NOC/oFQ PKC-dependent superoxide generation contributes to hypoxic-ischemic impairment of NMDA cerebrovasodilation

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Received 27 April 2000; accepted in final form 25 July 2000

Episodes of inadequate oxygen supply to the brain can result in significant neurological sequelae. Babies are frequently exposed to hypoxic-ischemic (H-I) 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 (19, 21, 22). Less, however, is known about the cerebrovascular consequences of combined H-I.

Glutamate is an important excitatory amino acid transmitter in the brain. It can bind to any of three different ionotropic receptor subtypes named after specific synthetic analogs: N-methyl-D-aspartate (NMDA), kainate, and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA). Activation of NMDA receptors has been observed to elicit cerebrovascular dilation and may represent one of the mechanisms for the coupling of local cerebral metabolism to blood flow (13). Several studies observed that NMDA-induced pial artery dilation was attenuated after global cerebral ischemia-reperfusion (I/R) (9, 31). Mechanisms for such altered dilation to NMDA after such an insult have been less well characterized.

During the last 5 years, several groups have isolated and cloned a new G protein-coupled receptor that showed high homology with opioid receptors (11, 15, 28). The peptide ligand for this receptor does not bind to classical opioid receptors (μ, δ, κ) and was named orphanin FQ by Reinscheid et al. (30) because its sequence begins with phenylalanine (F) and ends with glutamine (Q). The same peptide was called nociceptin by Meunier et al. (27) because it increased the reactivity to pain in animals in contrast with the analgesic effects of opioid drugs. Recently, nociceptin/orphanin FQ (NOC/oFQ) has been observed to elicit pial artery vasodilation in the newborn pig (1). However, little is known about the role of NOC/oFQ in the physiological or pathophysiological control of cerebral hemodynamics. Recent studies have shown that the cerebrospinal fluid (CSF) concentration of NOC/oFQ is elevated after H-I in the piglet (3). Interestingly, it has also been observed that NOC/oFQ can both inhibit the release of glutamate from rat cerebrocortical slices and inhibit glutamatergic transmission in the rat spinal cord as well as have its own signaling modulated by NMDA (12, 29, 36). Because of the latter observations, more recent studies were designed to investigate the interaction between NOC/oFQ and excitatory amino acids in the piglet cerebral circulation. Results of these
Studies show that coadministration of NOC/oFQ, in a concentration similar to that in CSF after HI, diminished NMDA and glutamate-induced pial dilation under non-HI conditions (4). Additionally, NOC/oFQ antagonist pretreatment partially restored decremented NMDA and glutamate dilation after HI (4). These data then suggest that NOC/oFQ release contributes to impaired excitatory amino acid-induced cerebrovasodilation after HI.

The present study, therefore, was designed to determine a potential mechanism whereby NOC/oFQ might contribute to HI-impaired NMDA cerebrovasodilation. Importantly, NOC/oFQ has been observed to activate protein kinase C (PKC) (26), and PKC activation has also been observed to generate superoxide anion (O$_2^-$) (2). This study was then designed to determine whether NOC/oFQ generates O$_2^-$ in a PKC-dependent manner and whether such O$_2^-$ production contributes to HI impairment of NMDA-induced pial artery dilation.

METHODS

Newborn (1–5 days 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 minimum alveolar concentration). Anesthesia was maintained with α-chloralose (30–50 mg/kg, supplemented with 5 mg·kg$^{-1}$·h$^{-1}$ iv). A catheter was inserted into a femoral artery to monitor blood pressure and to sample for blood gases 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 MgCl$_2$, 1.5 CaCl$_2$, 132 NaCl, 6.6 urea, 3.7 dextrose, and 24.6 NaHCO$_3$. This artificial CSF was warmed to 37°C and had the following chemistry: pH 7.33, PCO$_2$ 46 mmHg, and PO$_2$ 43 mmHg, which is 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.

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 HI could be identified. Pial arterial vessel diameter was determined every minute for a 10-min exposure period after infusion onto the exposed pial arterial cortex of artificial CSF before drug application and after infusion of artificial CSF containing a drug. Typically, 2–3 ml of CSF were flushed through the window over a 30-s period, and excess CSF was allowed to run off through one of the needle ports.

Techniques for induction of total cerebral ischemia in the piglet have been well documented (19, 21, 22). Briefly, total cerebral ischemia was accomplished by infusing artificial CSF into a hollow bolt in the cranium to maintain an intracranial pressure 15 mmHg greater than the numerical mean of systolic and diastolic arterial blood pressure (22). Intracranial pressure was monitored via a sidearm of the cranial window. Blood flow in pial arterioles, viewed with a microscope and video monitor, stopped completely on elevation of intracranial pressure and did not resume until the pressure was lowered (22). To prevent the arterial pressure from rising inordinately (Cushing response), venous blood was withdrawn as necessary to maintain mean arterial pressure no greater than 100 mmHg. As the cerebral ischemic response subsided, the shed blood was returned to the animal. Cerebral ischemia was maintained for 20 min. In combined HI/R animals, hypoxia (PO$_2$ < 100 mmHg) was produced for 10 min before ischemia by decreasing the inspired O$_2$ via inhalation of N$_2$, which was immediately followed by the total ischemia protocol as described above after concomitantly restoring room air.

