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## Astrocyte-derived CO is a diffusible messenger that mediates glutamate-induced cerebral arteriolar dilation by activating smooth muscle cell $K_{Ca}$ channels

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### Abstract

Astrocyte signals can modulate arteriolar tone, contributing to regulation of cerebral blood flow, but specific intercellular communication mechanisms are unclear. Here we used isolated cerebral arteriole myocytes, astrocytes, and brain slices to investigate whether carbon monoxide (CO) generated by the enzyme heme oxygenase (HO) acts as an astrocyte-to-myocyte gasotransmitter in the brain. Glutamate stimulated CO production by astrocytes with intact HO-2, but not those genetically deficient in HO-2. Glutamate activated transient  $K_{Ca}$  currents and single  $K_{Ca}$  channels in myocytes that were in contact with astrocytes, but did not affect  $K_{Ca}$  channel activity in myocytes that were alone. Pre-treatment of astrocytes with chromium mesoporphyrin (CrMP), a HO inhibitor, or genetic ablation of HO-2 prevented glutamate-induced activation of myocyte transient  $K_{Ca}$  currents and  $K_{Ca}$  channels. Glutamate decreased arteriole myocyte intracellular  $Ca^{2+}$  concentration and dilated brain slice arterioles and this decrease and dilation were blocked by CrMP. Brain slice arteriole dilation to glutamate was also blocked by L-2-alpha amino adipic acid, a selective astrocyte toxin, and paxilline, a  $K_{Ca}$  channel blocker. These data indicate that an astrocytic signal, notably HO-2 derived CO, is employed by glutamate to stimulate arteriole myocyte  $K_{Ca}$  channels and dilate cerebral arterioles. Our study explains the astrocyte and HO dependence of glutamatergic functional hyperemia observed in the newborn cerebrovascular circulation in vivo.

### Keywords

newborn; cerebrovascular circulation; functional hyperemia; heme oxygenase

### INTRODUCTION

Local cerebral arteriole smooth muscle tone is temporally and spatially coordinated to match blood flow to neuronal activity. This vascular response is termed functional hyperemia or neurovascular coupling<sup>1, 2</sup>. In the cerebrovascular circulation, signals to vascular smooth muscle derive from endothelium, nerves, astrocytes, or pericytes, which interact to form a neurovascular unit<sup>3</sup>. Astrocytes relay signals to neurons<sup>4</sup> and other astrocytes<sup>5, 6</sup>. Parenchymal arterioles are ensheathed by astrocyte endfeet<sup>7, 8</sup>. Pial arterioles lay on a bed of astrocytes, the glia limitans, and are coated by astrocyte processes and endfeet<sup>9–11</sup>. Recent

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**DISCLOSURES:** none

papers have reviewed astrocytes functioning as intermediaries between neurons and cerebral blood vessels to adjust cerebral blood flow to neuronal activity<sup>3, 7, 8</sup>. Potential astrocyte-derived vasoactive mediators of cerebrovascular responses include epoxyeicosatrienoic acids<sup>12, 13</sup>, K<sup>+</sup><sup>14, 15</sup> and ATP<sup>16</sup> which appear to perform in concert<sup>8</sup>.

The principle excitatory cerebral neurotransmitter is glutamate that dilates cerebral arterioles to match blood flow to neural activity. Specific cells and mechanisms involved in coupling the glutamate release to arteriolar smooth muscle relaxation are uncertain. Astrocytes are uniquely positioned for sensing neuronal activity and regulating cerebral blood flow<sup>7, 8, 17–19</sup>. They possess both metabotropic glutamate receptors (mGluRs) and ionotropic glutamate receptors (iGluRs). Glutamate stimulation of astrocytic mGluR and iGluR produces intracellular Ca<sup>2+</sup> oscillations and localized and global elevations of astrocyte intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>)<sup>20–24</sup>. In brain slices, glutamate elevates astrocyte [Ca<sup>2+</sup>]<sub>i</sub> but reduces adjacent vascular smooth muscle cell [Ca<sup>2+</sup>]<sub>i</sub> that produces vasodilation<sup>22, 25</sup>. However, the signaling mechanisms between astrocytes and vascular smooth muscle that result in this dilation to glutamate remain uncertain with eicosanoids<sup>17, 25</sup>, K<sup>+</sup><sup>2, 14</sup>, and Ca<sup>2+</sup><sup>24</sup> among the mechanisms considered.

CO could be a glutamate-induced, diffusible messenger employed by astrocytes to signal for arteriole dilation. This hypothesis is rational because glutamatergic dilation of newborn pig cerebral arterioles, *in vivo*, is blocked by inhibition of HO that produces CO<sup>26, 27</sup>. Furthermore, astrocyte injury, *in vivo*, blocks piglet pial arteriolar dilation to glutamate<sup>9</sup> making it reasonable to propose that astrocytes are the source of vasoregulatory CO involved in excitatory amino acid-induced cerebral vasodilation. CO dilates cerebral arterioles by activating smooth muscle cell large conductance Ca<sup>2+</sup> activated potassium channels (K<sub>Ca</sub> channels)<sup>28, 29</sup>. Therefore, we investigated whether astrocyte-derived CO increases smooth muscle cell K<sub>Ca</sub> channel activity, providing a mechanism by which glutamate can cause arteriole dilation. Specifically, the present study tested the hypothesis that glutamate stimulates astrocyte production of CO that increases arteriole smooth muscle cell K<sub>Ca</sub> currents, leading to a decrease in intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) and vasodilation.

## MATERIALS AND METHODS

### Piglet cerebral arteriole smooth muscle cell isolation

Procedures were approved by the University of Tennessee Health Science Center Animal Care and Use Committee. Newborn pigs (1–3 days old, 1–2.5 kg) were anesthetized with ketamine hydrochloride (33 mg/kg im) and acepromazine (3.3 mg/kg im). The brain was removed and placed into ice-cold HEPES-buffered physiological saline solution (PSS). For electrophysiological studies, arterioles (50–200 μm in diameter) were dissected from the cerebral cortical surface. Individual vascular smooth muscle cells were enzymatically dissociated from cerebral arterioles using a procedure previously described<sup>30</sup>.

### Astrocyte culture

Piglets were anesthetized as above, and adult HO-2<sup>+/+</sup> and HO-2<sup>-/-</sup> mice were killed by peritoneal injection of sodium pentobarbital (130 mg/kg) (Mouse procedures were approved by New York Medical College Animal Care and Use Committee). Brains were removed and placed in ice-cold DMEM with antibiotic/antimycotic (100 U/ml penicillin, 100 mg/ml streptomycin, and 2.5 mg/ml amphotericin B). Astrocytes were isolated, grown, and identified as described previously<sup>9</sup>. Primary culture astrocytes were dislodged by trypsin-EDTA and either transferred to DMSO (10%)/FBS (90%) and frozen in liquid nitrogen for future use or directly plated on glass cover slips (4mm × 4mm) and grown to confluence. No differences were detectable in growth or phenotype between directly plated and frozen

astrocytes. Astrocytes on cover slips were used for experiments with freshly isolated smooth muscle cells.

### Astrocyte culture on Cytodex microcarrier beads

Confluent mouse HO-2<sup>+/+</sup> and HO-2<sup>-/-</sup> astrocytes were dislodged with trypsin-EDTA and mixed with Cytodex microcarrier beads (175  $\mu$ m diameter, denatured collagen chemically coupled to a matrix of cross-linked dextran, GE Healthcare), transferred into spinner flasks and stirred intermittently (20 rpm 30 min on and 3 h off) at 37 °C and 5% CO<sub>2</sub> in air. After 12 h, the beads were stirred continuously (20 rpm) for 4–6 days before experiments. Cell density was approximately 10<sup>6</sup> cells/ml beads.

