Role of cAMP and K\(^+\) channel-dependent mechanisms in piglet hypoxic/ischemic impaired nociceptin/orphanin FQ-induced cerebrovasodilation

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Abstract

This study was designed to determine the role of altered cAMP and K\(^+\) channel-dependent mechanisms in impaired pial artery dilation to the newly described opioid, nociceptin/orphanin FQ (NOC/oFQ) following hypoxia/ischemia in newborn pigs equipped with a closed cranial window. Recent studies have observed that NOC/oFQ elicits pial dilation via release of cAMP, which, in turn, activates the calcium sensitive (K\(_a\)) and the ATP-dependent K\(^+\) (K\(_{\text{ATP}}\)) channel. Global cerebral ischemia (20 min) was induced via elevation of intracranial pressure, while hypoxia (10 min) decreased pO\(_2\) to 35±3 mmHg with unchanged pCO\(_2\). Topical NOC/oFQ (10\(^{-8}\), 10\(^{-6}\) M) induced vasodilation was attenuated by ischemia/reperfusion (I/R) and reversed to vasoconstriction by hypoxia/ischemia/reperfusion (H/I/R) at 1 h of reperfusion (control, 9±1 and 16±1%; I/R, 3±1 and 6±1%; H/I/R, -7±1 and -12±1%). Such altered dilation returned to control values within 4 h in I/R animals and within 12 h in H/I/R animals. NOC/oFQ dilation was associated with elevated CSF cAMP in control animals but such biochemical changes were attenuated in I/R animals and reversed to decreases in cAMP concentration in H/I/R animals (control, 1037±58 and 1919±209 fmol/ml; I/R, 1068±33 and 1289±30 fmol/ml; H/I/R, 976±36 and 772±27 fmol/ml for absence and presence of NOC/oFQ 10\(^{-6}\) M, respectively). Topical 8-Bromo cAMP (10\(^{-8}\), 10\(^{-6}\) M) pial dilation was unchanged by I/R but blunted by H/I/R (control, 10±1 and 20±1%; I/R, 11±1 and 20±2%; H/I/R, 0±1 and 0±2%). Pituitary adenylate cyclase activating polypeptide and cromakalim, adenylate cyclase and K channel activators, respectively, ATP elicited dilation that was blunted by both I/R and H/I/R while NS1619, a K\(_a\) channel activator, elicited dilation that was unchanged by I/R but blunted by H/I/R. These data indicate that impaired NOC/oFQ dilation following I/R results from altered adenylate cyclase and K\(_{\text{ATP}}\) channel-dependent mechanisms. These data further indicate that impaired NOC/oFQ dilation following H/I/R results not only from altered adenylate cyclase and K\(_{\text{ATP}}\) channel but also from altered cAMP and K\(_a\) channel-dependent mechanisms. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Episodes of inadequate oxygen supply to the brain can result in significant neurological sequelae. Babies are frequently exposed to hypoxic/ischemic insults during the perinatal period. One contributor to neurological damage is thought to be cerebrovascular dysfunction. Global cerebral ischemia results in reductions in pial artery diameter and cerebral blood flow as well as impaired cerebrovascular control during hypotension and hypercapnia in a newborn pig model [17–19]. Less, however, is known about the cerebrovascular consequences of combined hypoxia/ischemia. The membrane potential of vascular smooth muscle is a major determinant of vascular tone and activity of potassium (K\(^+\)) channels is a major regulator of membrane potential [23]. Activation or opening of these channels increases potassium efflux, thereby producing hyperpolarization...
zation of vascular muscle. Membrane hyperpolarization closes voltage-dependent calcium channels, causing relaxation of vascular muscle [22]. Several types of K⁺ channels, including ATP-sensitive (K_{ATP}), calcium-sensitive (K_{Ca}), delayed rectifier and inward rectifier K⁺ channels have been identified. Pharmacological studies using activators and inhibitors have additionally provided functional evidence that K⁺ channels, especially K_{ATP} and K_{Ca} channels, regulate tone of cerebral blood vessels in vitro and in vivo [10,16,22,23]. In the piglet, cAMP elicits pial artery dilation predominantly via activation of the K_{Ca} channel with a more minor K_{ATP} channel contributing component [3,4].

