Endothelium-Dependent Relaxation of Canine Basilar Arteries

Part 1: Difference Between Acetylcholine- and A23187-Induced Relaxation and Involvement of Lipoxygenase Metabolite(s)

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Vascular responses to acetylcholine (ACh) and the calcium ionophore A23187 were studied in rings of canine basilar arteries. In preparations that were precontracted to a stable plateau by $3 \times 10^{-6}$ M prostaglandin $F_2\alpha$ (PGF$_2\alpha$), $10^{-7}$ to $10^{-5}$ M A23187 elicited significant relaxation of the basilar arteries if the endothelium was intact. Judging from histologic findings, the ability of a ring to relax in this manner is due to the presence of the endothelium. The same concentration of A23187 did not relax vascular tissues in which the endothelium was purposely disrupted. Although $10^{-7}$ to $10^{-3}$ M ACh did not sufficiently produce endothelium-dependent relaxation of canine basilar artery rings, ACh in the same concentration did produce significant relaxation in canine femoral rings. Our results suggest that the sensitivity of the muscarinic receptor of cerebral arteries appears to be appreciably different from that of peripheral (femoral) arteries. Pretreatment with $1.5 \times 10^{-5}$ M indomethacin, a cyclooxygenase inhibitor, potentiated the contractile responses produced by PGF$_2\alpha$ in intact rings. Preincubation with the lipoxygenase inhibitors nordihydroguaiaretic acid (NDGA) at $1.5 \times 10^{-5}$ M or AA861 at $10^{-5}$ M prevented A23187-induced relaxation. The same concentration of NDGA and AA861 did not affect endothelium-independent relaxation induced by glyceryl trinitrate. We suggest that endothelium-dependent relaxation of the canine basilar artery by A23187 may be mediated by noncyclooxygenase metabolite(s).

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Endothelial cells play a major role in mediating the inhibitory effects of acetylcholine (ACh) and A23187 in isolated arteries. Studies have shown that the vasodilator action of these agents is mediated by release of a relaxing substance termed endothelium-derived relaxing factor (EDRF) from the endothelial cells. It has also been shown that in rabbit aortas, the relaxation response to metacholine and A23187 can be reversed by eicosatetraenoic acid and nordihydroguaiaretic acid (NDGA), but not by indomethacin. As a result, it has been suggested that an unstable oxidation product of arachidonic acid that is formed via a lipoxygenase pathway mediates endothelium-dependent relaxation in rabbit aortas. While the exact nature of the signal generated by the endothelial cells is undetermined, the release of EDRF induced by some agents may depend on the species and anatomic sites of the blood vessels.

To our knowledge, there have been few reports regarding EDRF induced by ACh or A23187 in canine cerebral arteries, although EDRF in canine femoral arteries has been reported.

The present study was designed to demonstrate EDRF in canine basilar arteries using ACh and A23187, to clarify differences in the response of canine basilar and femoral arteries to ACh and A23187, and to elucidate the basic pharmacologic properties of EDRF in canine basilar arteries.

Materials and Methods

Preparation of Rings, Mounting, and Apparatus

Twenty-three mongrel dogs of either sex were anesthetized with 30 mg/kg i.v. sodium pentobarbital and killed. The brains and femoral arteries were rapidly removed. The basilar arteries were isolated from the brains, and the basilar and femoral arteries were quickly immersed in Krebs-Henseleit solution (KHS) equilibrated with 95% O$_2$ and 5% CO$_2$ at room temperature. The millimolar composition of the KHS was 115.0 NaCl, 4.7 KCl, 2.5 CaCl$_2$, 1.2 MgCl$_2$, 25.0 NaHCO$_3$, 1.2 KH$_2$PO$_4$, and 10.0 glucose. The arteries were then cut into rings 2 mm long according to the method of Furchgott and Zawadzki and mounted vertically between small hooks in a water-jacketed tissue bath containing 20 ml KHS maintained at 37 $\pm$ 0.5°C and bubbled with 95% O$_2$ and 5% CO$_2$ at room temperature. The upper end of the ring was connected to the lever of a Nihon Kohden Kogyo Model TB-612T force-displacement transducer (Tokyo, Japan). The optimal resting tensions applied to the rings were 0.5 g for basilar and 2.0 g for femoral artery rings. Isometric contractions were recorded on a Nihon Kohden Kogyo Model WT-68SG writing oscillograph (Tokyo, Japan).