### Patch-clamp electrophysiology

A glass coverslip with or without a confluent layer of astrocytes was placed in an experimental chamber. Freshly isolated cerebral arteriole smooth muscle cells were added to the chamber and allowed to settle for 10 min. Potassium currents were measured by using the whole cell perforated-patch-clamp configuration<sup>28</sup>. Transient K<sub>Ca</sub> currents were measured at a steady membrane potential of -40 mV and calculated from 5 min of continuous gap-free data. At -40 mV, a transient K<sub>Ca</sub> current was defined as the simultaneous opening of three K<sub>Ca</sub> channels. Single K<sub>Ca</sub> channel currents were recorded at a steady membrane potential of 0 mV in the presence of thapsigargin (100 nmol/L).

### CO measurement

Following overnight incubation in L-glutamine and serum free DMEM, astrocytes grown on Cytodex microcarrier beads (1ml beads diluted to 1.7 ml with Krebs solution) were placed into amber vials with <sup>13</sup>C<sup>18</sup>O (<sup>31</sup>CO, ISOTECH) as the internal standard. Following 1h incubation at 37°C, 100 $\mu$ l of head space gas was collected for CO detection by gas chromatography/mass spectrometry as described before<sup>31, 32</sup>.

### Detection of HO-2 expression

Proteins from Laemmli buffer solubilized samples were separated by 12 % SDS PAGE, transferred to PVDF membranes and blocked with 10 % BSA in PBS-Tween 20 (0.1%). Immunoblotting was performed using the following antibodies: primary 1:2500 rabbit anti-mouse HO-2 antibodies (Stressgen), secondary 1:5000 goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology); primary 1:5000 mouse anti-mouse  $\alpha$ -actin (Sigma Aldrich), secondary 1:10,000 goat anti-mouse IgG-HRP (Santa Cruz Biotechnology). The immunocomplexes were visualized with ECL Plus Western Blot detection reagent (GE Healthcare) and quantified by digital densitometry using Gel-Pro Analyzer software (Media Cybernetics).

### Brain slice preparation, [Ca<sup>2+</sup>]<sub>i</sub>, and arteriolar diameter measurements

Newborn pig brains were removed and placed in cold (4–8°C) M199 with 10 mmol/L HEPES, and 10 mmol/L glucose, pH 7.37. Tangential slices were cut (200  $\mu$ m thick) from the cerebral cortical surface and placed in M199. Diameters of cortical surface arterioles were measured in bicarbonate-buffered PSS (21% O<sub>2</sub>, 5% CO<sub>2</sub>) superfused (1.5 ml/min, 35°C) brain slices using an inverted microscope, CCD camera, and edge detection software (IonWizard, IonOptix, Milton, MA). [Ca<sup>2+</sup>]<sub>i</sub> was measured in cerebral arterioles of piglet brain slices using fura-2 with a method published previously<sup>33</sup>. The cerebral arteriolar wall in the brain slice was selected for data acquisition.

To selectively injure astrocytes, slices were incubated with L-2-alpha amino adipic acid (L-AAA, 2 mmol/L). We<sup>9</sup>, and others<sup>10, 11</sup>, have shown previously that L-AAA treatment *in*

*vivo* produces histological evidence of injury to the superficial glia limitans and loss of astrocyte-dependent cerebrovascular responses without altering responses in general. Control slices were incubated with D-AAA, the inactive isomer.

## Chemicals

Cr (III) mesoporphyrin IX chloride (CrMP) was purchased from Frontier Scientific (Logan, Utah). Papain was purchased from Worthington Biochemical (Lakewood, NJ). All other chemicals were obtained from Sigma Chemical (St. Louis, MO) unless otherwise stated.

## Statistical analysis

Values are means  $\pm$  SEM. For comparisons among more than 2 groups, results were subjected to a one-way ANOVA for repeated measures with Tukey's post hoc test to isolate differences between groups. Student *t*-tests were used for comparison between each two groups of paired or unpaired data, as appropriate.  $P < 0.05$  was considered significant.

## RESULTS

Cerebral arteriole smooth muscle cells were allowed to settle either on astrocytes or empty coverslips prior to measurement of whole cell currents. Using patch-clamp electrophysiology, transient  $K_{Ca}$  current frequency and  $K_{Ca}$  channel activity were recorded in smooth muscle cells. At  $-40$ mV, a physiological membrane potential, glutamate ( $20\mu\text{mol/L}$ ) reversibly increased ( $\sim 75\%$ ) transient  $K_{Ca}$  current frequency in cerebral arteriole smooth muscle cells that were in contact with astrocytes (Fig.1). Conversely, glutamate had no effect on the transient  $K_{Ca}$  current frequency of smooth muscle cells that were not in contact with astrocytes (Fig.1).

Transient  $K_{Ca}$  current amplitudes are not normally distributed (failed Kolmogorov and Smirnov for a Gaussian distribution at  $P < 0.0001$ ). Therefore, transient  $K_{Ca}$  currents were divided into groups that are logical based on numbers of simultaneous channel openings (Table 1): small transient  $K_{Ca}$  currents (3–7 channel openings [1 channel =  $2.8\text{pA}^{34}$ ]), mid-sized transient  $K_{Ca}$  currents (8–14 channel openings) and large transient  $K_{Ca}$  currents (15–30 channel openings). As can be seen in the table, the distribution is skewed toward smaller transient  $K_{Ca}$  currents, then medium sized transient  $K_{Ca}$  currents, and fewer large transient  $K_{Ca}$  currents. Glutamate increased the number of small (1.6 fold), medium (1.7 fold), and large (3.1 fold) transient outward currents in smooth muscle cells in contact with astrocytes, but did not change the number of outward currents of any amplitude in smooth muscle cells that were alone.

To investigate  $K_{Ca}$  channel regulation by glutamate, transient  $K_{Ca}$  currents were inhibited by depleting SR  $\text{Ca}^{2+}$  with thapsigargin ( $100\text{nmol/L}$ ), a SR  $\text{Ca}^{2+}$  ATPase inhibitor that blocks  $\text{Ca}^{2+}$  sparks<sup>30</sup>. Glutamate increased ( $\sim 65\%$ ) single  $K_{Ca}$  channel activity when smooth muscle cells were in contact with astrocytes (Fig.2). In contrast, glutamate did not change  $K_{Ca}$  channel activity in smooth muscle cells without astrocytes.

To investigate the contribution of astrocyte HO to the responses, astrocytes were pretreated with the HO inhibitor CrMP ( $20\mu\text{mol/L}$ , 1h). Coverslips with CrMP-treated astrocytes were rinsed and then added to the recording chamber to avoid exposure of smooth muscle cells to the HO inhibitor. Cerebral arteriole smooth muscle cells were allowed to settle on CrMP-treated astrocytes. In contrast to the effect of glutamate on vascular smooth muscle cells in contact with untreated astrocytes, where transient  $K_{Ca}$  current frequency increased  $\sim 75\%$  and single  $K_{Ca}$  channel open probability increased  $\sim 65\%$ , glutamate changed neither transient  $K_{Ca}$  current frequency nor  $K_{Ca}$  channel activity of smooth muscle cells in contact with CrMP-pretreated astrocytes (Fig. 3).

