

Distinct effects of intravascular and extravascular angiotensin II on cerebrovascular circulation of newborn pigs

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Abstract

Angiotensin II (AngII) is important in regulation of vascular resistance and control of blood flow among organs and tissues. The effect of AngII on the cerebral microvasculature may be mediated or altered by endothelial-derived signals. The aim of this study was to test the hypothesis that blood AngII dilates neonatal pial arterioles via an endothelial-dependent mechanism but brain AngII can constrict pial arterioles by activating smooth muscle AT₁ receptors. Studies used anesthetized newborn pigs with surgically implanted closed cranial windows. AngII was given either by infusion into the carotid artery ipsilateral to the cranial window or topically. Intracarotid infusion of AngII dilated pial arterioles. The dilation was blocked by systemic administration of the AT₁-receptor antagonist, losartan, but unaffected by topical losartan. Topical AngII also caused dilation, but this dilation was converted to constriction by topical losartan. In piglets pretreated with the angiotensin-converting enzyme (ACE) inhibitor, enalapril, topical AngII constricted, rather than dilated, pial arterioles. In enalapril-treated piglets, light/dye endothelial injury blocked dilation to intracarotid AngII but did not affect constriction to topical AngII. Either indomethacin or L-nitroarginine methyl ester blocked the dilation to intraluminal AngII, but neither affected constriction to topical AngII. Chromium mesoporphyrin, that inhibits heme oxygenase, did not affect responses to either topical or intravascular AngII. These data are consistent with the hypotheses that: (a) circulating AngII dilates pial arterioles via endothelial AT₁ receptor-derived relaxing factors, notably prostanooids and nitric oxide; (b) direct AT₁ receptor activation on the brain side of the blood–brain barrier by AngII causes AT₁ receptor-mediated constriction that can mask underlying AT₁ receptor-independent dilation when ACE is inhibited. Clinical manipulation of the renin–angiotensin system will have disparate actions on cerebral circulation depending on the functional integrity of the intima and ACE.

Keywords: endothelium, AT₁ receptor, prostaglandin cyclooxygenase, nitric oxide synthase, angiotensin-converting enzyme

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Introduction

Angiotensin II (AngII) is important in the regulation of vascular resistance and control of blood flow among organs and tissues. The strong constrictor action of AngII on systemic vascular resistance beds makes manipulation of the renin–angiotensin system a target in the treatment of congenital and acquired heart disease. In patients with biventricular and univentricular heart disease this system is commonly medically antagonized. Inhibition of the vasoconstrictive effects of AngII decreases the amount of work the heart must perform.¹ Conversely, AngII infusion could be employed to increase vascular resistance and mean circulatory filling pressure to elevate both cardiac output and blood pressure, but seldom has been reported.² Medical antagonism of the renin–angiotensin system may affect cerebral blood flow differently depending on the extent of

blood–brain barrier (BBB) integrity. Cerebral blood flow also factors into treatment of some congenital heart disease. In children born with only a single ventricle, part of the surgical palliation includes routing systemic venous return directly to the lungs, bypassing the heart,³ to reduce volume loading of the ventricle and increase effective pulmonary blood flow.⁴ An intermediate stage in this procedure is the superior cavopulmonary anastomosis in which the superior vena cava is directly anastomosed to the pulmonary arteries providing the only source of pulmonary blood flow.⁵ With such circuitry, pulmonary blood flow is largely dependent on cerebral blood flow as the major contribution to superior caval venous return. Thus, understanding actions of the renin–angiotensin system manipulations on cerebrovascular circulation is critical for clinical management of these babies.

Between any circulating agent and the cerebral vascular smooth muscle lie the endothelial cells and internal elastic lamina that comprise the BBB. The effect of any intravascular agent on the cerebral microvasculature may be mediated or altered by endothelial-derived signals. For example, norepinephrine constricts newborn pial arterioles via α -adrenergic receptors when applied from the brain side of the BBB,⁶ but from the vascular side it dilates cerebral arterioles, increasing cerebral blood flow via β -adrenergic receptors.⁷

There are four identified AngII receptors, although little is known about AT₃ and AT₄. The AT₁ receptor mediates most of the vasoactive effects of AngII.⁸ AT₂ is involved in organ development and can cause vasodilation.⁹ The AT₁ receptor antagonist, losartan, causes systemic vasodilation altering vascular resistance and blood pressure.¹⁰ Conversely, intravascular AngII infusion causes vasoconstriction in most systemic vascular beds via direct smooth muscle contraction of resistance arterioles¹¹ elevating vascular resistance and blood pressure.

As introduced above, there is reason to believe that the endothelium may change the cerebrovascular response to peripherally circulating AngII. AngII has receptors in the brain,¹² is known to release the prostaglandin precursor arachidonic acid from membrane phospholipids and *in vivo* can result in endothelial-dependent vasodilation of rat cerebral arteries.¹³ Nitric oxide (NO) has been reported as a mediator of cerebrovascular dilation to topical AngII via the AT₂ receptor in newborn pigs.¹⁴ NO-mediated reduction of systemic vascular resistance is increased with inhibition of angiotensin-converting enzyme (ACE)¹⁵ or AT₁ receptor blockade.¹⁶ Vasodilation following blockade of AT₁ receptors may be due to the action of unmasked AT₂ receptors and/or elevation of bradykinin.¹⁶ In addition, endothelial-derived hyperpolarizing factor(s) (EDHF) has been shown to be involved in AngII dilation of isolated, pressurized, perfused rat cerebral arteries that remain following inhibition of nitric oxide synthase (NOS) and cyclooxygenase (COX).¹⁷

The aim of this study is to test the hypothesis that blood AngII dilates neonatal pial arterioles via an endothelial-dependent mechanism but brain AngII can constrict pial arterioles by activating smooth muscle AT₁ receptors. Thus, we anticipated that intravascular AngII would dilate pial arterioles when the endothelial barrier is intact, but cause constriction following endothelial injury. Furthermore, topical AngII was expected to produce constriction via activation of AT₁ receptors on vascular smooth muscle, but it could cause dilation via AT₂ receptors or endothelial AT₁ receptors.

Methods

The University of Tennessee Health Science Center's Animal Care and Use Committee approved all animal procedures.

Newborn pigs (1–5 days old) (1–3.5 kg) were anesthetized with ketamine hydrochloride (33 mg/kg intramuscular) and acepromazine (3.3 mg/kg intramuscular);

sedation was maintained with α -chloralose (50 mg/kg intravenous). The animals were intubated via tracheostomy and ventilated with air. The femoral vein was cannulated for anesthesia, fluid and drug injections. The femoral artery was cannulated for continuous blood pressure monitoring and drawing samples for blood gas and pH analysis. The carotid artery ipsilateral to the cranial window was cannulated for antegrade saline and AngII infusion. Blood gases, pH and body temperature were maintained within reference ranges. Blood gases were obtained prior to initiation of the protocol and following completion of each major segment of the protocol and at protocol completion.

