A BRIEF COMMUNICATION

Age and Species Dependence of Pial Arteriolar Responses to Topical Carbon Monoxide *In Vivo*

DAVID C. HOLT, ALEXANDER L. FEDINEC, ASHLEY N. VAUGHN, AND CHARLES W. LEFFLER[1]

Laboratory for Research in Neonatal Physiology, Department of Physiology, University of Tennessee Health Science Center, Memphis, Tennessee 38163

In newborn pigs, carbon monoxide (CO) contributes to regulation of cerebrovascular circulation. Results from isolated adult cerebral arteries suggest CO may have less dilatory potential in mature animals. However, few data are available on the direct effects of CO on cerebrovascular circulation *in vivo* except for those from newborn pigs. Therefore, we tested the hypothesis that i) rat cerebral arterioles dilate to CO *in vivo* and ii) CO-induced cerebrovascular dilatory responses are age dependent in pigs. Also, we examined whether the permissive role of nitric oxide in CO-induced dilation observed in piglets is present in older pigs and rats. Experiments used anesthetized newborn, 7-week-old, and juvenile (3- to 4-month-old) pigs and 3- to 4-month-old rats with closed cranial windows and topical applications of CO and sodium nitroprusside (SNP). Dilations to SNP were not different at different ages in pigs or between pigs and rats. CO produced pial arteriolar dilations in all groups. Dilation to 10^{-5} *M* CO was reduced in juvenile pigs as compared to newborn and 7-week-old pigs, and tended to less at 10^{-6} *M* CO. Dilations of rat pial arterioles to all concentrations were less than those of newborn and 7-week-old pigs, but not different from those of juvenile pig pial arterioles. In newborn and 7-week-old pigs, L-nitro-arginine (LNA) inhibited the dilation to CO, an effect reversed by a constant background of SNP. In contrast, LNA did not reduce dilation to CO in juvenile pigs or rats. In conclusion, rat pial arterioles like those in piglets dilate to CO *in vivo*, but there are age and species differences with regard to reactivity and interaction with NO. Exp Biol Med 232:1465–1469, 2007

Key words: postnatal development; cerebrovascular circulation; species dependence

Introduction

Carbon monoxide (CO) is an endogenous gaseous autocrine/paracrine messenger analogous to nitric oxide (NO; Ref. 1). CO is a major component in the regulation of cerebrovascular circulation in newborn pigs because it is involved in responses to excitatory amino acids, hypotension, and hypoxia (1). Results from excised adult arteries suggest CO may have less dilatory potential in older animals when compared to newborns (1). However, though data are available from newborn pigs, there are few data regarding the effects of topical CO on the cerebral vasculature of older individuals of any species *in vivo*. Thus, the question remains open whether CO could be a functional dilator in adult cerebral circulation, particularly in rodent models.

Therefore, we test the hypothesis that CO dilates pial arterioles of rats *in vivo*. We also address the hypothesis that maturation reduces the cerebrovascular responsiveness to CO in pigs. In addition, we examine the question of whether the permissive enabling role of NO for CO-induced dilation observed in the newborn pig was present in older pigs and/or rats. These latter experiments were undertaken because all permissive interactions between NO and CO have been described in the newborn pig (1,2), but never examined at

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[1] To whom correspondence should be addressed at Department of Physiology, 894 Union Ave, Memphis, TN 38163. E-mail: cleffler@physio1.utmem.edu

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other ages or in other species, and contributions of NO to cerebrovascular control are age dependent (3–6).

Materials and Methods

The animal protocols were reviewed and approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center. Methods were as described in greater detail previously (e.g., Refs. 3–6). Male Sprague-Dawley rats (~350 g, 3–4 months old) and newborn (1–3 days old, 2.0 ± 0.5 kg), ~7-week-old (13 ± 2 kg), and 3- to 4-month-old (juvenile) (34 ± 1 kg) pigs were used. Each animal was first anesthetized with ketamine (33 mg/kg, intramuscularly [im]) and acepromazine (3.3 mg/kg, im). Catheters were placed in a femoral artery and vein for blood pressure and blood gas monitoring and maintenance anesthesia (α-chloralose, 50 mg/kg), respectively. Newborn and 7-week-old pigs as well as rats were ventilated using a neonatal ventilator through a tracheotomy. The older pigs breathed spontaneously. Core temperature was maintained at 37° ± 1°C.