As an alternative approach for depression of astrocyte CO production, as well as for investigation of the HO subtype involved in glutamate-induced CO production, we used HO-2 knockout mice. Wild type (HO-2<sup>+/+</sup>) homogenates showed strong expression of HO-2, while there was no detectable HO-2 protein expression in HO-2 knockout (HO-2<sup>-/-</sup>) mice (Fig.4A). Glutamate more than doubled CO production by HO-2<sup>+/+</sup> astrocytes (Fig. 4B). In contrast, glutamate had no effect on CO production by HO-2<sup>-/-</sup> astrocytes (Fig.4B).

To examine the effect of HO-2 expression on the ability of astrocytes to activate K<sub>Ca</sub> channels in cerebral arterial smooth muscle cells, piglet cerebral arteriole smooth muscle cells were allowed to settle on either HO-2<sup>+/+</sup> or HO-2<sup>-/-</sup> mouse astrocytes. Transient K<sub>Ca</sub> current frequency and K<sub>Ca</sub> channel activity were recorded. As was the case when using piglet astrocytes, glutamate elevated transient K<sub>Ca</sub> current frequency (2.3 fold) as well as K<sub>Ca</sub> channel activity (2 fold) in piglet smooth muscle cells in contact with HO-2<sup>+/+</sup> mouse astrocytes (Fig. 5). In contrast, glutamate did not alter transient K<sub>Ca</sub> current frequency or K<sub>Ca</sub> channel activity in piglet smooth muscle cells in contact with HO-2<sup>-/-</sup> astrocytes (Fig. 5).

We used newborn pig cerebral cortex slices to examine contributions of HO and astrocytes to glutamate regulation of arteriolar [Ca<sup>2+</sup>]<sub>i</sub> and diameter in structurally intact brain. Glutamate (30μmol/L) decreased the fura-2 ratio (340/380 nm) in arterioles, indicating a decrease of [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 6). Pretreatment of slices with CrMP markedly inhibited the effect of glutamate on [Ca<sup>2+</sup>]<sub>i</sub>. In addition, glutamate dilated piglet neocortical slice arterioles (from 111±10 to 123±11 μm) (Fig. 7). In contrast, glutamate did not alter the diameters of arterioles in brain slices pretreated with CrMP, suggesting CO generated by HO is required for glutamate-induced vasodilation. CrMP-treated slice arterioles constricted normally to the thromboxane receptor agonist U46619 (data not shown). Paxilline, a selective K<sub>Ca</sub> channel blocker<sup>35</sup>, reversed glutamate-induced dilation, suggesting the dilation was mediated by K<sub>Ca</sub> channel activation. Also, following treatment of brain slices with L-AAA (2mmol/L, 2h), a selective astrocyte toxin, arterioles did not dilate to glutamate (Fig. 7D). In contrast, arterioles within slices incubated with the inactive isomer, D-AAA, dilated similarly to glutamate as did arterioles in untreated slices. L-AAA did not depress overall arteriolar reactivity as indicated by similar dilations to sodium nitroprusside of arterioles in L-AAA (12%) and D-AAA (15%) treated slices. These data show that astrocytes, HO-2, and K<sub>Ca</sub> channels are required for glutamate-induced cerebral arteriolar dilation in newborn pigs.

## DISCUSSION

Major findings of the present study are that: 1) Glutamate activates transient K<sub>Ca</sub> currents and K<sub>Ca</sub> channel activity in cerebral arteriolar smooth muscle cells that are in contact with astrocytes but not in smooth muscle cells alone; 2) Glutamate increases CO production by intact astrocytes but not by genetically HO-2 deficient astrocytes; 3) Pharmacological inhibition of HO or genetic ablation of astrocyte HO-2 eliminates glutamate-induced K<sub>Ca</sub> channel activation in cerebral arteriolar smooth muscle cells in contact with these astrocytes; 4) Glutamate decreases brain slice arteriolar smooth muscle [Ca<sup>2+</sup>]<sub>i</sub> through a mechanism that is attenuated by pharmacological inhibition of HO, and 5) Glutamate-induced dilation of piglet neocortical brain slice arterioles is blocked by either HO inhibition, K<sub>Ca</sub> channel inhibition, or astrocyte injury.

Between the present and a recent study<sup>9</sup>, we use 3 approaches, isolated cells, brain slices, and cranial windows *in vivo*. Each has different strengths and weaknesses and the combination is mutually complimentary. Brain slices have no descending pressure or blood flow. But slices allow arteriole wall [Ca<sup>2+</sup>]<sub>i</sub> measurements and remove both neuronal influences from beyond the study area and blood borne mediators. The results from slices

mirror the *in vivo* experiments<sup>9</sup> that show glutamate causes arteriolar dilation and brain CO production<sup>9</sup> which are inhibited by astrocyte injury. *In vivo*, arterioles have normal pressure and flow and CO production can be measured, but specific cell types involved in communication or CO production, channels involved, and  $[Ca^{2+}]_i$  measurements are not possible. Isolated cells allow specific communication between myocytes and astrocytes to be investigated, inhibitor treatment of only astrocytes, using HO-2 knockout astrocytes with wild type arteriolar smooth muscle, and measurement of CO production specifically by astrocytes. Thus, the combined 3 approaches lead to a more complete picture of the functional control mechanisms.

CO is produced physiologically by catabolism of heme to CO, iron, and biliverdin<sup>36</sup>. HO catalyzes this reaction with oxidation of NADPH. HO is expressed as three known isoforms: HO-1, HO-2 and a third isoform (HO-3) with much lower heme degrading activity and expression<sup>37</sup>. The present study shows that glutamate increases CO production by HO-2<sup>+/+</sup> mouse astrocytes but not HO-2<sup>-/-</sup> astrocytes that do not express HO-2. CO reduces vascular smooth muscle tone both *in vitro*<sup>38,39</sup> and *in vivo*<sup>27, 40</sup>. Indications of the functional significance of CO are supported by alterations of responses to important physiological cerebrovascular regulatory stimuli upon HO inhibition. In the newborn pig *in vivo*, pial arteriolar dilations to glutamate, hypotension, and hypoxia are inhibited following topical treatment with CrMP that blocks CO production<sup>26, 41</sup>.

While CO has been demonstrated to increase transient  $K_{Ca}$  currents when applied directly to isolated vascular smooth muscle cells or arterioles, the present study is the first to show that a HO-2 product produced by another cell, an astrocyte, can activate transient  $K_{Ca}$  currents in an associated arteriolar smooth muscle cell. Because HO products other than CO do not activate  $K_{Ca}$  channels<sup>28</sup>, the HO-2 product activating the  $K_{Ca}$  channel must be CO. Of additional significance, the signal, glutamate, is a primary mediator of brain function that does not directly affect  $K_{Ca}$  currents in cerebrovascular myocytes (Fig 1). The signal from astrocytes is necessary to provide the functional hyperemic response.

Our studies using cranial windows *in vivo*, found that either astrocyte injury or inhibition of CO production blocks dilation of newborn pig pial arterioles to topical glutamate application<sup>9, 26</sup>. In the present study, we show that in the absence of astrocytes or with astrocytes pretreated with CrMP, glutamate does not increase either smooth muscle cell transient  $K_{Ca}$  currents or  $K_{Ca}$  channel activity. To confirm that HO-2 is involved in this signaling pathway, piglet smooth muscle cells were allowed to contact mouse HO-2<sup>+/+</sup> and HO-2<sup>-/-</sup> astrocytes. Glutamate increased transient  $K_{Ca}$  currents as well as  $K_{Ca}$  channel activity of smooth muscle cells attached to HO-2<sup>+/+</sup> astrocytes, but not those attached to HO-2<sup>-/-</sup> astrocytes. We show that glutamate-induced dilation of brain slice arterioles is abrogated by either blocking the enzyme that produces CO or by blocking the  $K_{Ca}$  channel that CO activates. Further, HO inhibition prevented glutamate-induced reduction of  $[Ca^{2+}]_i$  in brain slice arterioles. All these data are consistent with the hypothesis that glutamate stimulates astrocyte HO-2, producing the diffusible gasotransmitter, CO, which activates  $K_{Ca}$  channels in adjacent cerebral microvascular smooth muscle cells.