Cranial windows

The scalp was retracted and an opening 2 cm in diameter was created in the skull over the parietal cortex. The dura was cut without touching the brain, and cut edges retracted over the bone so that the periarachnoid space was not exposed to bone or damaged membranes. A stainless steel and glass cranial window was fitted in the hole and cemented in place with dental acrylic. The windows had side needle ports so fluid under the window could be exchanged and test compounds administered topically to the cerebral vessels. The space under the window was filled with artificial cerebrospinal fluid (aCSF) equilibrated with 6% CO₂ and 6% O₂ producing gases and pH within the reference range for CSF (pH ~7.34, P_{CO₂} and P_{O₂} ~43 mmHg). Pial vessels were observed through the window with a dissecting microscope. Arteriole diameters were measured with a video micrometer coupled to a television camera mounted on the microscope and a video monitor. Following cannulation, piglets in most groups were given a single dose of intravenous enalapril (50 μ g/kg), an ACE inhibitor, to minimize native AngII and to emulate clinical practice. In another group, responses to topical AngI and AngII were measured in piglets not treated with enalapril. All pharmaceutical agents except losartan (Merck Pharmaceuticals, Whitehouse Station, NJ, USA) were obtained from Sigma Chemicals (St Louis, MO, USA).

Intravascular AngII

AngII was dissolved in normal saline to 50 μ g/mL concentration. This solution was infused antegrade into the carotid artery ipsilateral to the cranial window. Initial rate was 5 μ g/kg/min and incrementally increased at five-minute intervals to 10, 20 and 50 μ g/kg/min. These doses were selected because the 50 μ g/kg had been shown to improve cerebral blood flow during cardiopulmonary resuscitation in immature pigs.¹¹ Blood pressure was continually monitored and kept stable by controlled exsanguination to avoid arteriole diameter changes due to autoregulation. Exsanguinated blood was mixed with heparin and kept warm, and then returned to the animal following completion of AngII infusion. To control for effects of flow and/or pressure due to the infusion, normal saline was infused into the carotid artery prior to administration of AngII. The rate of saline infusion was slightly higher than

the maximum rate to be used for infusion of AngII for each animal.

Topical AngII

AngII was dissolved in aCSF to concentrations of 10^{-8} , 10^{-7} and 10^{-6} mol/L. Beginning with the 10^{-8} mol/L, each concentration was sequentially instilled under the cranial window in a volume sufficient to fill the space. Washout with aCSF free of AngII was done after each concentration. Vessel diameter was measured and recorded following each instillation to generate a dose-response curve.

AT₁-blockade

To differentiate between effects of AngII receptors, the AT₁-specific antagonist, losartan, was administered to two groups of piglets. The first group was administered a single 3 mg/kg dose intravenously; this dose is hemodynamically significant in pigs.¹⁸ Ten minutes were allowed to elapse to stabilize blood pressure and pulse. A separate group had 10^{-5} mol/L losartan dissolved in aCSF instilled under the cranial window in a volume sufficient to fill the space. During the topical protocol, losartan was re-instilled with the AngII to ensure the presence of both. Intravenous and topical AngII were then administered to both groups as described.

Endothelial injury

To delineate the role of vascular endothelium in effects of AngII in piglets, the endothelium was damaged *in vivo*. Sodium fluorescein (160 mg/kg in 8 mL/kg volume) was injected intravenously and activated with exposure to appropriately filtered light from a mercury arc lamp producing endothelial injury.¹⁹ Following injection, 30 min elapsed to allow uptake by the endothelial cells and the light was directed through the cranial window for eight minutes. In addition to topical and systemic AngII, piglets in this group were administered topical 10^{-6} mol/L isoproterenol and 10^{-6} mol/L bradykinin, before and after light-dye treatment. Washout with aCSF was done prior to each being instilled. Vasodilation due to bradykinin requires a functional endothelium while the effect of isoproterenol is by direct stimulation of smooth muscle beta-receptors, independent of endothelial cells.²⁰ Loss of dilation to bradykinin showed loss of endothelial function while persistent arteriole reactivity was confirmed with isoproterenol. To ensure fluorescein alone did not affect endothelial function, a group of animals were injected with fluorescein but not exposed to light.

Prostanoids

To block prostanoid synthesis, indomethacin trihydrate was dissolved in normal saline and 10 mg/kg was given intravenously; a dose sufficient to block dilation to topical arachidonic acid²¹ and constriction to topical acetylcholine,²² suggesting sufficient passage across the BBB to be effective. Intravenous and topical AngII were given as above, before

and after indomethacin administration. Isoproterenol at 10^{-6} mol/L was applied as part of the topical protocol to assure generalized alteration of vascular responsiveness had not occurred.

The same protocol was repeated in another group of piglets with indomethacin, at 10^{-4} mol/L in aCSF, applied topically.

Nitric oxide

L-nitroarginine methyl ester (L-NAME), at 10^{-3} , 10^{-5} and 10^{-6} mol/L in aCSF, was administered topically to inhibit NOS. The protocol administering AngII and isoproterenol was repeated before and after administration of L-NAME.

Carbon monoxide

Chromium mesoporphyrin (CrMP), dissolved to 2×10^{-5} mol/L in aCSF, was applied topically to inhibit production of CO via heme oxygenase (HO) in another group of piglets. This concentration has been shown to inhibit HO activity in neonatal piglets.²³ This solution was protected from light by wrapping aluminum foil around the syringe during administration to prevent inactivation. The protocol administering AngII and isoproterenol was repeated prior to and following CrMP. CrMP (2×10^{-5} mol/L) inactivated via exposure to light for >24 h was used to act as an additional control.

Statistical analysis

Data were analyzed using analysis of variance for repeated measures and Bonferroni *post hoc* analysis to compare mean arteriolar diameter. Statistical analysis was performed using InStat software (GraphPad Software, Inc, LaJolla, CA, USA).

Results

Control

Infusion of normal saline into the carotid artery, at a rate slightly greater than the maximal infusion rate of AngII, had no effect on the diameter of pial resistance arterioles (Figure 1a 'Baseline' versus 'Control'). Similarly, replacement of aCSF without additional agents under the cranial window did not affect pial arteriolar diameter.

Angiotensin II

Intracarotid infusion of AngII dilated pial arterioles (Figure 1a). The dilation was blocked by systemic administration of the AT₁-receptor antagonist, losartan (Figure 1b), but unaffected by topical losartan (Figure 1c).

Conversely, in piglets pretreated with the ACE inhibitor, enalapril, application of AngII directly on surface pial arterioles resulted in vasoconstriction (Figure 2a). In contrast, when piglets were not given enalapril, topical AngII caused vasodilation (Figure 2b). On the other hand, topical losartan not only blocked the