A 2-cm diameter hole was cut in the skull overlying the parietal cortex of the pigs. A smaller hole, approximately 1 cm in diameter, was used for rats. The dura was cut and reflected over the bone edges. A stainless steel and glass cranial window was placed in the hole and cemented in place with dental acrylic. The space under the window was filled with 37°C artificial cerebrospinal fluid (aCSF; pH 7.33; $P_{CO_2} = 46$ mm Hg, $P_{O_2} = 43$ mm Hg; Refs. 5, 6).

Pial arterioles were observed *via* a dissecting microscope with a mounted video camera. Diameters were measured with a video micrometer. All measurement periods were 5 mins in duration, with the maximal diameter reported. Fresh aCSF, with or without experimental treatment, was placed beneath the window to begin the period. The responses to sodium nitroprusside (SNP; 10^{-6} *M*) were measured, the window was flushed and filled with aCSF and control pial arteriolar diameter reestablished, and then responses to ascending concentrations of CO (10^{-7}, 10^{-6}, and 10^{-5} *M*) were measured. These concentrations cover a range from just above the threshold to near maximal. CO solutions were produced by saturation of water with CO (10^{-3} *M*) with dilations made in gas-tight containers without a gaseous interface.

L-Nitro-arginine (LNA; 10^{-3} *M*) was added to the aCSF to inhibit NO synthase. Responses to SNP and CO, as above, were measured before and in the presence of LNA alone and then with the addition of 2×10^{-7} *M* SNP to the aCSF.

Values for each variable are presented as mean ± the standard error of the mean (SEM). Comparisons among populations within each age/species group used analysis of variance (ANOVA) with repeated measures. For comparison among species and age, ANOVA without repeated measures was used. The Tukey-Kramer Multiple Comparisons test or the Bonferroni multiple comparison test for selected groups was used to isolate differences between groups as appropriate for the design structure.

Results

Throughout the experiments, there were no significant changes in arterial blood gases, pH, or pressure when the values were compared at the beginning and end of the experiments. Combined control pial arteriole diameters for all experiments were: newborn pigs, 50 ± 3 μm; 7-week-old pigs, 52 ± 5 μm; juvenile pigs, 65 ± 6 μm; and adult rats, 52 ± 4 μm.

Figure 1 shows the effect of SNP (10^{-6} *M*) and ascending doses of CO (10^{-7}, 10^{-6}, and 10^{-5} *M*) on pial arteriolar diameter. The dilations to SNP were not different across ages or species. CO produced pial arteriolar dilations in newborn, 7-week-old, and juvenile pigs and in rats. In pigs, there was no difference in dilation in response to CO when comparing newborn and 7-week-old pigs. Pial arteriolar dilation to 10^{-5} *M* CO was less in juvenile pigs than newborn and 7-week-old pigs and tended to be less to 10^{-6} *M* CO ($P = 0.057$ compared to newborn). Pial arteriolar dilations in response to CO at all concentrations in rats were less than those of newborn and 7-week-old pigs, but not different from those of juvenile pigs. Comparisons between the responsiveness of pial arterioles to CO and NO should not be made from these experiments because the concentration of NO produced by the 10^{-6} *M* SNP is not known.

LNA (10^{-3} *M*) was used to inhibit NO synthase, thereby blocking NO production. In both newborn and 7-week-old pigs, LNA inhibited the dilation to CO (Fig. 2). In contrast, LNA did not significantly reduce dilation to CO in juvenile pigs or adult rats. Addition of a constant background concentration of SNP restored dilation to CO in newborn and 7-week-old pigs, but had no effect on the dilation to CO in either juvenile pigs or rats.

Discussion

Summarized findings are i) CO dilates pial arterioles of baby and juvenile pigs and young adult rats *in vivo*, ii) responsiveness of cerebral arterioles to CO is greater in baby pigs than in older pigs, iii) pial arterioles of rats and pigs of all ages studied dilate similarly to topical NO, iv) rat arterioles show a reduced dilator response to CO when compared to newborn and baby, but not juvenile, pigs, and v) a basal level of NO is necessary to permit dilation in response to CO in newborn and baby pigs, but this role for NO is not seen in older pigs or rats.