In contrast to rat hypothalamus<sup>42</sup> and newborn pig cerebral cortex<sup>26</sup>, the sole reported influence of the HO/CO system on vascular tone in adult rat cerebral cortex has been constrictor<sup>43</sup>. Endogenous CO appears to have a tonic inhibitory effect on NO production in rat cerebrum because the HO inhibitor, zinc protoporphyrin (ZnPP) dilated pial arterioles over a 1h period and enhanced cerebral NO production as estimated using diaminofluorescein-2<sup>43</sup>. The dilation was blocked by L-NAME and by addition of CO to the superfusion solution. Conceivably, contributions of CO to cerebrovascular regulation could be biphasic with basal production providing tonic inhibition of NOS with resultant increased

tone, while acute CO elevations in response to stimulation lead to dilation. Also, available data on responses of isolated adult cerebral arteries to CO suggest that species differences exist. Dilation to exogenous CO added to the bathing solution has not been detected in rabbit, rat, or mouse cerebral arteries *in vitro*<sup>44, 45</sup>. On the other hand, CO dilates dog basilar artery segments<sup>46</sup>. In addition, in pigs, responses of pial arterioles to CO are age-dependent with juvenile pigs, and adult rats, having reduced dilations compared to baby pigs<sup>47</sup>.

CO causes dilation by activating smooth muscle cell  $K_{Ca}$  channels<sup>26,29, 48</sup>. Absence of dilation of rat cerebral arteries and arterioles to CO *in vitro* (above) is surprising because CO dilates peripheral rat arterioles via  $K_{Ca}$  channel activation<sup>49,50</sup>. In vascular smooth muscle cells, several  $K_{Ca}$  channels are activated by spatially localized intracellular  $Ca^{2+}$  transients, termed  $Ca^{2+}$  sparks, that elevate the local  $[Ca^{2+}]_i$  into the micromolar range<sup>51</sup>. Transient  $K_{Ca}$  currents induce membrane hyperpolarization that reduces voltage-dependent  $Ca^{2+}$  channel activity, and thus decreases global  $[Ca^{2+}]_i$ , producing dilation. CO increases transient  $K_{Ca}$  currents by enhancing the coupling of  $Ca^{2+}$  sparks to  $K_{Ca}$  channels<sup>28</sup>. The  $K_{Ca}$  channel  $\alpha$ -subunit contains a heme-binding pocket and binding of heme inhibits  $K_{Ca}$  channel activity<sup>52</sup>. CO, by binding to channel-bound heme, changes the association of heme with the  $K_{Ca}$  channel and causes activation<sup>48</sup>. Therefore, the  $K_{Ca}$  channel is functionally a heme-protein, with heme acting as the binding site for CO<sup>48</sup>.

Species and/or age differences exist in  $Ca^{2+}$  spark-to- $K_{Ca}$  channel coupling efficiency that could influence arteriole smooth muscle sensitivity to CO and possibly even the role of CO in regulation of cerebrovascular circulation. In adult rat cerebrovascular smooth muscle cells, virtually all  $Ca^{2+}$  sparks produce transient  $K_{Ca}$  currents<sup>53, 54</sup>. Conversely, not all  $Ca^{2+}$  sparks produce transient  $K_{Ca}$  currents in either newborn pig<sup>28, 55</sup> or adult human<sup>56</sup> cerebral arterioles. Therefore, the ability of CO to hyperpolarize and dilate rat cerebral arterioles may be less<sup>44</sup> than in newborn pig cerebral arterioles because CO increases the coupling of  $Ca^{2+}$  sparks to  $K_{Ca}$  channels and rat  $Ca^{2+}$  spark-to- $K_{Ca}$  channel coupling is essentially unitary at physiological membrane potentials.

In the present study, rather than report transient  $K_{Ca}$  current amplitude as the mean of all transient  $K_{Ca}$  currents, we divided transient  $K_{Ca}$  currents into smaller (7–20 pA), larger (21–40 pA), and largest (>40 pA) currents. As can be seen in Table 1, transient  $K_{Ca}$  current frequency in all size categories increased upon treatment with glutamate. In piglets, smaller amplitude  $Ca^{2+}$  sparks do not cause transient  $K_{Ca}$  currents<sup>28,34</sup>. CO increases the number of smaller amplitude transient  $K_{Ca}$  currents because smaller, uncoupled  $Ca^{2+}$  sparks become coupled to  $K_{Ca}$  channels<sup>28</sup>. The addition of these smaller currents depresses the overall mean, which explains the minimal change in mean transient  $K_{Ca}$  current amplitude, even though large amplitude transient  $K_{Ca}$  currents are markedly increased. An accurate depiction of the effect of glutamate on transient  $K_{Ca}$  current amplitude requires accounting for the increased number of smaller currents. Glutamate increases frequencies of transient  $K_{Ca}$  currents across the amplitude spectrum because glutamate uses CO to increase  $Ca^{2+}$  spark-to- $K_{Ca}$  channel coupling.

The present study shows glutamate increases CO production by astrocytes, but coupling mechanisms are not known. Stimulation of mGluR and iGluR increase  $Ca^{2+}$  oscillations in astrocytes (20–24), electrical field stimulation increases brain slice astrocyte endfoot  $[Ca^{2+}]_i$  (14,22), and inhibition of calmodulin blocks glutamate stimulation of CO production in cerebral microvessels and cortical neurons (57,58). Therefore, it is reasonable to speculate that glutamate stimulates CO production in astrocytes via elevation of astrocytic  $[Ca^{2+}]_i$ . Increasing  $[Ca^{2+}]_i$  could increase HO-2 activity directly to elevate CO production or by a variety of indirect actions including delivery of electrons from NADPH via cytochrome P450 reductase, delivery of heme to HO-2, or activation of kinases that elevate HO-2

activity. Glutamate also could increase HO-2 activity via  $\text{Ca}^{2+}$  independent kinase pathways. In endothelium, vascular smooth muscle, and neurons HO-2 activity can be increased by phosphorylation (31, 58). mGluR elevates diacylglycerol that stimulates protein kinase C, which could phosphorylate and activate HO-2.

In summary, the present study indicates that the neurotransmitter glutamate activates transient  $\text{K}_{\text{Ca}}$  currents and  $\text{K}_{\text{Ca}}$  channel activity in newborn pig cerebral arteriole smooth muscle cells by increasing CO production by associated astrocytes. Either inhibition of CO production or treatment with astrocyte toxin prevents glutamate-induced arteriolar dilation in piglet brain slices. These data support the conclusion that astrocytes employ CO as a diffusible messenger to cause cerebral arteriole dilation and enhance local blood flow to match increased glutamatergic neuronal activity.