Removal of Endothelial Cells

The endothelial cells of the arterial rings were mechanically removed by a standard brief, gentle rubbing
of the intimal surface with a 25–30-gauge stainless steel rod having a diameter equivalent to the lumen of the arteries as described by Lee. Control rings were not rubbed and are termed intact.

**Scanning Electron Microscopy**

Segments of the freshly dissected arteries and those from the in vitro tissue bath were fixed in 2.5% glutaraldehyde in 0.07 M phosphate buffer (pH 7.4), dehydrated in graded alcohols and acetone, and dried by the critical-point method. Specimens were mounted on metal stubs, coated with gold, and viewed in a JSM-T200 scanning electron microscope (Tokyo, Japan).

**Arterial Relaxation Response to ACh and A23187**

The basic experimental protocol employed a control period during which the intact arterial rings were contracted with an approximate ED50 concentration of prostaglandin F2α (PGF2α) and relaxed by the cumulative addition of A23187. At the end of each series of experiments, 10−4 M papaverine was added to attain maximum relaxation according to the report by Toda et al. Values relative to the papaverine-induced relaxation are presented in the text, table, and figures. Preparations had been treated for 20 minutes with blocking agents before the response to ACh or A23187 was obtained.

**Statistical Analysis**

The ED50 was obtained from a plot of percent response vs. log concentration of the agonist and expressed as the negative logarithm (pD2). The results are expressed as mean ± SD, and compared using Student’s t test. A probability of ≤0.05 was considered significant.

**Results**

**Scanning Electron Microscopy**

The presence or absence (disruption) of the endothelium in arterial rings was ascertained with scanning electron microscopy. In Figure 1 Left, oval elevations are portions of the endothelial cells overlying nuclei in the endothelium of a control artery. Rubbing the endothelium with a stainless steel rod completely removed the endothelial cells (Figure 1 Right). Residual debris of the rubbed endothelial cells is also seen in Figure 1 Right.

**Relaxation Response to A23187 and ACh**

Rings of intact basilar arteries were contracted with PGF2α, and the addition of 10−9 to 10−7 M A23187 caused a dose-dependent relaxation (Figure 2a). However, even at high concentrations ACh did not produce significant relaxation in the intact canine basilar arteries (Figure 2b). A23187-induced relaxations relative to those induced by 10−4 M papaverine in the intact basilar artery rings are summarized in Table 1 (control values). Both ACh and A23187 induced noticeable relaxation in the intact femoral artery rings (Figure 2, c and d). Dose–response curves for A23187 and ACh in the intact basilar and femoral artery rings are shown in Figure 3. A23187 produced significantly greater relaxation in the femoral than in the basilar artery rings (Figure 3a). It was remarkable that the same was true of ACh (Figure 3b). Therefore, consecutive experiments were performed using A23187. Figure 4 shows the cumulative dose–response relation, in both the presence and absence of endothelium, to A23187-stimulated relaxation of the basilar artery rings that had been precontracted with PGF2α. This figure clearly demonstrates that relaxation of the canine basilar artery by the calcium ionophore A23187 is completely endothelial-cell–dependent. A23187 elicited maximal relaxation, 69.30 ± 9.31% of the plateau tension induced by PGF2α (Table 1). As shown in Table 1, pD2 for A23187-stimulated relaxation is 7.57 ± 0.32 (control).

**Mechanism of Endothelium-Dependent Relaxation of Canine Basilar Artery**

To rule out the possibility that this relaxation might be caused by A23187-induced production of prostacyclin in the endothelial cells, a cyclooxygenase inhibitor, indomethacin, was used. The effect of $1.5 \times 10^{-3}$ M indomethacin added to the incubating bath for 20 minutes before and during the contraction–relaxation
cycle is shown in Figure 5a. This concentration of indomethacin had no significant effect on the A23187 dose–response relation. The contractile responses to $3 \times 10^{-6}$ M PGF$_{2\alpha}$ were augmented by indomethacin as shown in Table 1. However, there were no significant differences between $pD_2$ in the presence or absence of indomethacin (Table 1). In any case, the dilatations observed in the present experiments were not mediated by prostacyclin as the response was not inhibited.