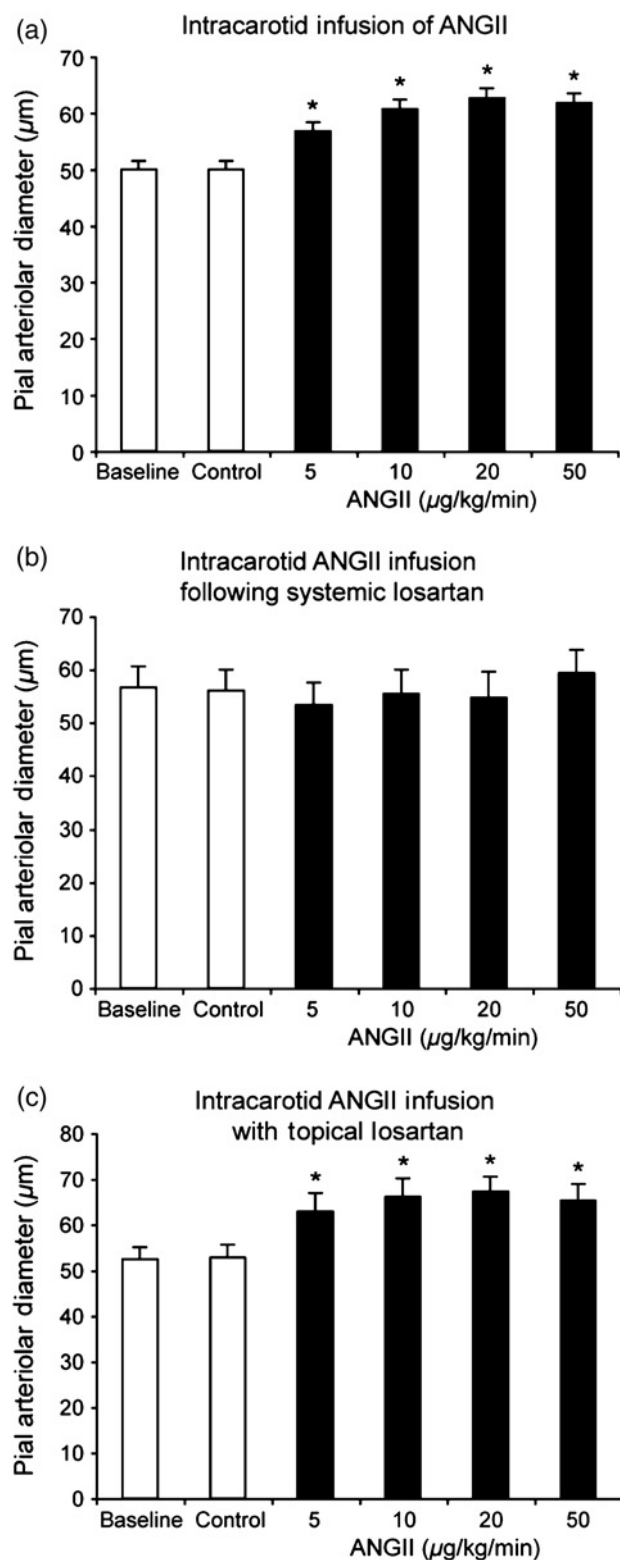


Figure 1 Effect of intracarotid AngII infusion on pial arteriolar diameters of newborn pigs. Baseline is before and Control is during saline vehicle infusion. (a) Without losartan. (b) Following 3 mg/kg losartan, intravenous. (c) During topical losartan (10^{-5} mol/L). Mean $\pm$ SEM. $n = 6, 6$ and 5 piglets in (a), (b) and (c), respectively. * $P < 0.05$ from control. Ang, angiotensin

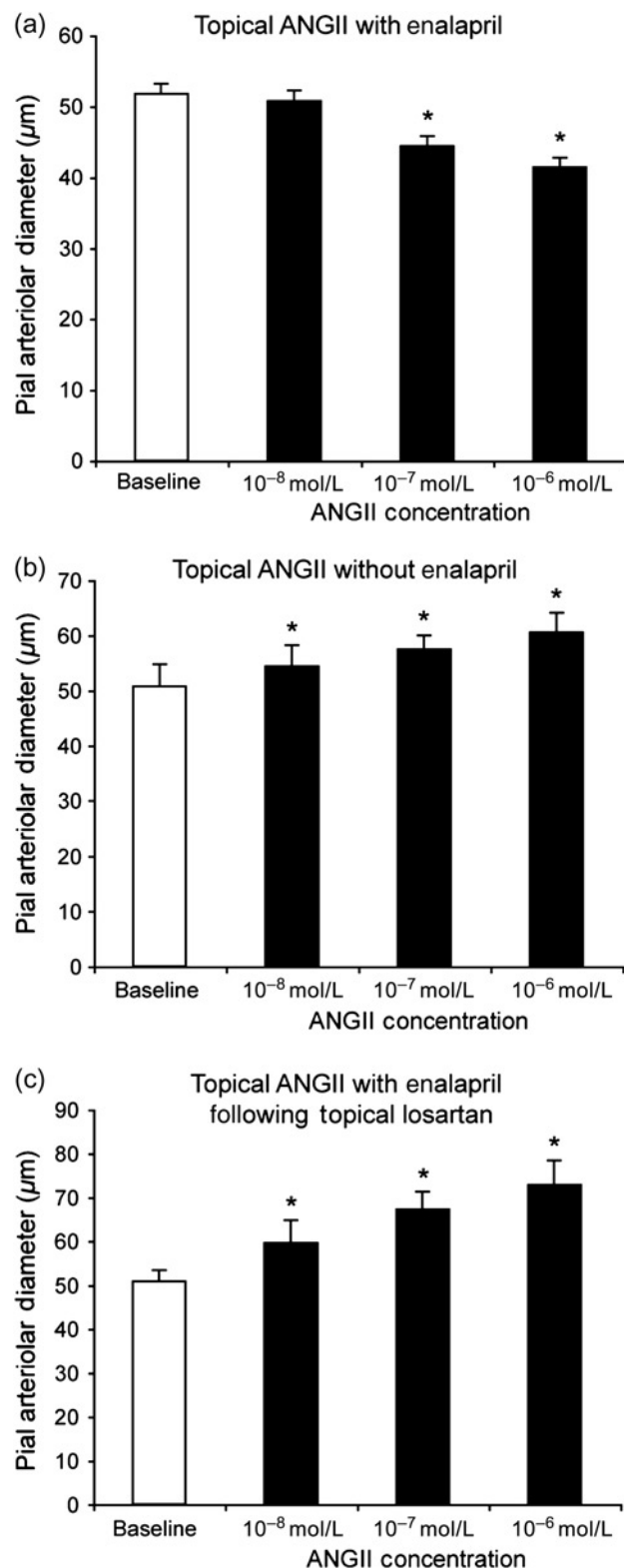


Figure 2 Effect of topical AngII on pial arteriolar diameters of newborn pigs. (a) Piglets pretreated with enalapril ($50 \mu\text{g/kg}$, intravenous). (b) Untreated piglets. (c) Piglets treated with enalapril ($50 \mu\text{g/kg}$, intravenous) and topical losartan (10^{-5} mol/L). Mean $\pm$ SEM. $n = 6, 4$ and 6 piglets in (a), (b) and (c), respectively. * $P < 0.05$ from baseline. Ang, angiotensin

constrictor response of pial arterioles to AngII in piglets treated with enalapril but converted the response to concentration-dependent dilation (Figure 2c). All other piglets were studied in the presence of enalapril because we were pursuing mechanisms behind the divergent responses of pial arterioles to intra-vascular versus topical AngII that were revealed by ACE inhibition.

Topical AngI (10^{-6} mol/L) had no effect on pial arteriolar diameter even in piglets not treated with enalapril (51 ± 3 and 51 ± 3 μm , before and during AngI, respectively, $n = 4$ piglets).

Endothelial injury

Both isoproterenol and bradykinin caused pial arteriolar dilation before light-dye treatment (Figure 3). Following light-dye, dilation in response to bradykinin was blocked indicating that the endothelium was non-functional. Dilation to isoproterenol was unchanged indicating the arterioles retained the ability to dilate when given a stimulus that acts directly on the smooth muscle. In the animals given fluorescein dye only (i.e. without light activation), there was no change in the response to either bradykinin or isoproterenol (Figure 3).

In piglets with confirmed light-dye injury as in Figure 3, the endothelial injury did not affect the constrictor response to topical AngII (Figure 4a). In contrast, the vasodilatory response to intracarotid infusion of AngII was converted to constriction following light-dye endothelial injury (Figure 4b).