Pial arterioles of both rats and pigs dilated to topical CO *in vivo*. In contrast, most prior studies, all performed *in vitro* on adults, have been unable to detect CO-induced dilation of cerebral arteries. For example, CO did not alter the tone of excised adult white rabbit cerebral arteries (7). Additionally, Andresen *et al*. (8) concluded that CO, at physiological concentrations, is not a dilator of rat or mice pressurized and

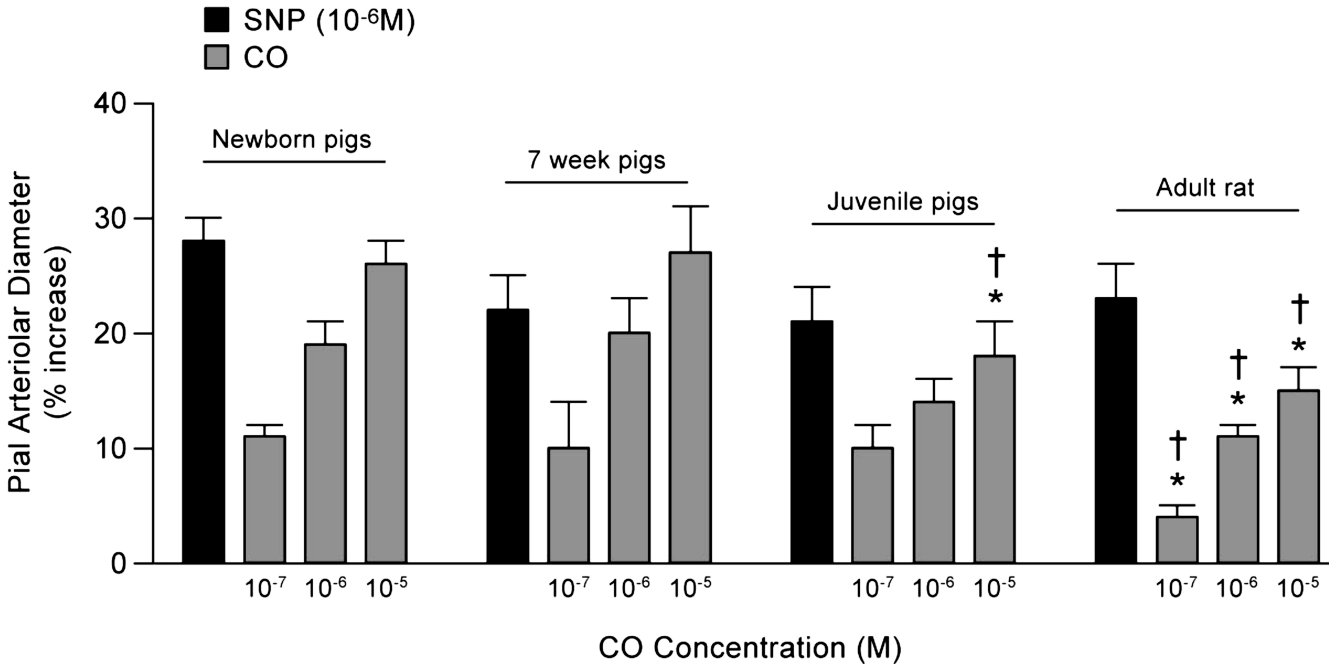

Figure 1. Dilation of pial arterioles in response to SNP (10^{-6} *M*) and CO. Values are mean ± SE. * and + are $P < 0.05$ compared to newborn and 7-week-old pig, respectively. Increases in diameters at all CO concentrations in pigs of all ages and rats are significant at $P < 0.05$. Dilations of juvenile pig and rat pial arterioles to CO at each concentration are not different ($P > 0.05$). $N = 28$, 10, 15, and 13, newborn, 7-week-old, juvenile pigs, and rats, respectively.

perfused cerebral arteries. However, dog basilar artery segments did dilate to CO at about the highest concentration used in the present study (9). The dog arteries were denuded of endothelium, suggesting dog cerebral arteries, like those of rats and juvenile pigs in the present study, do not require the permissive signal from the endothelium necessary in the newborn pig (2). Pressurized pial arterioles of newborn pigs *in vitro* do dilate to a light-activated CO-releasing molecule in the superfusion solution (2). The present data show rat arterioles *in vivo* can dilate to CO. Therefore, endogenously produced CO could contribute to cerebrovascular dilatory responses in adult rats. It is uncertain why rat cerebral vessels do not dilate to CO *in vitro* but do show a dilatory response *in vivo*. Because CO is applied to the intact cortical surface *in vivo*, contributions to the arteriolar responses by other cell types not present with isolated arteries and arterioles, including neurons, astrocytes, and even blood cells, could result in differences between *in situ* and *ex vivo* data. Furthermore, such variables as vessel size and tone must be considered.