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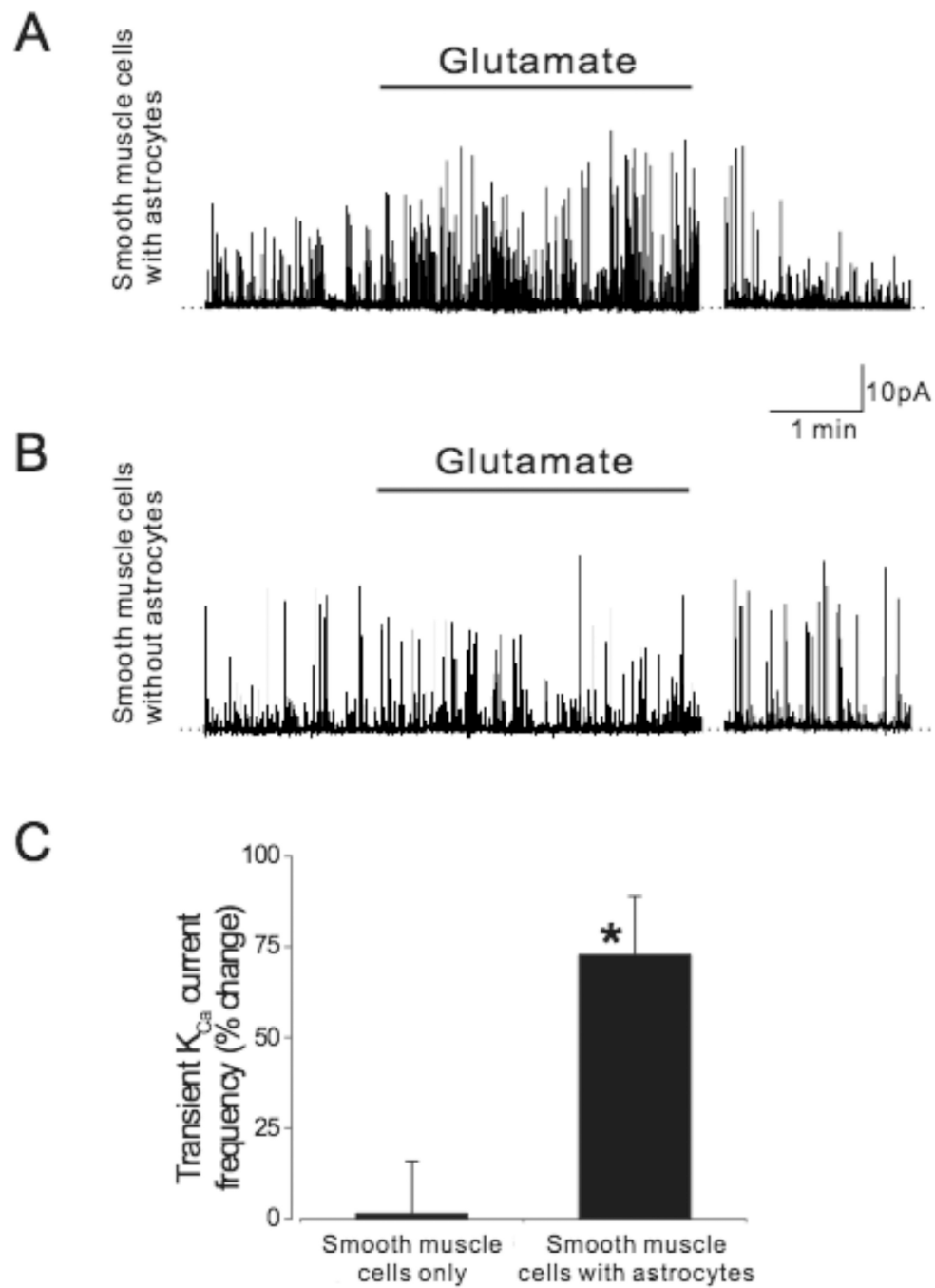
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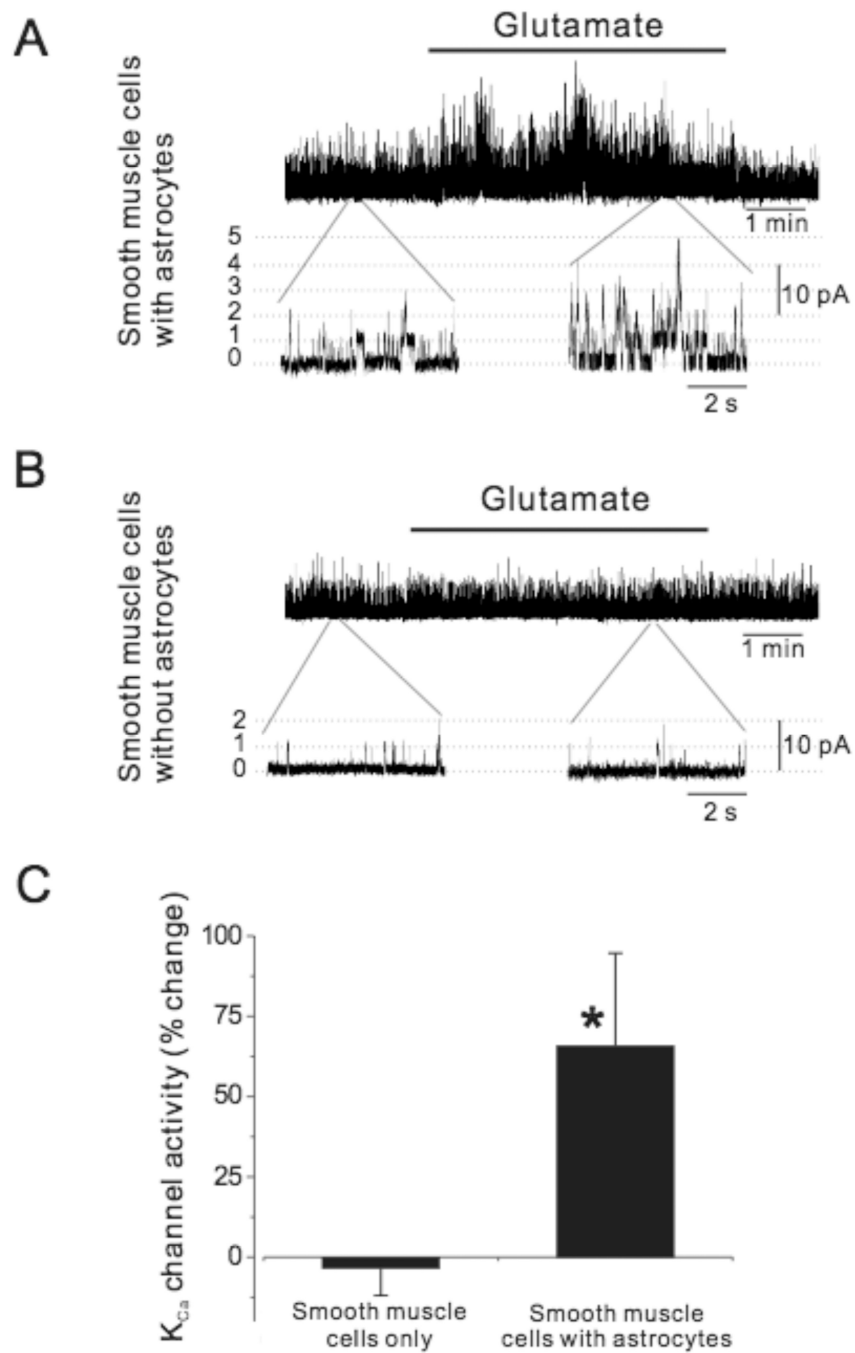
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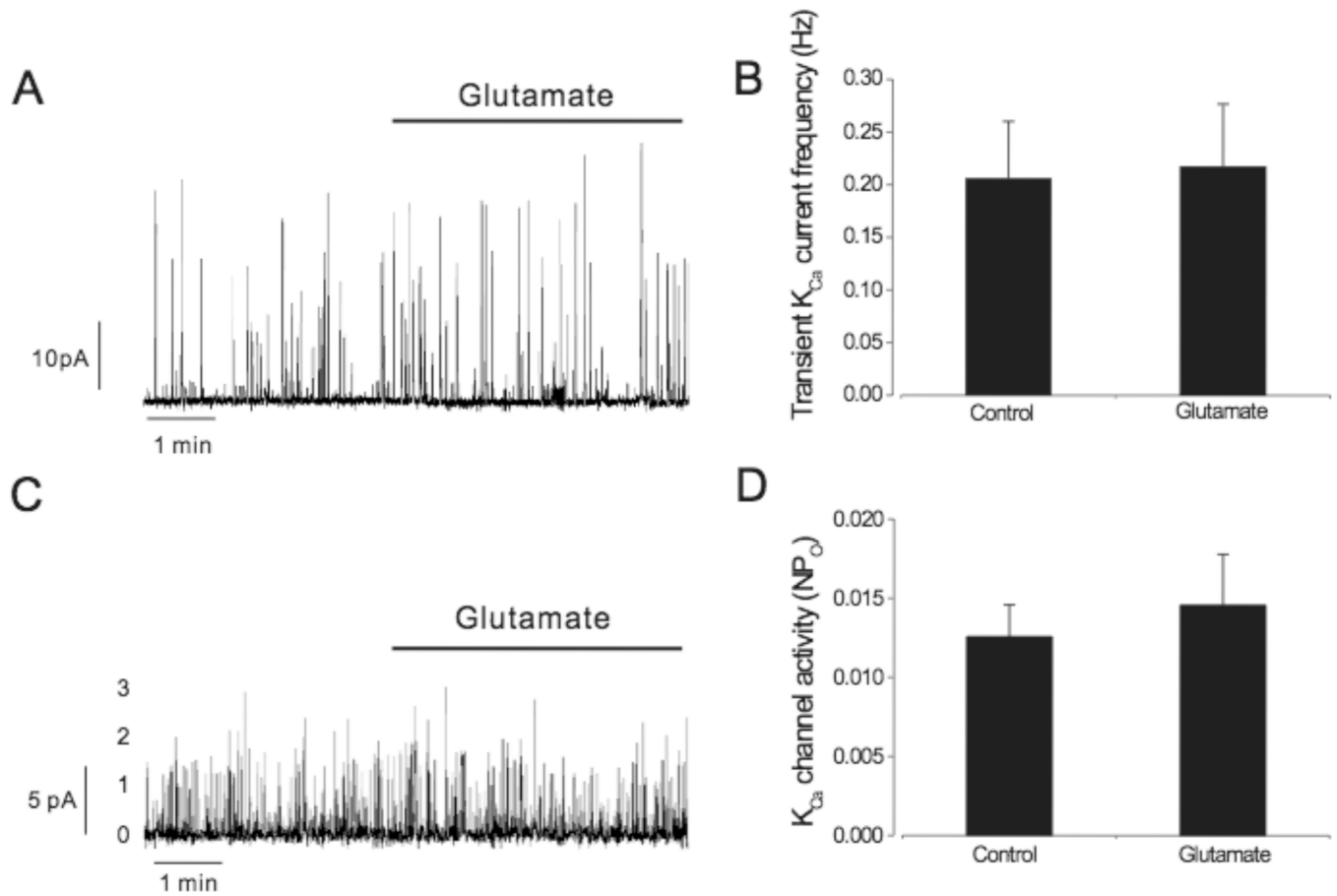
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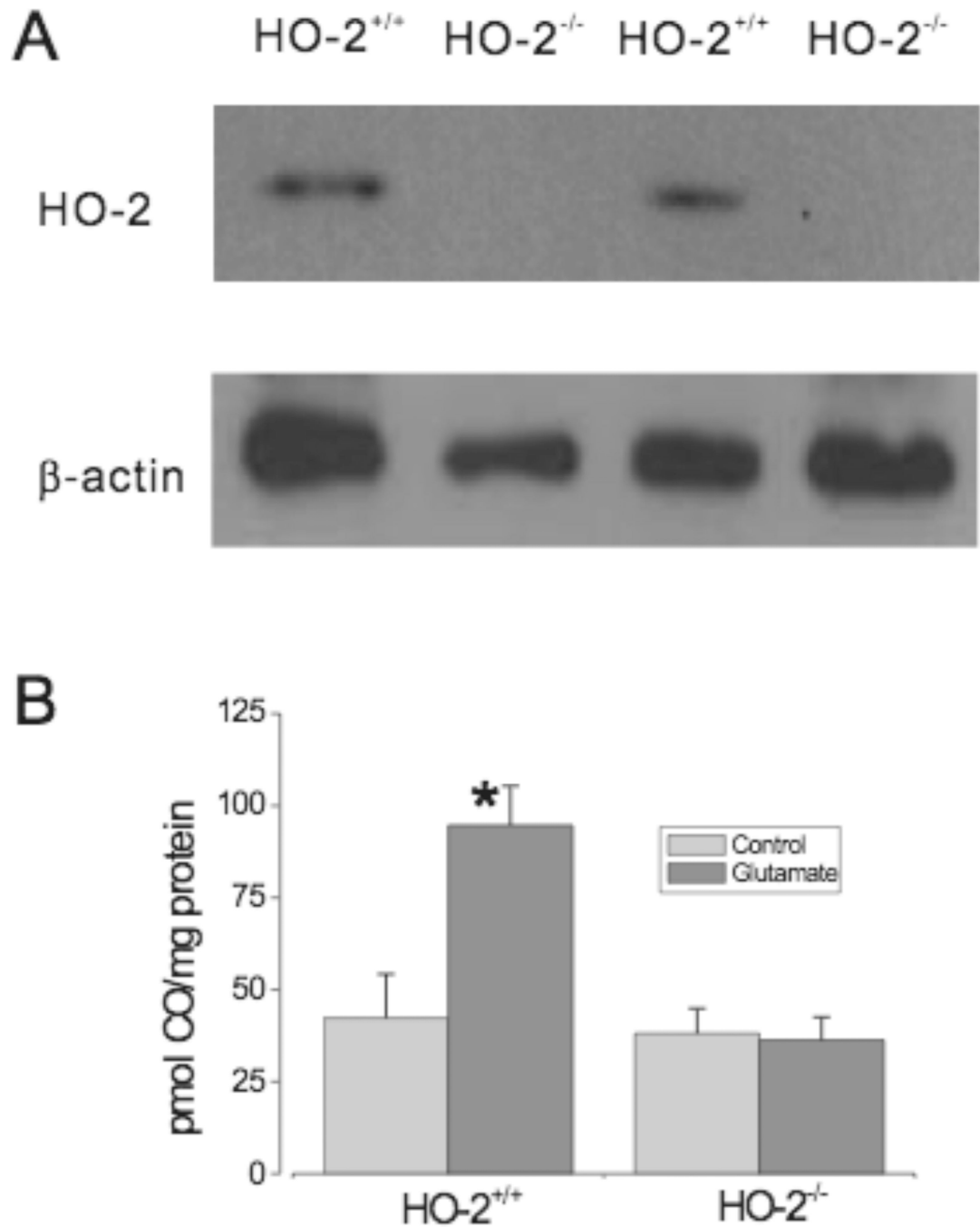
**Fig. 1.** Glutamate (20 $\mu$ mol/L) elevates transient  $K_{Ca}$  current frequency in cerebrovascular smooth muscle cells in contact with astrocytes (A) but not in cerebrovascular smooth muscle cells alone (B). Summarized data are shown in C. n=7 and n=12 for smooth muscle cells alone and with astrocytes, respectively. \*P<0.05 compared to zero.