Opioids have been observed to be important in the control of the cerebral circulation of the piglet during physiological and pathological conditions [5]. During the last 5 years, several groups have isolated and cloned a new G-protein-coupled receptor that showed high homology with opioid receptors. This opioid-like receptor, however, displayed no affinity for opioid ligands and remained an ‘orphan’ until late 1995 [15]. At that time, two independent groups [21,24] identified a 17-amino acid peptide that did not bind to the classical opioid receptors (μ, δ, κ) but activated the orphan receptor in a nanomolar concentration range and could therefore be considered the endogenous ligand for the orphan receptor. This peptide was named orphanin FQ by Reinscheid et al. [24] because its sequence begins with phenylalanine (F) and ends with a glutamine (Q). The same peptide was called nociceptin by Meunier et al. [21] because it increased the reactivity to pain in animals in contrast with the analgesic effects of opioid drugs. The orphan receptor therefore will be referred to as ORL-1 (for opioid receptor-like 1) and its endogenous ligand, NOC/oFQ (for nociceptin/orphanin FQ). In situ hybridization studies have demonstrated localization of ORL-1 in several regions of the central nervous system including the cerebral cortex, thalamus, and hypothalamus [11–13]. A similar distribution has been observed for NOC/oFQ. It has therefore been suggested that this opioid system may play a role in memory, nociception, learning, and emotion [20]. Recently, NOC/oFQ has been observed to elicit pial artery vasodilation in the newborn pig [2]. The mechanism for such pial dilation involved the release of cAMP, and subsequent activation of the K_{ATP} and K_{Ca} channel [2]. However, little is known about the role of NOC/oFQ in the physiological or pathophysiological control of cerebral hemodynamics.

CSF NOC/oFQ concentration has been recently observed to increase following I+R in the piglet [1]. Additionally, NOC/oFQ-induced pial artery vasodilation is attenuated following I+R and reversed to vasoconstriction following combined hypoxia/ischemia/reperfusion (H+I+R) [1]. Such impaired NOC/oFQ-mediated pial dilation appears to contribute to reduced cerebral blood flow and pial artery vasoconstriction observed following hypoxia/ischemia [1]. However, the mechanism for impaired NOC/oFQ-induced pial dilation following hypoxia/ischemia is unknown. Interestingly, it has been observed that K_{ATP} channel function is impaired following global cerebral ischemia in the piglet [9]. Such studies did not observe any effect of that insult on K_{Ca} or cAMP agonist mediated dilation [8]. However, those studies did not investigate the effects of combined hypoxia/ischemia on cAMP, K_{ATP} and K_{Ca} channel function.

Therefore, the present study was designed to determine the role of altered cAMP and K⁺ channel-dependent mechanisms in impaired pial artery dilation to NOC/oFQ observed following hypoxia/ischemia.

2. Methods

Newborn (1–5-day-old, 1.3–2.1 kg) pigs of either sex were used in these experiments. All protocols were approved by the Institutional Animal Care and Use Committee. Piglets were initially anesthetized with isoflurane (1–2 MAC). Anesthesia was maintained with α-chloralose (30–50 mg/kg, supplemented with 5 mg/kg per h i.v.). A catheter was inserted into a femoral artery to monitor blood pressure and to sample for blood gas tensions and pH. Drugs to maintain anesthesia were administered through a second catheter placed in a femoral vein. The trachea was cannulated, and the animals were mechanically ventilated with room air. A heating pad was used to maintain the animals at 37–39°C.