Prior to the present study, all *in vivo* data available on immediate cerebrovascular responses to direct application of CO were from newborn pigs. In the rat pial circulation *in vivo*, endogenous CO has a tonic inhibitory effect on NO production (10). Thus, the heme oxygenase (HO) inhibitor zinc protoporphyrin (ZnPP) progressively dilated pial arterioles over 60 mins. This dilation was blocked by L-N^G-nitroarginine methyl ester (L-NAME) and by addition of CO to the superfusion solution. Further, ZnPP enhanced cerebral NO production as estimated using diaminofluorescein-2. No direct effect was detected following 60 mins superfusion with CO (10^{-5} *M*). These results, coupled with the present report, suggest that CO may have time-dependent actions; CO produced by acute activation of HO functions as a dilatory gasotransmitter, whereas basal CO production and prolonged elevation can produce tonic inhibition of NO production and increase cerebrovascular tone. Our previous results in piglets indicate that endogenously produced CO stimulated by heme and inhibited by HO blockers causes dilation (reviewed in Ref. 1). However, the affects of prolonged application of heme to the brain surface in piglets, pigs, or rats are unknown.

Postnatal development appears to alter CO-induced dilation of pial arterioles. We found a reduction of the dilatory response to CO in the oldest pigs studied when compared to newborn and baby pigs. The juvenile pigs, though large animals and the same absolute age as the rats, are immature as compared to the rats. Whether mature adult pigs (6 months) that weigh more than 100 kg or even older larger pigs might show further reduced responses to CO or whether newborn rats may show increased responses is not known. However, our previous work found marked shifts in cerebrocirculatory control mechanisms in pigs over the developmental period represented in the present study. For example, acetylcholine produces pial arteriolar dilation in 3- to 4-week-old pigs (4), similar to adults of various species (11), but not in newborn pigs (4). In addition, cerebrovascular dilation to hypercapnia in newborn pigs is largely NO independent, whereas NO assumes an increasing role in