To ensure that uptake of fluorescein did not affect the function of the endothelial cells, a separate group of

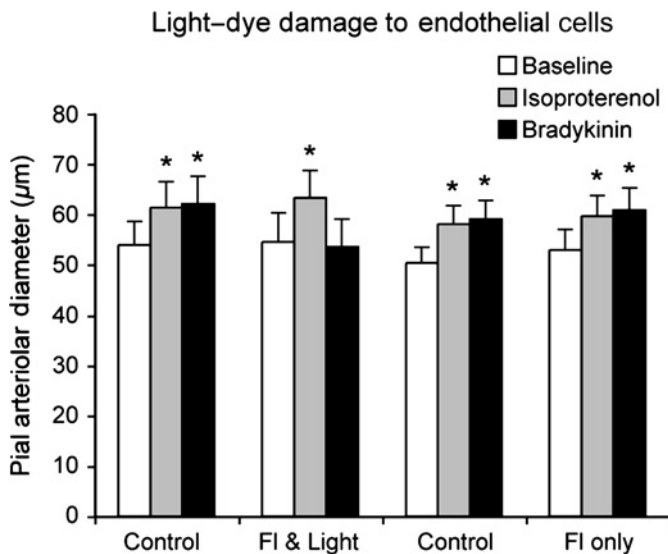


Figure 3 Effect of sodium fluorescein (FI) (160 mg/kg, intravenous) and light from a 100 W mercury arc lamp filtered for maximal transmission between 420 and 430 nm for eight minutes (light) on pial arteriolar responses to topical isoproterenol (10^{-6} mol/L) and bradykinin (10^{-6} mol/L). Controls were before treatment and light/dye (FI & Light) is following dye and light ($n = 3$). In separate animals ($n = 4$), responses were measured before and after fluorescein alone without light activation (FI only). Mean $\pm$ SEM. * $P < 0.05$ from respective baseline

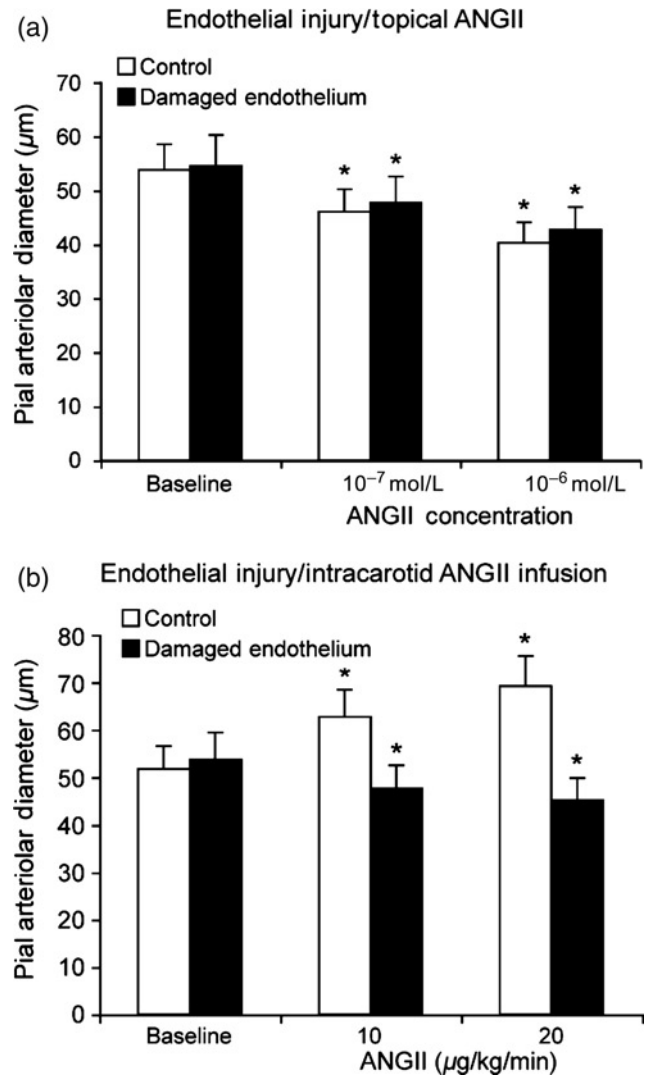


Figure 4 Effect of light-dye endothelial injury on pial arteriolar response to AngII. AngII at increasing concentration was (a) applied topically or (b) infused intracarotidly prior to and following exposure to fluorescein and light. Mean $\pm$ SEM. $n = 3$ piglets. * $P < 0.05$ from baseline. Ang, angiotensin

animals was injected with fluorescein intravenously but protected from light to avoid activating the fluorescein (Figure 3). The same period of time was allowed to elapse between injection of fluorescein and exposure to AngII as in the animals exposed to light. The response to intracarotid infusion of AngII was no different before and after dye-only treatment (50.8 ± 3.6 – 61.6 ± 3.5 μm before and 51.4 ± 3.6 – 62.0 ± 4.1 μm after dye only in response to 20 μg AngII/kg/min infusion and 50.3 ± 3.1 – 40.6 ± 2.7 μm before and 52.9 ± 4.8 – 42.5 ± 3.7 μm after dye only in response to topical AngII [10^{-6} mol/L], respectively).

Characterization of endothelial-derived relaxing factor

Because the data presented above indicate that endothelial-derived relaxing factor(s) (EDRF) cause dilatory responses to AngII in piglet pial arterioles, we investigated potential EDRF(s) involved. Previous data indicate that prostanoids, NO and CO are EDRFs in the newborn cerebral

