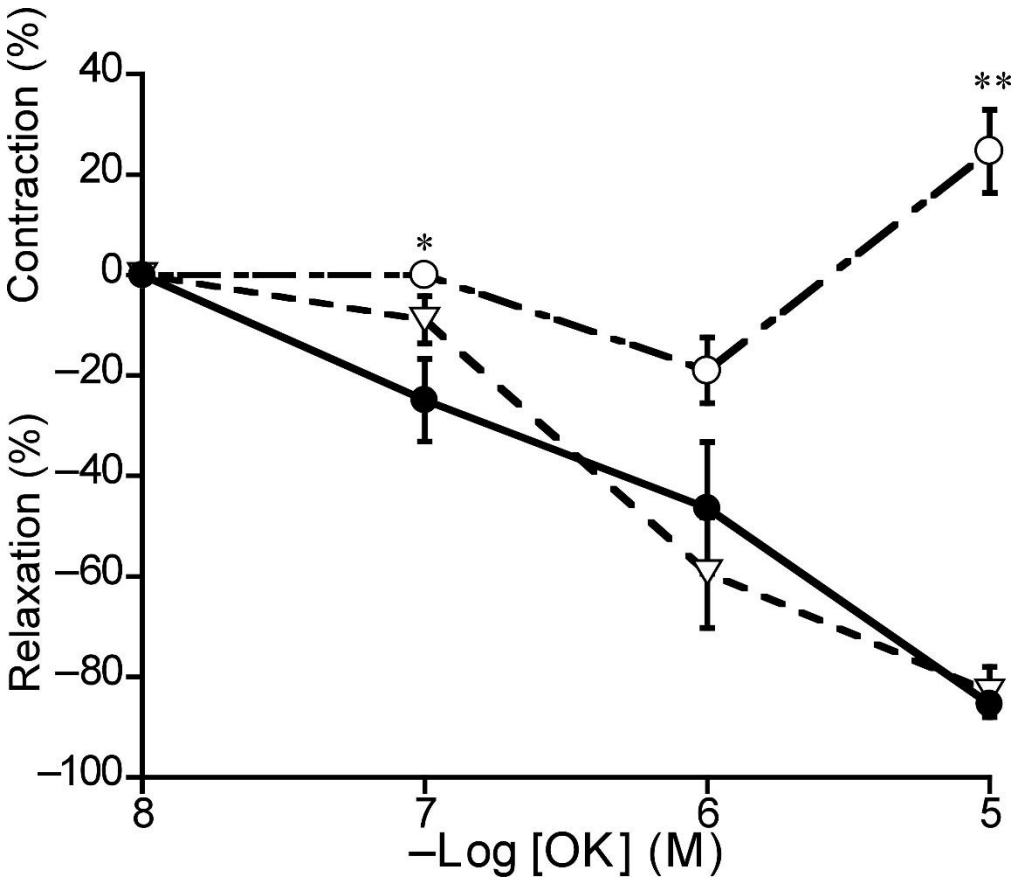

Figure 8. Effects of L-NNA (○: 10^{-4} M) and L-NNA plus indomethacin (10^{-5} M) (▽) on ornithokinin (OK)-induced relaxation (●) in duck basilar arteries. The contraction induced with 60 mM KCl and the relaxation induced with 10^{-4} M sodium nitroprusside were taken as 100% contraction and relaxation, respectively. Each point represents the mean ± SEM of 4 ducks. (*$p < 0.05$, **$p < 0.01$ vs. control).