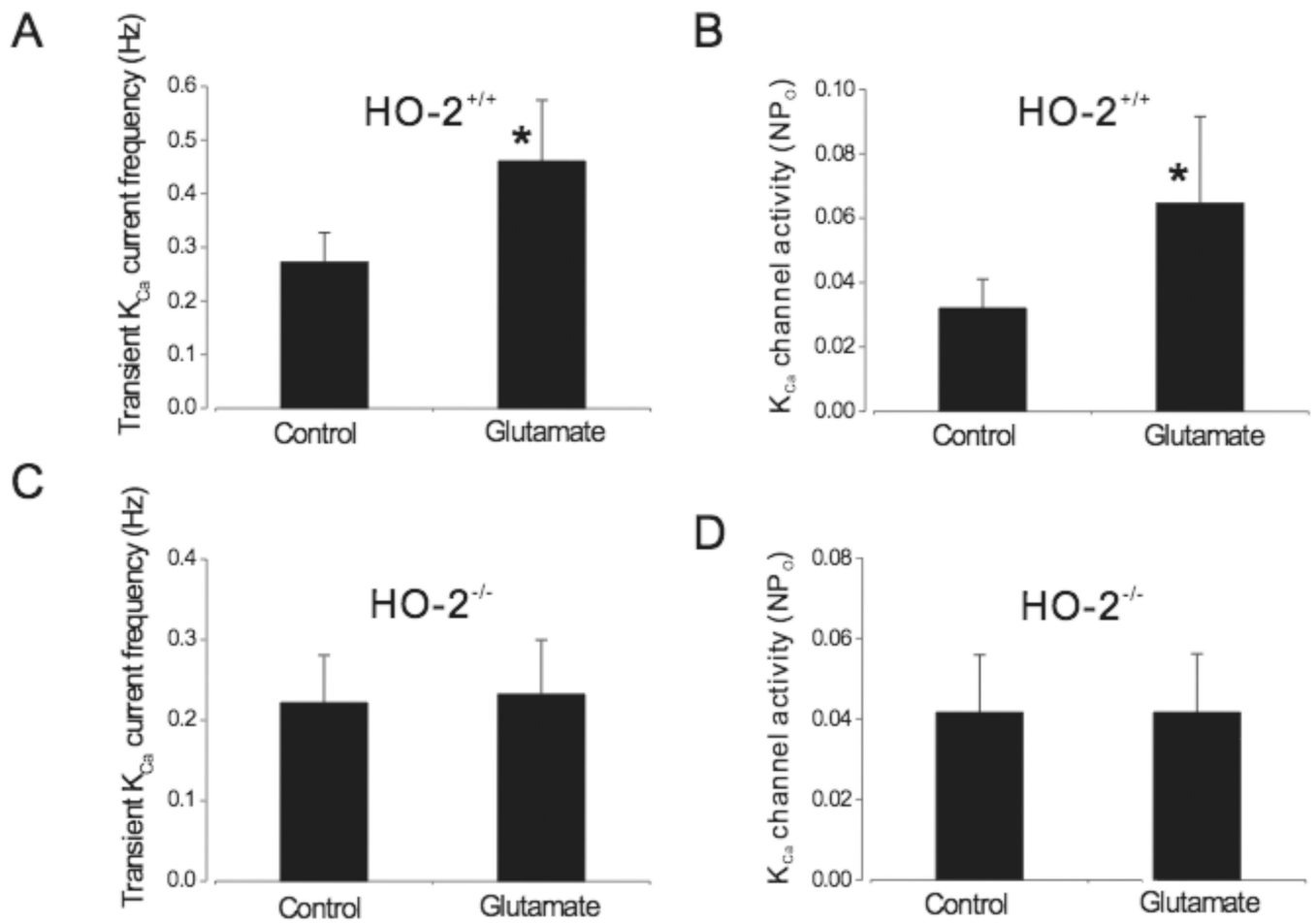
**Fig. 2.** Glutamate (20  $\mu$ mol/L) activates  $K_{Ca}$  channels in cerebrovascular smooth muscle cells in contact with astrocytes (A) but not in cerebrovascular smooth muscle cells alone (B). Summarized data are shown in C.  $n=6$  and  $n=13$  for smooth muscle cells alone and with astrocytes, respectively. \* $P < 0.05$  compared to zero.