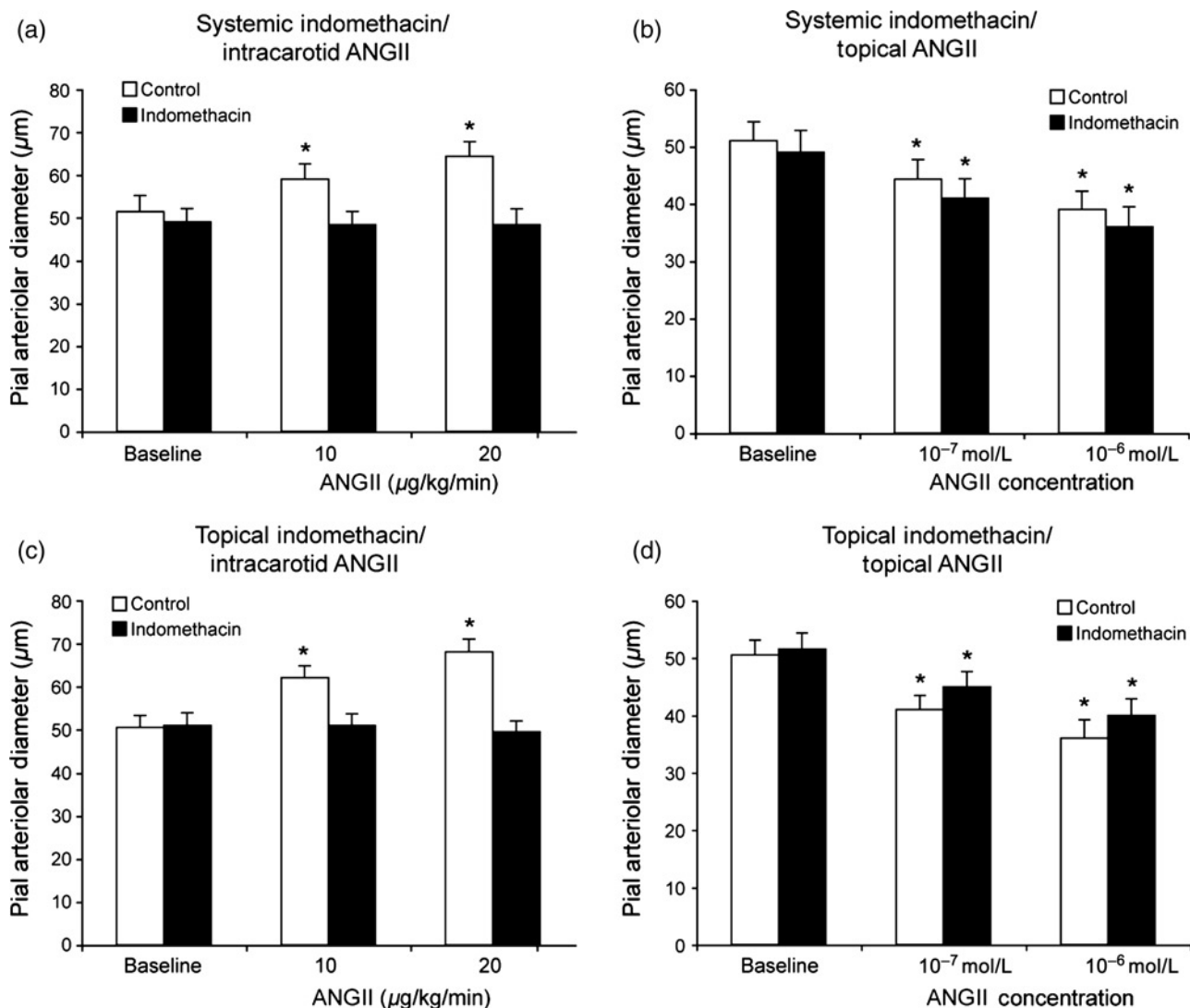


Figure 5 Effect of indomethacin on pial arteriolar responses to AngII. Indomethacin was administered intravenously (10 mg/kg) (a and b, $n = 6$) or topically (10^{-4} mol/L) (c and d, $n = 5$). AngII was infused intracarotidally (a and c) or applied topically (b and d). Mean $\pm$ SEM. * $P < 0.05$ from baseline. Ang, angiotensin

circulation,^{14,20,21,23–26} so the COX/prostanoid, NOS/NO and HO/CO systems were addressed.

Prostanoids

Indomethacin, both systemic and topical, blocked the response to intracarotid infusion of AngII (Figures 5a and c). Conversely, the constrictor response to topical AngII was not altered by either systemic or topical indomethacin (Figures 5b and c). There was no change in the vasodilatory response to topical isoproterenol before and after treatment with either systemic or topical indomethacin (data not shown).

Nitric oxide

Similarly to indomethacin, topical L-NAME (10^{-3} mol/L) blocked the vasodilatory response to intracarotid AngII infusion (Figure 6a). In contrast, 10^{-6} mol/L L-NAME had no effect on pial arteriolar dilation to intracarotid AngII

infusion (at 0, 10 and 20 $\mu\text{g/kg/min}$ AngII: 59 ± 8 , 66 ± 8 and 71 ± 8 μm before and 60 ± 6 , 68 ± 8 and 69 ± 8 μm after 10^{-6} mol/L L-NAME, respectively). However, a 10-fold higher L-NAME concentration (10^{-5} mol/L) did inhibit dilation in response to intracarotid infusion of AngII (at 0, 10 and 20 $\mu\text{g/kg/min}$ AngII: 58 ± 10 , 62 ± 12 and 66 ± 12 μm before and 57 ± 11 , 57 ± 11 and 57 ± 11 μm after 10^{-5} mol/L L-NAME, respectively).

Pial arteriolar constriction to topical AngII administration was unchanged when NOS was inhibited (Figure 6b).

L-NAME (10^{-3} mol/L) had no effect on the vasodilation response to topical isoproterenol (data not shown).

Carbon monoxide

Inhibition of CO production with CrMP (2×10^{-5} mol/L, topically²³) had no effect on pial arteriolar responses to either topical or intracarotid AngII (Figure 7). As a control, CrMP inactivated by light was administered topically.

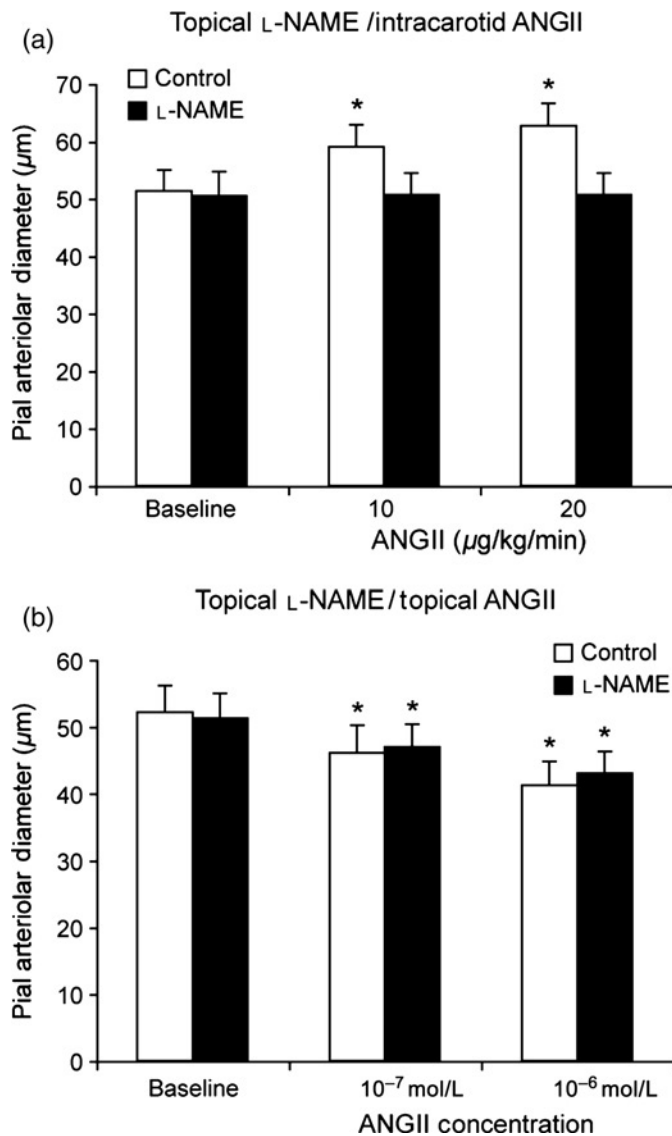


Figure 6 Effect of topical L-NAME (10^{-3} mol/L) on pial arteriolar responses to (a) intracarotid AngII and (b) topical AngII. Mean $\pm$ SEM. $n = 3$ piglets. * $P < 0.05$ compared with appropriate baseline. Ang, angiotensin; L-NAME, L-nitroarginine methyl ester

Piglets were administered AngII topically and then systemically, first in the presence of inactivated CrMP, and then following washout with aCSF free of additional substances, with active compound, CrMP.