General discussion

Chapter 1 of Study 1 focused on characterizing the adrenergic receptor (AR) subtypes responsible for modulating vascular responses in chicken basilar arteries, crucial for regulating blood supply to the brainstem. The study revealed the presence of both alpha and beta (α and β) receptor subtypes, with noradrenaline inducing concentration-dependent contraction and relaxation. A noteworthy discovery was the predominant role of β_3-ARs on endothelial cells in mediating the relaxation response, providing novel insights into avian basilar arteries. The investigation also explored the potency ranking of β-AR agonists, identifying isoproterenol as the most potent inducer of relaxation. Schild regression analysis, involving a range of β-AR antagonists against isoproterenol, unveiled the involvement of multiple β-AR subtypes, with β_3-ARs emerging as the predominant subtype. Subsequent experiments with the β_3-AR agonist SR 58611A demonstrated its capacity to induce relaxation, a response inhibited by the nitric oxide synthase (NOS) inhibitor L-NNA. This highlighted the significant role of β_3-ARs in mediating vasodilation through nitric oxide (NO) release.

The study's findings contribute valuable insights into the AR subtypes present in chicken basilar arteries, emphasizing the distinctive contribution of β_3-ARs, especially within endothelial cells. This knowledge expands our comprehension of avian vascular reactivity and holds potential implications for poultry health, particularly in contexts involving viral infections that may impact endothelial cell function. The results also lay the groundwork for future investigations into the specific mechanisms governing β_3-AR-mediated vasodilation in avian species.

Chapter 2, study 2 focused on characterizing the responsiveness of duck basilar arteries (BAs) to vasoactive substances. Ducks, known for their resistance to HPAIV compared to chickens, play a crucial role in understanding the vascular dynamics associated with viral infections. The study revealed that the endothelium of duck BAs, responsible for relaxation through nitric oxide (NO) release, exhibits reduced involvement compared to chickens. This finding suggests a potential explanation for the lower lethality observed in ducks during HPAIV infection, where endothelial apoptosis is

a significant factor in chickens.

The investigation further explored the receptor subtypes involved in duck BA responses to various substances. In terms of vasoactive substances, 5-HT and histamine (His) induced concentration-dependent contraction, with 5-HT_1 and H_1 receptors identified as dominant. This contrasts with chickens, where both H_1 and H_2 receptors contribute to relaxation. Moreover, adrenergic receptors in ducks exhibited differences, with no significant role for α receptors. Interestingly, β_3-ARs were identified as mediators of relaxation in both ducks and chickens, highlighting a potentially vital role in avian vascular physiology. The study ranked β-AR agonists, revealing isoproterenol as the most potent inducer of relaxation. Schild regression analysis, involving a range of β-AR antagonists against isoproterenol, emphasized the dominance of β_3-ARs. Notably, the study unveils that L-NNA does not inhibit the relaxation induced by isoproterenol, indicating the non-involvement of NO in β_3-AR-mediated vasodilation.

The findings contribute novel insights into the distinctive vascular reactivity of duck BAs, emphasizing the diminished role of endothelial NO. These physiological differences may underlie the resilience of ducks to HPAIV, providing valuable information for understanding avian vascular responses to viral infections. The study sets a foundation for future research on the specific mechanisms governing β_3-AR-mediated vasodilation in avian species, opening avenues for potential applications in poultry health and viral infection scenarios.

General conclusion

In conclusion, the dual investigation covering Chapter 1 of Study 1, centered on chicken basilar arteries (BAs), and Chapter 2 of Study 2, focused on duck BAs, collectively unveils the responsiveness of avian basilar arteries to vasoactive substances, providing significant implications for our understanding of the physiological characteristics of avian cerebral arteries. The study on chicken BAs was the first to report the presence of β_3-adrenergic receptors, especially in endothelial cells. The study on duck BAs suggests that duck BAs are characterized by a smaller endothelial release of nitric oxide (NO) and a reduced degree of endothelial involvement in reactivity compared to chicken BAs. The involvement of 5-HT_1 and H_1 receptors in arterial contraction, as well as β_3 and M_3 receptors in relaxation, was highlighted. Notably, NO plays a role with M_3 but not β_3-adrenergic receptors. These physiological differences may offer insights into why severe effects, including death, from HPAIV infection are observed in chickens but not in ducks.

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