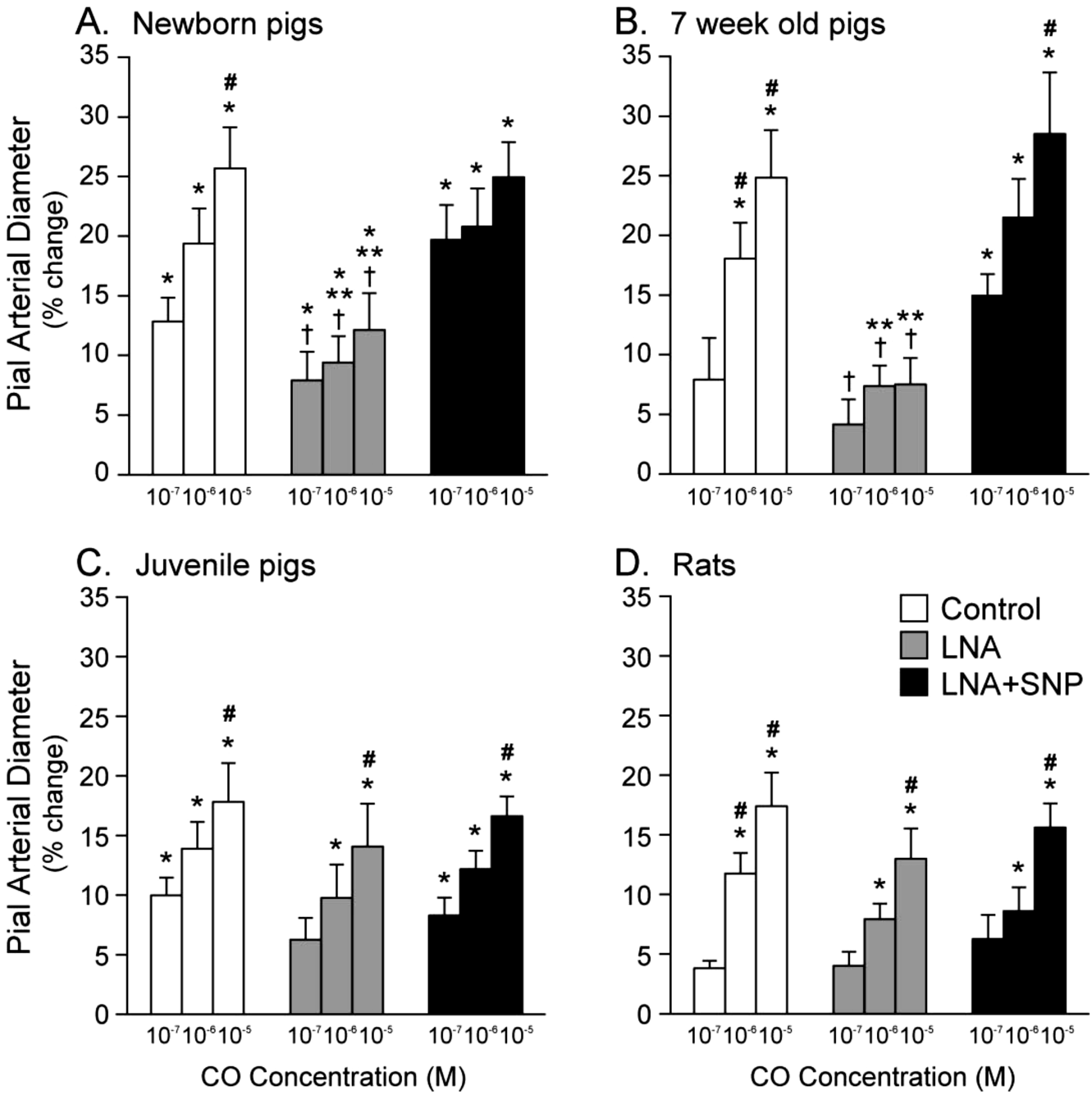

Figure 2. Dilation of pial arterioles in newborn pigs ($n = 9$) (A), 7-week-old pigs ($n = 6$) (B), juvenile pigs ($n = 7$) (C) and adult rats ($n = 10$) (D) to ascending doses of CO alone, with LNA (10^{-3} *M*), and with LNA plus SNP (2×10^{-7} *M*). Values are mean ± SE and *, #, **, and † are $P < 0.05$ compared to zero % change, 10^{-7} *M* CO, without LNA, and LNA + SNP, respectively.

maturation within the age range used in the present study (3, 6).

The mechanisms involved in the diminished responses of older pigs and rat pial arterioles as compared to newborn and baby pigs require further investigation. It does not appear that a reduction in vasoreactivity in general is involved, because pial arterioles of rat and pigs of all ages responded similarly to the NO source, SNP, that increases cyclic guanosine monophosphate (11). CO dilates *via* activation of large conductance Ca^{2+}-activated K^+ channels (K_{Ca} channels)(1, 12). Ca^{2+} transients termed Ca^{2+} sparks activate adjacent K_{Ca} channels (13), leading to a transient outward K^+ current. In adult rats, virtually all Ca^{2+} sparks produce transient K^+ currents in cerebral vascular smooth muscle cells (14). However, in newborn pigs only about 60% of sparks cause K_{Ca} channel currents (15). CO increases Ca^{2+} sparks and increases coupling of Ca^{2+} sparks to K_{Ca} channels (16, 17). It is possible that the ability of CO

to dilate rat arterioles is less than in piglets because rat basal K_{Ca} channel activity is higher, and all Ca^{2+} sparks induce K_{Ca} channel currents in the basal state.

NO provides a permissive signal for CO-induced vasodilation in newborn and baby pigs. As shown in newborn pigs before, LNA blocks dilation to CO and that dilation is restored by a constant, minimally vasodilator concentration of NO (1). In contrast, in the older pigs and rats, the dilation to CO was not diminished by the addition of LNA nor increased by the addition of SNP, suggesting the absence of this signal with advancing age. Similarly, in the rat hypothalamus, the contribution of CO to the regulation of vascular tone became more evident in the absence of NO (18). Of note, the similarity of responses to CO of LNA-treated baby pigs, juvenile pigs, and rats suggests that the permissive NO signal that markedly accentuates the response of the baby cerebral microvasculature to CO is absent in the older animals. This absence could contribute to the reduced response of the intact pial arterioles compared with those of babies.

Overall, we find that the gasotransmitter CO dilates arterioles on the cerebral cortical surface of both pigs and rats. However, pial arteriolar dilator reactivity to CO in older pigs and adult rats is less than that of newborn and baby pigs. In addition, postnatal maturation appears to be accompanied by alteration in interactions between CO and NO in the control of cerebral arteriolar tone.

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