A cranial window was placed in the parietal skull of these anesthetized animals. This window consisted of three parts: a stainless steel ring, a circular glass coverslip, and three ports consisting of 17-gauge hypodermic needles attached to three precut holes in the stainless steel ring. For placement, the dura was cut and retracted over the cut bone edge. The cranial window was placed in the opening and cemented in place with dental acrylic. The volume under the window was filled with a solution, similar to CSF, of the following composition (in mM): 3.0 KCl, 1.5 MgCl₂, 1.5 CaCl₂, 132 NaCl, 6.6 urea, 3.7 dextrose, and 24.6 NaHCO₃. This artificial CSF was warmed to 37°C and had the following chemistry: pH 7.33, pCO₂ = 46 mmHg, and pO₂ = 43 mmHg, which was similar to that of endogenous CSF. Pial arterial vessels were observed with a dissecting microscope, a television camera mounted on the microscope, and a video output screen. Vascular diameter was measured with a video microscaler. For production of cerebral ischemia, a hollow stainless steel bolt was implanted in a small (2 mm) hole in the skull.

2.1. Protocol

Two types of pial arterial vessels, small arteries (resting diameter 120–160 μm) and arterioles (resting diameter 50–70 μm), were examined to determine whether segmental differences in the effects of hypoxia/ischemia could be
assay determines cyclic nucleotide concentration for binding to an antiserum that has a high specificity for the cyclic nucleotide. The antibody-bound cyclic nucleotide is then reacted with an anti-rabbit second antibody bound to fluoromicrospheres. Labeled cyclic nucleotide bound to the primary rabbit antibody can then be measured by determining the amount of light emitted by the fluoromicrospheres. All unknowns were assayed at two dilutions, with the lower limit of detection being 100 fmol/ml. The concentration of the unlabeled cyclic nucleotides is calculated from the standard curve via linear regression analysis.

2.3. Statistical analysis

Pial artery diameter, systemic arterial pressure, and cyclic nucleotide values were analyzed using repeated measures of analysis or t-tests where appropriate. If the F value was significant, the Fisher test was performed on all data analyzed by repeated measures. An α level of P<0.05 was considered significant in all statistical tests. The n values reflect data for one vessel in each animal. Values are represented as means±S.E.M. of absolute values or as percentages of change from control values.

3. Results

3.1. Influence of I+R and H+I+R on NOC/oFQ pial artery reactivity and release of cAMP

Topical NOC/oFQ (10⁻⁸, 10⁻⁶ M) elicited reproducible pial small artery (120–160 μm) and arteriole (50–70 μm) dilation over a 12-h period in sham control animals (data not shown). NOC/oFQ-induced pial small artery dilation was diminished within 1 h but returned to control value with 4 h of reperfusion in I+R animals (Fig. 1A). Similar changes were observed in pial arterioles. Such NOC/oFQ-induced vasodilation was associated with elevated cortical periarachnoid CSF cAMP concentration (Fig. 2A). At 1 h of reperfusion, this NOC/oFQ-induced increase in CSF cAMP was attenuated, but such biochemical responses were restored to control (pre-ischemia) value within 4 h of reperfusion (Fig. 2A).

In contrast, NOC/oFQ-induced vasodilation was reversed to pial artery vasoconstriction at both 1 and 4 h of reperfusion after H+I+R (Fig. 1B). At 8 h of reperfusion such vasoconstriction had returned to modest vasodilation, whereas at 12 h of reperfusion NOC/oFQ dilation was not different from that observed before the insult (Fig. 1B). Similar changes were observed in pial arterioles. NOC/oFQ associated elevation in CSF cAMP was blocked and, in fact, reversed to small stimulus-induced decreases in CSF cAMP at 1 and 4 h of reperfusion in such animals (Fig. 2B). At 8 h of reperfusion, NOC/oFQ-induced increases in CSF cAMP once again, though attenuated compared to sham control. Finally, at 12 h of reperfusion,
NOC/oFQ-induced elevated CSF cAMP was no different than that observed in sham control animals (Fig. 2B). Baseline CSF cAMP was unchanged by either 1+R or H+1+R in the absence of NOC/oFQ administration (Figs. 2A,B).