Discussion

Novel findings of the present investigation in newborn pigs are as follows: (1) intravascular AngII dilates pial arterioles via an endothelial and AT_1 receptor-dependent mechanism; (2) topical AngII causes pial arteriole dilation in untreated piglets, but AT_1 receptor-dependent constriction following ACE inhibition; (3) either COX or NOS inhibition blocks pial arteriolar dilation to intraluminal AngII, but neither affects vasoconstriction to topical AngII; and (4) heme oxygenase inhibition has no effect on pial arteriolar responses to either intraluminal or topical AngII. These data are

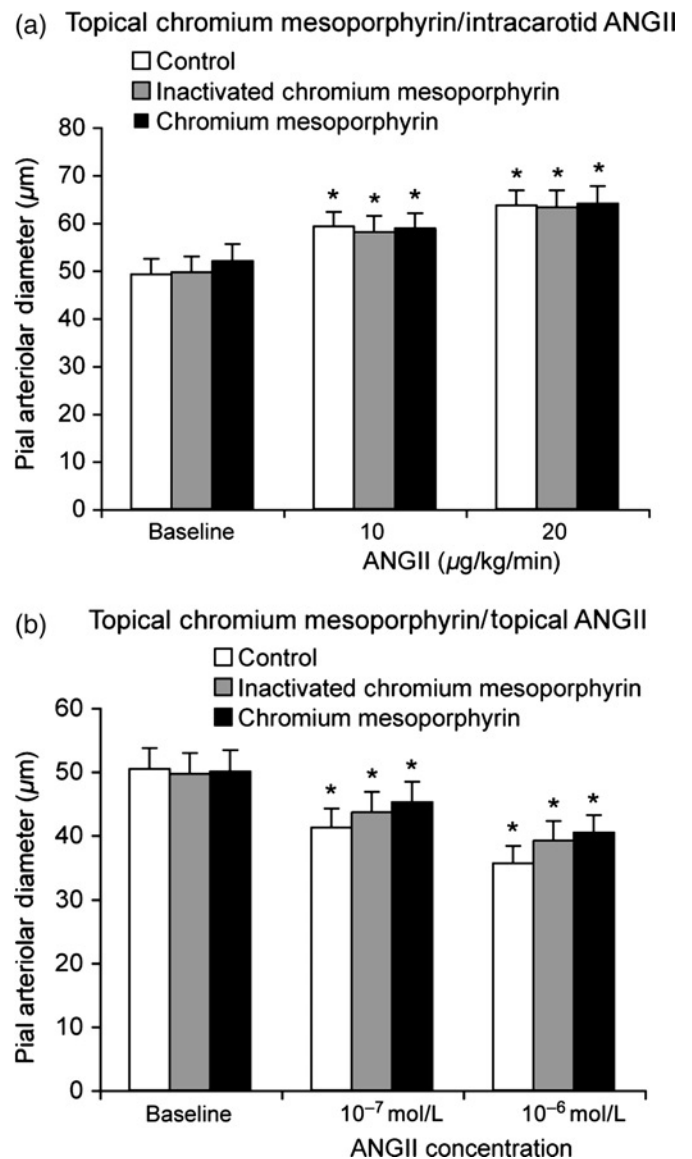


Figure 7 Effect of inhibition of HO with topical chromium mesoporphyrin (2×10^{-5} mol/L, CrMP) on piglet pial arteriolar responses to (a) intracarotid AngII or (b) topical AngII. The effect of inactivated CrMP was determined before active CrMP in the same piglets. Mean $\pm$ SEM. $n = 5$. * $P < 0.05$ from baseline. Ang, angiotensin; HO, heme oxygenase; CrMP, chromium mesoporphyrin

consistent with the hypotheses that: (a) circulating AngII dilates pial arterioles via endothelial AT_1 receptor-derived relaxing factors, notably prostanoids and NO; and (b) direct AT_1 receptor activation on the brain side of the BBB by AngII causes AT_1 receptor-mediated constriction that can mask underlying AT_1 receptor-independent dilation.

These are the first data regarding the effects of intra-arterial infusion of AngII on cerebrovascular circulation *in vivo*. Our results suggest that AT_1 receptor inhibition to decrease after-load on a failing heart (Introduction) may block AngII-induced cerebral arteriole dilation potentially decreasing cerebral blood flow. This is especially pertinent in children whose pulmonary blood flow is heavily dependent on cerebral venous return. In addition, cerebrovascular endothelial injury, as can be caused by numerous

pathological conditions involved in hemodynamic instability in pediatric critical care settings, will likely block the AT₁ receptor-induced cerebrovascular dilation, causing AngII-induced constriction and compromising cerebral blood flow.

Previous studies have reported on the effect of topical AngII on pial resistance arterioles. Similarly to the present report in piglets without ACE inhibition, Meng and Busija²⁷ and Baranov and Armstead^{14,24} found that topical AngII caused pial arteriolar dilation. This dilation was blocked by inhibition of AT₂ receptors.¹⁴ These studies also reported contributions of prostanoids and NO to AngII-induced pial arteriolar dilation.^{14,24,27} We found dilation mediated by relaxing factors also, but vasoconstriction in response to topical AngII application following ACE inhibition with enalapril. Present data indicate that this constriction is not mediated by the endothelium. Neither indomethacin nor L-NAME affected the constriction to topical AngII in enalapril-treated piglets. In contrast, both indomethacin and L-NAME inhibited the endothelium-mediated dilator response to systemic AngII.

The appropriate dose of L-NAME for topical application to the brain *in vivo* is difficult to determine. We have typically used 10⁻³ mol/L because it blocks dilation to L-NAME without altering dilation to hypercapnia or isoproterenol, suggesting selectivity.²⁸ Similarly, Pelligrino *et al.*²⁹ used 10⁻³ mol/L in adult rats that blocks dilation to acetylcholine but does not change dilation to either SNP or moderate hypoxia. Others have used 10⁻⁶ mol/L (*N*ω-nitro-L-arginine [L-NNA] rather than L-NAME)¹⁴ or 10⁻⁴ mol/L.³⁰ Of applicability to the present discussion, Faraci and Heistad³¹ found concentration-dependent, 10⁻⁵ versus 10⁻⁴ mol/L L-NAME, inhibition of basilar artery dilation to acetylcholine, suggesting that topical 10⁻⁵ mol/L L-NAME was insufficient to maximally inhibit NOS of a large cerebral artery *in vivo*. In the present study, 10⁻³ and 10⁻⁵ mol/L, but not 10⁻⁶ mol/L L-NAME, inhibited dilation to intravascular AngII but did not alter basal pial arteriolar diameter or dilation to isoproterenol. The difference between the inhibition of dilation to AngII by L-NAME in the present study and the lack of effect of L-NNA observed by Baranov and Armstead¹⁴ could result from the different concentrations of the NOS inhibitors used rather than the route of AngII administration.