A series of experiments using NDGA, a reported lipoxygenase inhibitor, were conducted after NDGA was found to significantly inhibit A23187-induced relaxation of precontracted rabbit aorta rings in a dose-dependent manner. Pretreatment with $1.5 \times 10^{-3}$ M NDGA significantly reduced the maximal relaxation to A23187 (Table 1). As shown in Figure 5b, pretreatment with NDGA prevented the relaxation in response to most concentrations of A23187 tested but did not alter the contractile responses to $3 \times 10^{-6}$ M PGF$_{2\alpha}$ (Table 1). AA861 has been reported to inhibit 5-lipoxygenase activity specifically. Pretreatment with $10^{-3}$ M AA861 significantly reduced the maximal relaxation to A23187 (Figure 5c) without affecting the contractile response to $3 \times 10^{-6}$ M PGF$_{2\alpha}$ (Table 1).

To determine the selectivity of the inhibitory effects of NDGA and AA861, we examined the effects of these agents against the relaxation induced by another agent, glyceryl trinitrate (GTN), which occurs independent of any action on the endothelial cells. Control rings contracted to a plateau tension by PGF$_{2\alpha}$ were relaxed 76.07 ± 8.58% by GTN. The $pD_2$ for this agonist was 5.44 ± 0.30. Pretreatment with $1.5 \times 10^{-3}$ M NDGA or $10^{-3}$ M AA861 had no effect on relaxation induced by $10^{-7}$ to $10^{-4}$ M GTN (Figure 6).

### Table 1. Effects of Indomethacin, NDGA, and AA861 on PGF$_{2\alpha}$-Induced Contraction and A23187-Induced Relaxation in Canine Basilar Artery Rings

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>n</th>
<th>Developed tension (mg)</th>
<th>A23187-induced relaxation</th>
<th>$pD_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (control)</td>
<td>9</td>
<td>353.2 ± 48.4</td>
<td>69.30 ± 9.31</td>
<td>7.57 ± 0.32</td>
</tr>
<tr>
<td>$1.5 \times 10^{-5}$ M Indomethacin</td>
<td>5</td>
<td>420.5 ± 35.3*</td>
<td>67.80 ± 6.13</td>
<td>7.41 ± 0.13</td>
</tr>
<tr>
<td>$1.5 \times 10^{-5}$ M NDGA</td>
<td>9</td>
<td>365.8 ± 34.2</td>
<td>20.98 ± 4.07*</td>
<td>—</td>
</tr>
<tr>
<td>$10^{-5}$ M AA861</td>
<td>10</td>
<td>393.2 ± 118.5</td>
<td>32.81 ± 11.3*</td>
<td>—</td>
</tr>
</tbody>
</table>

NDGA, nordihydroguaiaretic acid; PGF$_{2\alpha}$, prostaglandin F$_{2\alpha}$. PGF$_{2\alpha}$ at $3 \times 10^{-6}$ M. $pD_2$, negative logarithm.

*Different from control ($p < 0.01$).
Discussion

The experiments reported here support the hypothesis, originally put forth by Furchgott and Zawadzki,\textsuperscript{1} that endothelium-dependent relaxation evoked by A23187 is mediated by a noncyclooxygenase metabolite of arachidonic acid. Whereas prostacyclin can be produced in canine basilar arteries,\textsuperscript{14-16} based on negative results with indomethacin (Figure 5a), it is unlikely that prostacyclin is the active dilator substance produced in response to A23187. Indomethacin reportedly potentiates the arachidonic-acid-induced relaxation in intact rabbit aorta rings.\textsuperscript{17} Unlike relaxation stimulated by arachidonic acid,\textsuperscript{11} that stimulated by A23187 was not significantly potentiated by indomethacin pretreatment (Figure 5a).