### 3.2. Influence of 1+R and H+1+R on pial artery vasodilation induced by 8-bromo cAMP, Sp 8-Bromo cAMPs, PACAP, cromakalim, and NS1619

Topical 8-Bromo cAMP, Sp 8-Bromo cAMPs, PACAP, cromakalim, and NS1619 (10⁻⁸, 10⁻⁶ M) elicited reproducible pial small artery and arteriole dilation (data not shown). After 1 h of reperfusion, in 1+R animals, pial small artery dilation induced by the cAMP analogues 8-Bromo cAMP and Sp 8-Bromo cAMPs was unchanged (Figs. 3 and 4). However, pial small artery dilation induced by the adenylate cyclase activator PACAP and the K<sub>ATP</sub> channel activator cromakalim was attenuated within 1 h of reperfusion in 1+R animals (Figs. 5 and 6). NS1619-induced pial small artery dilation, though, was unchanged in 1+R animals (Fig. 7). Similar observations were made for agonist reactivities in pial arterioles.

In contrast, pial small artery dilation induced by 8-Bromo cAMP and Sp 8-Bromo cAMPs was blocked in H+1+R animals within 1 h of reperfusion (Figs. 3 and 4). PACAP- and cromakalim-induced pial small artery dilation was modestly, though nonsignificantly, attenuated to a greater extent in H+1+R versus 1+R animals at 1 h of reperfusion (Figs. 5 and 6). Finally, NS1619-induced pial

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Fig. 1. (A) Influence of NOC/oFQ (10⁻⁸, 10⁻⁶ M) on pial small artery diameter before (control) and at 1 and 4 h post I+R. (B) Influence of NOC/oFQ on pial small artery diameter before (control) and at 1, 4, 8, and 12 h post H+1+R, n=7. *P<0.05 compared to corresponding control.

Fig. 2. (A) Influence of NOC/oFQ (10⁻⁸, 10⁻⁶ M) on CSF cAMP (fmol/ml) in sham control animals and in 1+R animals at 1 and 4 h of reperfusion. (B) Influence of NOC/oFQ on CSF cAMP in sham control and in H+1+R at 1, 4, 8, and 12 h of reperfusion, n=7. *P<0.05 compared to corresponding 0 value; †P<0.05 compared to corresponding sham control value.
Fig. 3. Influence of 8-Bromo cAMP ($10^{-8}, 10^{-6}$ M) on pial small artery and arteriole diameter in sham control animals and in I+R and H+I+R animals at 1 h of reperfusion, $n=7$. *$P<0.05$ compared to corresponding control value.

Fig. 4. Influence of Sp 8-Bromo cAMPS ($10^{-8}, 10^{-6}$ M) on pial small artery and arteriole diameter in sham control animals and in I+R and H+I+R animals at 1 h of reperfusion, $n=7$. *$P<0.05$ compared to corresponding control value.

Fig. 5. Influence of PACAP ($10^{-8}, 10^{-6}$ M) on pial small artery and arteriole diameter in sham control animals and in I+R and H+I+R animals at 1 h of reperfusion, $n=7$. *$P<0.05$ compared to corresponding control value.

Fig. 6. Influence of cromakalim ($10^{-8}, 10^{-6}$ M) on pial small artery and arteriole diameter in sham control animals and in I+R and H+I+R animals at 1 h of reperfusion, $n=7$. *$P<0.05$ compared to corresponding control value.
CSF cAMP concentration in sham control animals similar to previous observations [2], but new data in this study show that such stimulated cAMP release was attenuated at 1 h but returned to sham control induced release within 4 h of reperfusion. These data suggest that attenuated ability to elevate CSF cAMP contributes to impaired NOC/oFQ-induced pial artery dilation following ischemia/reperfusion. This insult, however, did not alter baseline CSF cAMP concentration.