The responses of pial arterioles to agonists, inhibitors and receptor blockers are dependent on the side of the BBB to which they are applied and the integrity of the barrier. Thus, intravascular AngII dilated pial arterioles and this dilation could be blocked by systemic losartan but not by topical losartan, suggesting the AT₁ receptors providing the dilatory signal may be on the luminal endothelial face. While losartan can cross the BBB with chronic administration, it crosses the BBB more slowly than other more lipophilic AT₁ receptor blockers, requiring hours not minutes for efficacy.³²⁻³⁴ Losartan has no partial agonist effect and approximately 1000-fold greater affinity for AT₁ than AT₂.³⁵ The constriction to topical AngII seen in enalapril-pretreated piglets was reversed to dilation with topical losartan, a response identical to that seen in piglets not pretreated with the ACE inhibitor. The action of enalapril to

convert constriction to dilation must be on the endothelium because enalapril does not cross the BBB.^{36,37} That the endothelium generates the dilator signals was confirmed by converting the vasodilator response to intravascular Ang II to constriction by selective endothelial injury. Efficient endothelial injury in the pial arterioles under the cranial window was confirmed by total loss of dilation to the endothelial-dependent dilator, bradykinin,²⁰ but no effect on the dilation to endothelial-independent dilator, isoproterenol,¹⁹ that dilates via activating of vascular smooth muscle β-adrenergic receptors. Consistent with the present functional studies, Wackenfors *et al.*¹⁷ detected AT₁ and AT₂ receptors on the endothelium. AngII receptors appear to be on the vascular smooth muscle also because AngII causes constriction following endothelial injury (Figure 4).

The mechanism(s) by which enalapril alters the pial arteriolar responses to AngII on the brain side of the BBB are not known. ACE inhibitors are known to effect cerebral autoregulation in the short term, decreasing the lower limit and decreasing or leaving unchanged the upper limit.³⁸ Enalapril increases cerebral blood flow in adult patients in heart failure without an increase in cardiac output.³⁹ While we controlled for the effect of autoregulation by keeping systemic mean arterial pressure constant, enalapril may effect local renin-angiotensin action that is somehow involved in the dilation. However, as the effect of ACE would be in the amount of AngII available to receptors, it seems unlikely that enzyme inhibition would change the response of a receptor when stimulated. The activation of AT₂ receptors can cause vasodilation independent of AT₁ and prostanoids in piglets.¹⁴ Therefore, stimulation of the AT₂ receptor may cause vasodilation in contrast to AT₁-mediated constriction, as found in the present study with topical application of AngII in losartan-treated piglets. Similarly, in adult mice, topical AngII caused AT₁ receptor-mediated pial arterial constriction, but vasodilation could be evoked by AT₂ receptor stimulation.⁴⁰ AngII can attenuate endothelial-derived vasodilator responses,⁴⁰ but the effect of the endothelium on action of AngII had not been previously clarified. ACE inhibition has been shown to amplify the endothelial-derived dilator responses in hypertensive rats reducing reactive oxygen species¹⁵ and could also involve elevations of bradykinin¹⁶ that dilate piglet pial arterioles via endothelial-produced prostacyclin that increases cAMP.²⁰

In the search for EDRFs involved in the dilatory action of intraluminal AngII, we found pathways either of whose inhibition totally blocked the pial arteriolar dilation to AngII, COX/prostanoid (presumably prostacyclin) and NOS/NO. The role of NO in dilation of piglet pial arterioles to AngII has been described before, and that was via AT₂ receptors.¹⁴ There are several reasonable possibilities for either COX or NOS inhibition alone to completely block dilation to AngII. COX products could stimulate NOS to elevate NO that produces dilation or conversely NO may stimulate COX. While possible, we have no evidence for this hypothesis. Also, the newborn cerebral circulation is very much prostanoid dominant with NO becoming of more significance with age.²⁰ It is conceivable that the effect of removal of one pathway is masked by increased

activity of the other. We do have evidence that removal of COX elevates the role of NO in the newborn cerebral circulation, but on a timescale of days not minutes.⁴¹ A third possibility has more supporting evidence. In the newborn pig cerebrovascular circulation CO is a functionally highly significant dilator.²⁶ However, either indomethacin or L-NAME will completely block dilation to CO.²⁵ Constant levels of either NO or prostacyclin will completely restore dose-dependent dilation to CO following indomethacin and/or L-NAME. These permissive roles of both PGI₂ and NO are accomplished via protein kinase G.^{26,42} However, in the present dilation to AngII, HO/CO is not involved because CrMP had no effect on the dilation to AngII. In addition to prostanoids, NO and CO, other endothelial-dependent dilator mechanisms potentially contribute to regulation of cerebrovascular circulation. Of particular note is (are) EDHF that cause(s) dilation of cerebral arteries and arterioles by hyperpolarization and involves K⁺ channel activation and gap junctions.⁴³ EDHF has been shown to be involved in AngII dilation of isolated, pressurized, perfused rat cerebral arteries that remain following inhibition of NOS and COX.¹⁷ In the present studies, the complete blockade of dilation to intravascular AngII by either L-NAME or indomethacin may suggest a permissive role for either or both of these mediators leaving open the possibility that additional endothelial-derived relaxant mechanisms, such as those underlying EDH activity, could also be involved in the AT₁-mediated dilation.

In summary, the endothelium alters the response of pial arterioles to AngII in neonatal piglets. Topical AngII can cause either constriction or dilation depending on whether ACE is blocked or not, respectively. Intraluminal AngII produces AT₁ receptor-mediated, endothelium-dependent dilation that is totally blocked by either COX or NOS, but not HO, inhibition. Clinical manipulation of the renin-angiotensin system will have disparate actions on cerebral circulation depending on the integrity of the BBB and ACE that requires further attention.