**Fig. 3.** Chromium mesoporphyrin ( $20\mu\text{mol/l}$ )(CrMP) pretreatment of astrocytes blocks glutamate ( $20\mu\text{mol/L}$ ) activation of transient  $K_{Ca}$  current frequency (A&B,  $n=8$ ) and  $K_{Ca}$  channel activity (C&D,  $n=6$ ) in smooth muscle cells in contact with astrocytes.

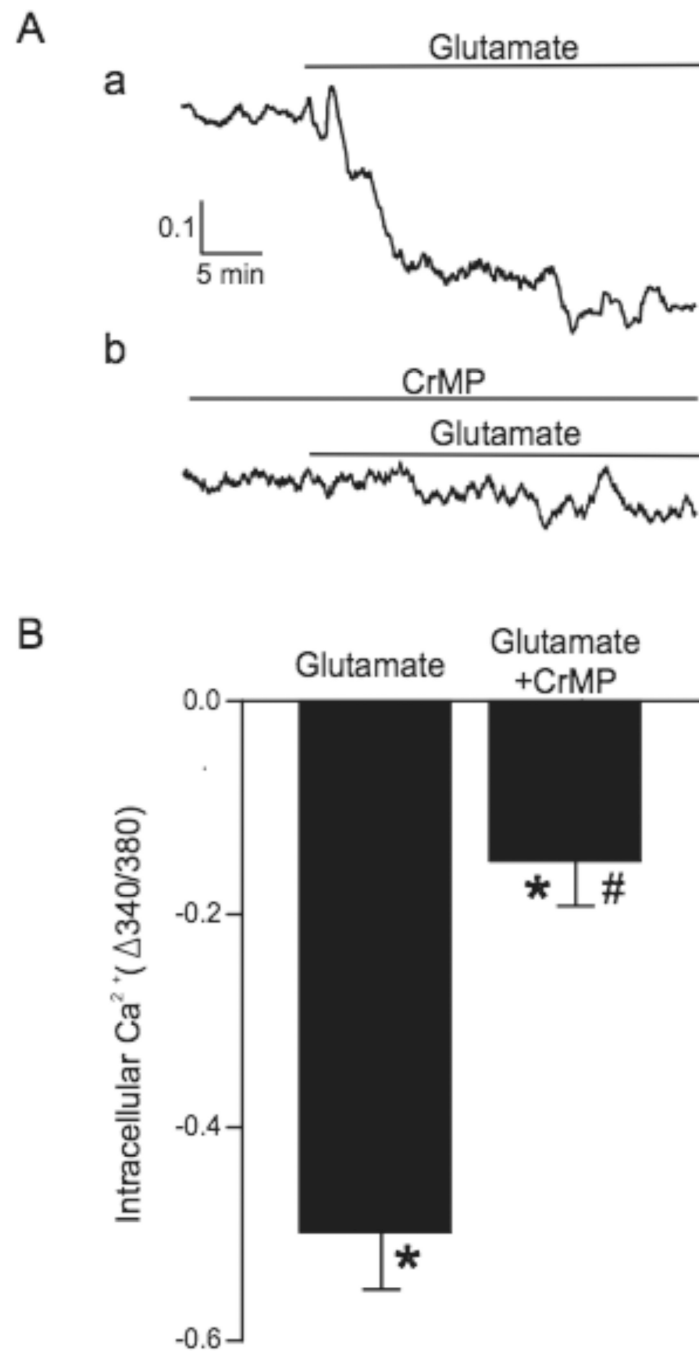


**Fig. 4.** A: HO-2 protein expression in HO-2<sup>+/+</sup> and HO-2<sup>-/-</sup> mice. B: Glutamate (20 $\mu$ mol/L) stimulates CO production by HO-2<sup>+/+</sup> mouse astrocytes but not by HO-2<sup>-/-</sup> mouse astrocytes (n=3 separate mouse brain preparations each for HO-2<sup>+/+</sup> and HO-2<sup>-/-</sup> with 5 or 6 tubes for each preparation). \*P<0.05 compared to basal production.