In contrast, several differences in the observed parameters described above were noted when the effects of ischemia/reperfusion were compared to that of hypoxia/ischemia/reperfusion. For example, NOC/oFQ-induced vasoconstriction was reversed to pial artery vasodilation at both 1 and 4 h of reperfusion following hypoxia/ischemia/reperfusion. At 8 h of reperfusion such vasodilation was returned to modest vasoconstriction, whereas at 12 h of reperfusion NOC/oFQ dilation was no different from that observed before the insult. Concomitantly, stimulated CSF cAMP release by NOC/oFQ was blocked, if not reversed to modest decreases in CSF cAMP concentration at 1 and 4 h post hypoxia/ischemia/reperfusion. At 8 h post reperfusion, NOC/oFQ stimulated release of cAMP once again, but such release was less than that in sham control animals. NOC/oFQ ability to stimulate cAMP release was not fully restored until 12 h of reperfusion. Similar to the ischemia/reperfusion insult, hypoxia/ischemia/reperfusion did not alter baseline non-agonist-stimulated CSF cAMP concentration. Taken together, these data suggest that the more profound impairment of NOC/oFQ-induced pial artery dilation following hypoxia/ischemia versus that observed following ischemia could relate to the potentiated inability of this agonist to elevate CSF cAMP concentration.

In order to more fully determine potential contributory mechanisms for the observed decrement in NOC/oFQ-induced pial vasodilation following hypoxia/ischemia, the effects of such insults on the ability of cAMP analogues, an adenylate cyclase activator, and activators of the K ATP and K ca channels to elicit vasodilation were explored. Results of these studies show that pial artery dilation induced by the cAMP analogues, 8-Bromo cAMP and Sp 8-Bromo cAMPs, was unchanged by ischemia/reperfusion, consistent with the observations of others who showed that the dilation to another analogue, dibutyryl cAMP, was similarly unchanged in a piglet global cerebral ischemia model [8]. In contrast, results of the present study show that hypoxia/ischemia produces blunted pial dilation to these same cAMP analogues. Such results extend those of previous investigations [8] and indicate that while cAMP-mediated dilation is resistant to influence by ischemia, such cyclic nucleotide vasodilation is susceptible to inhibition with a more robust insult like hypoxia/ischemia.

Additional results of the present study show that pial artery dilation in response to topical PACAP, an activator

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**Fig. 7.** Influence of NS1619 (10⁻⁵, 10⁻⁶ M) on pial small artery and arteriole diameter in sham control animals and in I+R and H+I+R animals at 1 h of reperfusion, n=7. *P<0.05 compared to corresponding control value.
of adenylate cyclase, were attenuated after both ischemic and hypoxia/ischemic insults. While uncertain as to the mechanism for diminished stimulated CSF concentrations of cAMP with NOC/oFQ following ischemia or hypoxia/ischemia, results of the latter studies suggest that an altered activation of adenylate cyclase might contribute to such diminished stimulated cAMP levels. These results are in contrast, however, to those observed for another adenylate cyclase activator, forskolin, whose dilation was unchanged following global cerebral ischemia in the piglet [8]. Reasons for such differences are uncertain but could relate to different pools/mechanisms for adenylate cyclase activation by these two substances. Alternatively, experimental differences related to duration of ischemia (20 min in the present study, 10 min in the other) could account for such a discrepancy.

Moreover, other results of the present study show that cromakalim, a K<sub>A</sub><sub>TP</sub> channel activator, elicited pial artery dilation that was blunted after both ischemia and hypoxia/ischemia. With respect to ischemia alone, these data are consistent with those previously published [9]. Present data, however, extend those previously published in that the effects of combined hypoxia/ischemia on K<sub>A</sub><sub>TP</sub> channel function had not been considered.

The final series of experiments in this study investigated the effects of ischemia and hypoxia/ischemia on vasodilation elicited by the K<sub>c</sub> channel activator, NS1619. Results of those studies show that such dilation was unchanged by ischemia, consistent with previous studies [8]. However, the observation that NS1619-induced pial vasodilation was blunted following combined hypoxia/ischemia is novel in that others had previously concluded that K<sub>c</sub> channel mechanisms were resistant to impairment [8]. Reasons for such impairment with hypoxia/ischemia and not ischemia alone are currently unknown.