The present study demonstrates that indomethacin pretreatment potentiates the contractile responses in intact canine basilar artery rings (Table 1). Although the exact explanation for this event cannot be given here, it seems that prostacyclin, a potent vasodilator produced by the activation of cyclooxygenase, is not produced by the administration of indomethacin.\textsuperscript{17} Considering the lack of effect of indomethacin on A23187-induced relaxation, the inhibition of endothelium-dependent relaxation by either NDGA or AA861 suggests that a noncyclooxygenase product or arachidonic acid metabolism may mediate or participate in the sequence of events resulting in relaxation. NDGA has been reported to inhibit lipoxygenase activity in other systems at concentrations similar to those we used.\textsuperscript{9,10} AA861 at 10\textsuperscript{-5} M selectively inhibits 5-lipoxygenase of guinea pig peritoneal polymorphonuclear leukocytes.\textsuperscript{12} This concentration is similar to that which we used. NDGA and AA861 did not affect the relaxation induced by GTN, suggesting that their inhibitory effects are specific for the endothelium-dependent vasodilator. However, the possibility that NDGA and AA861 inactivate the properties of A23187 by acting on it directly cannot be ruled out.

Most lipoxygenase metabolites of arachidonic acid described in the literature appear to be contractile agonists in vascular smooth muscle,\textsuperscript{18,19} and a recent study suggests that leukotrienes, lipoxygenase products, produce a constriction of the cerebral arterioles of mice.\textsuperscript{20} In canine basilar arteries, 15-hydroperoxymethachidonic acid produces significant constriction in either in vivo\textsuperscript{21} or in vitro\textsuperscript{22} studies. However, there have been several reports on lipoxygenase products with vasodilator activity. Bunting et al\textsuperscript{23} and, most recently, De Mey et al\textsuperscript{24} have reported that 15-hydroperoxymethachidonic acid relaxes vessels that have preexisting tone. Infusion of several hydroperoxy and hydroxy derivatives of arachidonic acid into isolated perfused stomach vasculature of rabbits and rats have also been reported to cause a marked decrease in perfusion pressure.\textsuperscript{25} Although the full spectrum of lipoxygenase me-

**Figure 3.** Dose-response curves showing relaxation of canine basilar (○) and femoral (●) artery rings with endothelium. a: A23187-induced relaxation during contractions induced by 3×10\textsuperscript{-6} M PGF\textsubscript{2α} for basilar and 10\textsuperscript{-7} M norepinephrine for femoral artery rings. b: Acetylcholine (ACh)-induced relaxation, same protocol as in a. Vertical bars are SD; numbers in parentheses are number of preparations; * p<0.05; † p<0.01.

**Figure 4.** Dose-response curves showing relaxation to A23187 in canine basilar artery rings with (●) and without (○) endothelium. * p<0.05.
tabolites and their biologic activities has not been discovered, lipoygenase is probably involved in the synthesis of EDRF as 7 chemically different lipoxygenase inhibitors were effective on the EDRF of rabbit aortas,36 and the 2 lipoxygenase inhibitors we tested also prevented the EDRF of canine basilar arteries.

A potent inducer of EDRF in canine basilar arteries, A23187 is a well-documented ionophore specific for divalent cations, especially calcium.27 In addition, the most effective stimulus for the formation of 5-lipoy-
genase products has been found to be this same diva-
lent cation ionophore.28 Although no precise role of 5-
ilipoygenase in living vascular tissue has yet been elucidated, it may have been involved in the A23187-
induced relaxation.

PGF$_{2\alpha}$ is suspected as a causative factor in experi-
mental vasospasm.29-31 In our experiment, EDRF strongly eliminated the contractile tensions induced by PGF$_{2\alpha}$, but whether an inhibition of EDRF takes place in cerebral vasospasm after aneurysmal rupture warrants further investigation. There have been numerous clinical and experimental studies of the morphologic changes in the endothelium of cerebral arteries after subarachnoid hemorrhage (SAH).32-38 For example, subarachnoid clot produces constriction of the major cerebral arteries with corrugation of the elastic lamina and opening of the tight junction.36 The corrugated lamina may squeeze the endothelial cells and inhibit their metabolism.36,39 Further, electron microscopic studies in dogs with SAH demonstrated degenerative changes of endothelial cells in the major arteries,36 and the synthetic activity of prostacyclin in canine basilar arteries exposed to subarachnoid blood injection remarkably diminished.14,16 Therefore, it is of interest to demonstrate whether endothelium-dependent relaxation is blocked by SAH. Such a study was conducted and is the subject of our next report.

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