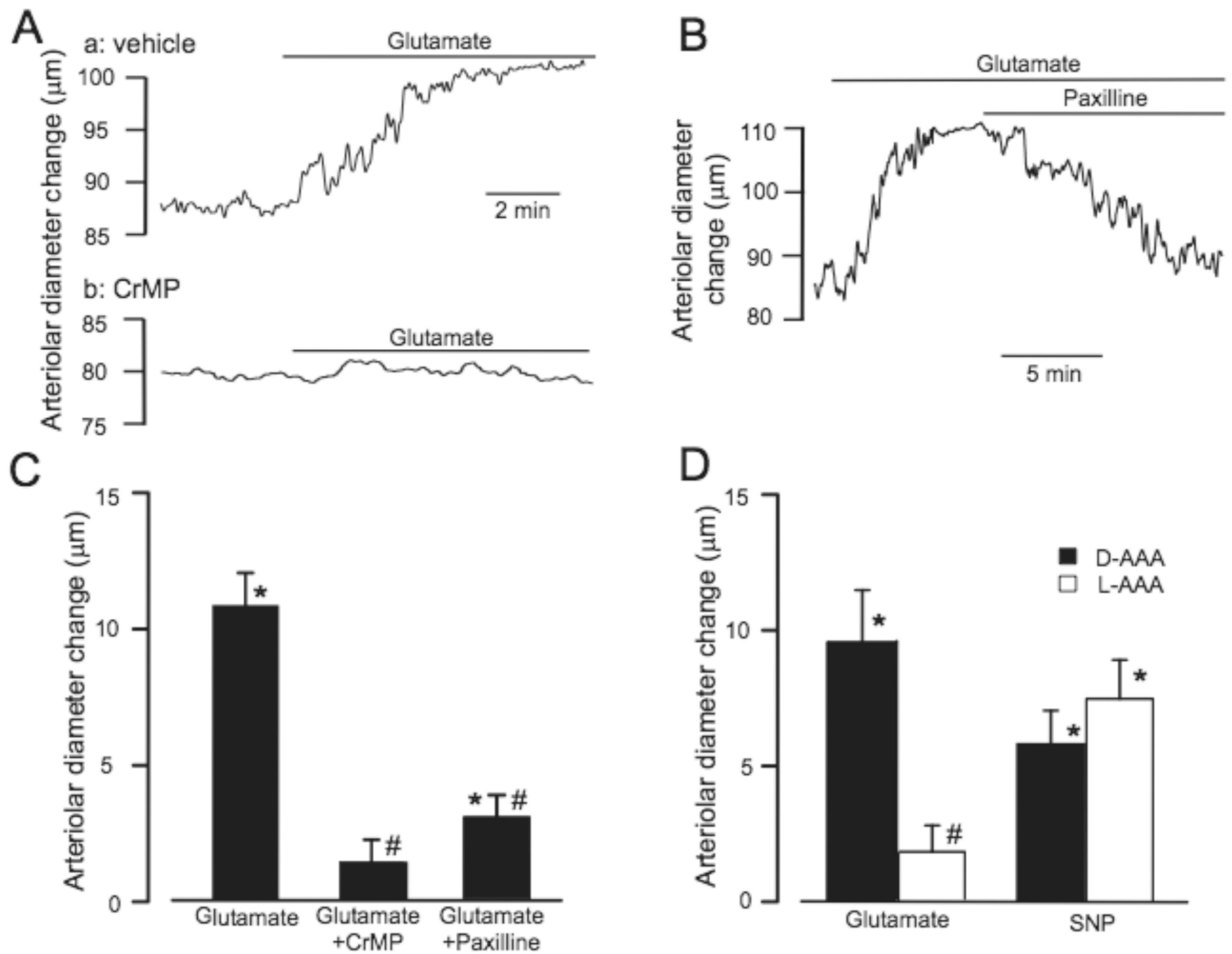


**Fig. 5.** Glutamate activates transient  $K_{Ca}$  current frequencies and single channel activity in piglet cerebral microvascular smooth muscle cells in contact with mouse HO-2<sup>+/+</sup> (A& B, n=8) but not HO-2<sup>-/-</sup> (C& D, n=6) astrocytes. \* $P < 0.05$  compared to without glutamate.





**Fig. 6.** Glutamate (30  $\mu\text{mol/l}$ ) decreases intracellular  $\text{Ca}^{2+}$  in brain slice arteriole smooth muscle and this effect is blocked by chromium mesoporphyrin (CrMP, 20  $\mu\text{mol/l}$ ). A. Representative traces of fura-2 fluorescence ratio in an arteriole of an untreated brain slice and a slice treated with CrMP. B. Collated data of brain slice arteriole responses to glutamate without and with CrMP (n=13 and 11, respectively). \*P<0.05 compared to diameter prior to glutamate. # P<0.05 compared to without CrMP.



**Fig. 7.** Chromium mesoporphyrin (CrMP, 20  $\mu\text{mol/l}$ ), paxilline (20  $\mu\text{mol/l}$ ), and L-2-alpha amino adipic acid (L-AAA, 2 mmol/l) block glutamate (30  $\mu\text{mol/L}$ )-induced dilation of piglet cerebral cortical brain slice arterioles. **A.** Representative trace illustrating the effect of glutamate on the diameter of an arteriole in an untreated slice (a) and a slice treated with CrMP (b). **B.** Representative trace of glutamate-induced arteriolar dilation and the effect of paxilline on that dilation. **C.** Collated data of brain slice arteriole responses to glutamate without and with CrMP or paxilline (n=26, 11, and 11, respectively). **D.** Brain slice arteriolar diameter responses to glutamate and sodium nitroprusside (SNP, 140  $\mu\text{mol/L}$ ) following treatment with D-AAA (n=10) or L-AAA (n=9). \*P<0.05 compared to control diameter. # P<0.05 compared to without CrMP, paxilline, or L-AAA, as appropriate.

**Table 1**Effect of glutamate on piglet cerebral arteriole smooth muscle cell (SMC) transient  $K_{Ca}$  currents.

Amplitude range	SMC with astrocytes		SMC alone	
	Control	Glutamate	Control	Glutamate
7–20pA	70±4	110±6 <sup>*</sup>	61±6	67±4
21–40pA	41±3	67±3 <sup>*</sup>	45±3	43±5
>40pA	6±1	19±2 <sup>*</sup>	4±1	3±1

Mean±SEM currents per cell in 10 min. N=7 cells with and without astrocytes.

<sup>\*</sup>P<0.05 compared to control.