With respect to an understanding of mechanisms involved in impairment of NOC/oFQ-induced vasodilation following ischemia alone, then, such impairment appears related to an attenuated ability to elevate CSF cAMP concentration, at least in part, due to impaired adenylate cyclase activation, as well as to an impairment of K<sub>A</sub><sub>TP</sub> channel function. Although cAMP elicits vasodilation via K<sub>A</sub><sub>TP</sub> channel activation [2], such a signal transduction linkage cannot explain impaired NOC/oFQ vasodilation, since cAMP analogue dilation was intact after ischemia. Therefore, cAMP independent contribution of K<sub>A</sub><sub>TP</sub> channel activation to NOC/oFQ dilation must be involved in the observed impairment following ischemia. Alternatively, a more marked inability to elevate CSF cAMP as well as impaired adenylate cyclase activation, cAMP analogue dilation, K<sub>A</sub><sub>TP</sub> and K<sub>c</sub> channel activation contribute to impaired NOC/oFQ-induced vasodilation following hypoxia/ischemia. NOC/oFQ-induced pial artery dilation is dependent on cAMP, K<sub>A</sub><sub>TP</sub> and K<sub>c</sub> channel-dependent mechanisms to elicit pial artery dilation [2], and interference with all of the above signal transduction pathways with hypoxia/ischemia presumably results in the more robust alteration of the vascular response with this insult.

The origin of the cAMP detected in CSF cannot be determined from the present experiments. Potential cellular sites of origin include neurons, glia, vascular smooth muscle, and endothelial cells. Previous studies have investigated the selectivity of the agents used as probes for the role of K<sub>A</sub><sub>TP</sub> and K<sub>c</sub> channel activation in impaired NOC/oFQ dilation post insult. Cromakalim-induced pial artery dilation has been observed to be blocked by glibenclamide and unchanged by iberiotoxin, K<sub>A</sub><sub>TP</sub> and K<sub>c</sub> channel antagonists, respectively [7]. Conversely, NS1619-induced pial artery dilation was blocked by iberiotoxin and unchanged by glibenclamide [4,6,7]. These data suggest that cromakalim and NS1619 are selective K<sub>A</sub><sub>TP</sub> and K<sub>c</sub> channel agonists in the piglet cerebral circulation. However, it has also been observed that NS1619 may additionally possess calcium channel antagonistic activity and, therefore, may not be useful as a probe for K<sub>c</sub> channel activation [14]. In contrast, recent observations in the piglet show that vasoconstrictor responses to the calcium channel agonist Bay K8644 were unchanged in the presence of NS1619 [4]. These results suggest that NS1619 has no calcium channel-blocking activity and, therefore, may be considered to be selective for activation of K<sub>c</sub> channels in the newborn pig.

Global cerebral ischemia in a piglet model has been previously observed to result in reductions in blood flow of the cerebrum and altered pial artery dilation to stimuli such as hemorrhagic hypotension and hypercapnia [17–19]. However, such ischemic effects are not nonselective in that although response to these stimuli were impaired, others (e.g., isoproterenol) were not [17,18]. Opioids are important contributors to the regulation of the newborn pig cerebral circulation during physiological and pathological conditions [5]. Because the present study did not characterize responses to NOC/oFQ after ischemia or hypoxia/ischemia in the juvenile or adult, it is uncertain whether similar results could be expected in the adult.

In conclusion, results of the present study show that impaired NOC/oFQ dilation following ischemia/reperfusion results from altered adenylate cyclase and K<sub>A</sub><sub>TP</sub> channel-dependent mechanisms. These data further indicate that impaired NOC/oFQ dilation following hypoxia/ischemia/reperfusion results only from altered adenylate cyclase and K<sub>A</sub><sub>TP</sub> channel but also from altered cAMP and K<sub>c</sub> channel-dependent mechanisms.

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