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REFERENCES

- Barrett KE, Barman SM, Boitano S, Brooks HL. *Ganong's Review of Medical Physiology*. 23rd edn. New York: McGraw-Hill, 2010, chapter 31
- Yunge M, Petros A. Angiotensin for septic shock unresponsive to noradrenaline. *Arch Dis Child* 2000;**82**:388-9
- Tweddell JS, Hoffman GM, Ghanayem NS, Frommelt MA, Mussatto KA, Berger MD. *Hypoplastic Left Heart Syndrome in Moss and Adams' Heart Disease in Infants, Children and Adolescents; Including the Fetus and Young Adults*. 7th edn. Philadelphia: Lippincott Williams and Wilkins, 2008
- Freedom RM, Nykanen D, Benson LN. The physiology of the bidirectional cavopulmonary connection. *Ann Thorac Surg* 1998;**66**:664-7
- Barron DJ, Kilby MD, Davies B, Wright JGC, Jones TJ, Brawn WJ. Hypoplastic left heart syndrome. *Lancet* 2009;**374**:551-64
- Busija DW, Leffler CW. Exogenous norepinephrine constricts cerebral arterioles via α_2 -adrenoreceptors in newborn pigs. *J Cereb Blood Flow Metab* 1987;**7**:184-8
- Rogers AH, Armstead WM, Mirro R, Busija DW, Leffler CW. Influence of intraarterial norepinephrine on cerebral hemodynamics of newborn pigs. *Proc Soc Exp Biol Med* 1989;**191**:174-8
- Weir MR, Dzau VJ. The renin-angiotensin-aldosterone system; a specific target for hypertension management. *Am J Hypertens* 1999;**12**:205S-13S
- Brewster UC, Setaro JF, Perazella MA. The renin-angiotensin-aldosterone system: cardiorenal effects and implications for renal and cardiovascular disease states. *Am J Med Sci* 2003;**326**:15-24
- Gottlieb SS, Dickstein K, Fleck E, Kostis J, Levine TB, LeJemtel T, DeKock M. Hemodynamic and neurohormonal effects of the angiotensin II antagonist losartan in patients with congestive heart failure. *Circulation* 1993;**88**:1602-9
- Little CM, Brown CG. Angiotensin II administration improves cerebral blood flow in cardiopulmonary arrest in swine. *Stroke* 1994;**25**:183-6
- Song K, Allen AM, Paxinos G, Mendelsohn FA. Angiotensin II receptor subtypes in rat brain and peripheral tissues. *Cardiology* 1991;**79**(suppl 1):45-54
- Haberl RL, Anneser F, Villringer A, Einhaupl KM. Angiotensin II induces endothelium-dependent vasodilation of rat cerebral arterioles. *Am J Physiol Heart Circ Physiol* 1990;**258**(6 Pt 2):H1840-6
- Baranov D, Armstead WM. Nitric oxide contributes to AT₂ but not AT₁ angiotensin II receptor-mediated vasodilation of porcine pial arteries and arterioles. *Eur J Pharmacol* 2005;**525**:112-16
- Lewis SJ, Hashmi-Hill MP, Owen JR, Sandock K, Roberson TP, Bates JN. ACE inhibition restores the vasodilator potency of the endothelium-derived relaxing factor, L-S-nitrosocysteine, in conscious spontaneously hypertensive rats. *Vasc Pharmacol* 2006;**44**:491-507
- De Gennaro Colonna V, Fioretti S, Rigamonti A, Bonomo S, Manfredi B, Muller EE, Berti F, Rossoni G. Angiotensin II type 1 receptor antagonism improves endothelial vasodilator function in L-NAME-induced hypertensive rats by a kinin-dependent mechanism. *J Hypertens* 2008;**24**:33-6
- Wackenfors A, Vikman P, Nilsson E, Edvinsson L, Malmjö M. Angiotensin II-induced vasodilation in cerebral arteries is mediated by endothelium-derived hyperpolarising factor. *Eur J Pharmacol* 2006;**531**:259-63
- Lankford SM, Plummer D, Helyer P, Christ DD, Bai SA. Pharmacokinetic-pharmacodynamic relations of losartan and EXP3174 in a porcine animal model. *J Cardiovasc Pharmacol* 1997;**30**:583-90
- Leffler CW, Mirro R, Shanklin DR, Armstead WM, Shibata M. Light/dye microvascular injury selectively eliminates hypercapnia induced pial arteriolar dilation in newborn pigs. *Am J Physiol Heart Circ Physiol* 1994;**266**:H623-30
- Willis AP, Leffler CW. Endothelial NO and prostanoid involvement in newborn and juvenile pig pial arteriolar vasomotor responses. *Am J Physiol Heart Circ Physiol* 2001;**281**:H2366-77
- Leffler CW, Busija DW. Arachidonic acid metabolites and perinatal cerebral hemodynamics. *Semin Perinatol* 1987;**11**:31-42
- Armstead WM, Mirro R, Busija DW, Leffler CW. Permissive role of prostanoids in acetylcholine-induced cerebral vasoconstriction. *J Pharmacol Exp Ther* 1989;**251**:1012-19

- 23 Leffler CW, Nasjletti A, Yu C, Johnson RA, Fedinec AL, Walker N. Carbon monoxide and cerebral microvascular tone in newborn pigs. *Am J Physiol Heart Circ Physiol* 1999;**276**:H1641–6
- 24 Baranov D, Armstead WM. Prostaglandins contribute to impaired angiotensin II-induced cerebral vasodilation after brain injury. *J Neurotrauma* 2002;**19**:1457–66
- 25 Leffler CW, Fedinec AL, Parfenova H, Jaggar JH. Permissive contributions of NO and prostacyclin in CO-induced cerebrovascular dilation in piglets. *Am J Physiol Heart Circ Physiol* 2005;**289**:H432–8
- 26 Leffler CW, Parfenova H, Jaggar JH, Wang R. Carbon monoxide and hydrogen sulfide: gaseous messengers in cerebrovascular circulation. *J Appl Physiol* 2006;**100**:1065–76
- 27 Meng W, Busija DW. Comparative effects of angiotensin-(1-7) and angiotensin II on piglet pial arterioles. *Stroke* 1993;**24**:2041–4
- 28 Busija DW, Leffler CW, Wagerle LC. Mono-L-arginine-containing compounds dilate piglet pial arterioles via an endothelium-derived relaxing factor-like substance. *Circ Res* 1990;**67**:1374–80
- 29 Pelligrino DA, Wang Q, Koenig HM, Albrecht RF. Role of nitric oxide, adenosine, N-methyl-D-aspartate receptors, and neuronal activation in hypoxia-induced pial arteriolar dilation in rats. *Brain Res* 1995;**704**:61–70
- 30 Colonna DM, Meng W, Deal DD, Busija DW. Nitric oxide promotes arteriolar dilation during cortical spreading depression in rabbits. *Stroke* 1994;**25**:2463–70
- 31 Faraci FM, Heistad DD. Role of ATP-sensitive potassium channels in the basilar artery. *Am J Physiol* 1993;**264**:H8–13
- 32 Bui JD, Kimura B, Phillipos MI. Losartan potassium, a nonpeptide antagonist of angiotensin II, chronically administered p.o. does not readily cross the blood–brain barrier. *Eur J Pharmacol* 1992;**219**:147–51
- 33 Polidori C, Ciccocioppo R, Pompei P, Cirillo R, Massi M. Functional evidence for the ability of angiotensin AT₁ receptor antagonists to cross the blood–brain barrier in rats. *Eur J Pharmacol* 1996;**307**:259–67
- 34 Wang JM, Tan J, Leenen FH. Central nervous system blockade by peripheral administration of AT₁ receptor blockers. *J Cardiovasc Pharmacol* 2003;**41**:593–9
- 35 *Physicians' Desk Reference*. 63rd edn. Montevale NJ: Physicians' Desk Reference, Inc, 2009
- 36 Cohen ML, Kurz K. Captopril and MK-421: stability on storage, distribution to the central nervous system, and onset of activity. *Fed Proc* 1983;**42**:171–5
- 37 Pipkin FB, Wallace CP. The effect of enalapril (MK421), an angiotensin converting enzyme inhibitor, on the conscious pregnant ewe and her foetus. *Br J Pharmacol* 1986;**87**:533–42
- 38 Paulson OB, Waldemar G, Andersen AR, Barry DI, Pedersen EV, Schmidt JF, Vorstrup S. Role of angiotensin in autoregulation of cerebral blood flow. *Circulation* 1988;**77**(suppl 1):I55–8
- 39 Kamishirado H, Inoue T, Fujito T, Kase M, Smimizu M, Sakai Y, Takayanagi K, Morooka S, Natsui S. Effect of enalapril maleate on cerebral blood flow in patients with chronic heart failure. *Angiology* 1997;**48**:707–13
- 40 Girouard H, Park L, Anrather J, Zhou P, Iadecola C. Angiotensin II attenuates endothelium-dependent responses in the cerebral microcirculation through nox-2-derived radicals. *Arterioscler Thromb Vasc Biol* 2006;**26**:826–32
- 41 Zhang Y, Leffler CW. Compensatory role of NO in cerebral circulation of piglets chronically treated with indomethacin. *Am J Physiol Regul Integr Comp Physiol* 2002;**282**:R400–10
- 42 Koneru P, Leffler CW. Role of cGMP in carbon monoxide-induced cerebral vasodilation in piglets. *Am J Physiol Heart Circ Physiol* 2004;**286**:H304–9
- 43 Andresen J, Shafi NI, Bryan RM Jr. Endothelial influences on cerebrovascular tone. *J Appl Physiol* 2006;**100**:318–27

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