Twenty major types of experiments were performed (all n = 7 animals): 1) generation of O$_2^-$ with NOC/oFQ, 2) generation of O$_2^-$ with NOC/oFQ in the presence of staurosporine, 3) generation of O$_2^-$ with NOC/oFQ in the presence of the NOC/oFQ receptor antagonist [F/G]NOC/oFQ(1–13)-NH$_2$, 4) generation of O$_2^-$ with NOC/oFQ in the presence of chelerythrine, 5) generation of O$_2^-$ with I/R, 6) generation of O$_2^-$ with I/R in staurosporine-pretreated animals, 7) generation of O$_2^-$ with I/R in chelerythrine-pretreated animals, 10) generation of O$_2^-$ with I/R in [F/G]NOC/oFQ(1–13)-NH$_2$-pretreated animals, 12) vascular responses to agonists in the absence of HI (sham control), 13) vascular responses to agonists after I/R, 14) vascular responses to agonists after I/R in staurosporine-pretreated animals, 15) vascular responses to agonists after I/R in [F/G]NOC/oFQ(1–13)-NH$_2$-pretreated animals, 16) vascular responses after I/R in polyethylene glycol (PEG) superoxide dismutase (SOD) and catalase (CAT)-pretreated animals, 17) vascular responses after H+I/R, 18) vascular responses after H-I/R in staurosporine-pretreated animals, 19) vascular responses after H+I/R in [F/G]NOC/oFQ(1–13)-NH$_2$-pretreated animals, and 20) vascular responses after H+I/R in SODCAT-pretreated animals.

In the first three series of experiments designed to investigate generation of O$_2^-$, NOC/oFQ (10$^{-10}$ M, Phoenix) was applied to the cerebral cortex for 20 min in either the absence or the presence of staurosporine (10$^{-7}$ M), [F/G]NOC/oFQ(1–13)-NH$_2$ (10$^{-6}$ M, Phoenix) or chelerythrine (10$^{-7}$ M). In the next three series of experiments, generation of O$_2^-$ 1 h after I/R or H+I/R was investigated in the absence and presence of staurosporine, [F/G]NOC/oFQ(1–13)-NH$_2$, and chelerythrine. In these experiments, staurosporine, [F/G]NOC/oFQ(1–13)-NH$_2$, and chelerythrine were administered 20 min before I/R or H+I/R. The NOC/oFQ antagonist staurosporine or chelerythrine was kept in constant contact with the cerebral cortex for the duration of the experiment.

In the vascular experiments, responses of arterial vessels to NMDA and glutamate (10$^{-8}$ and 10$^{-6}$ M; Sigma) were obtained before and 1 h after I/R or H+I/R either in the absence or presence of staurosporine, [F/G]NOC/oFQ(1–13)-NH$_2$, and SODCAT (1,000 and 10,000 U/kg of PEGSOD and CAT, respectively).
O₂ analysis. SOD-inhibitable nitroblue tetrazolium (NBT) reduction was determined as an index of O₂ generation, as previously described (2, 5, 17). Such reduction was determined by placing NBT (2.4 mM, Sigma) dissolved in artificial CSF under one window and NBT (2.5 mM) and SOD (Sigma, 60 U/ml) in artificial CSF under the other window 1 h after I/R or H₁I/R.

NBT is water soluble and forms a yellow solution that is converted to nitroblue formazan, an insoluble purple precipitate, in the presence of reducing agents, e.g., O₂. The SOD-inhibitable NBT reduction was determined by the difference in the quantities of nitroblue formazan precipitated on the brain surface under the two windows. Although NBT can be reduced by a variety of agents, SOD provides specificity for the assay. Details of this methodology have been published previously (2, 5, 18).

Statistical analysis. Pial arteriolar diameter, systemic arterial pressure, and NBT reduction values were analyzed using ANOVA for repeated measures or t-test where appropriate. If the value was significant, the data were then analyzed by Fisher’s protected least-significant difference test. An a-level of P < 0.05 was considered significant in all statistical tests. Values are represented as means ± SE of the absolute values or percent changes from control values.

RESULTS

Role of PKC activation in NOC/oFQ-induced O₂ generation during non-H-I and H-I conditions. Topical application of NOC/oFQ (10⁻¹⁰ M, concentration present in cortical periarachnoid CSF after I/R or H₁I/R) to the cerebral cortical surface of non-H-I animals increased SOD-inhibitable NBT reduction (Fig. 1A). This NBT reduction by NOC/oFQ was blunted by staurosporine (10⁻⁷ M) and by the NOC/oFQ receptor antagonist [F/G]NOC/oFQ(1–13)-NH₂ (10⁻⁶ M) (Fig. 1A). NBT reduction by NOC/oFQ was similarly blunted by chelerythrine, another PKC inhibitor (1 ± 1 to 20 ± 3 vs. 1 ± 1 to 7 ± 2 pmol NBT/mm² for absence and presence of chelerythrine, respectively). Under H-I conditions, SOD-inhibitable NBT reduction was increased 1 h after either I/R or H₁I/R (Fig. 1B). This enhanced NBT reduction after either insult was blunted by both staurosporine and [F/G]NOC/oFQ(1–13)-NH₂ (Fig. 1B). NBT reduction after H₁I/R was similarly blunted by chelerythrine (1 ± 1 to 15 ± 2 vs. 1 ± 1 to 6 ± 2 pmol NBT/mm² for the absence and presence of chelerythrine, respectively).

Role of NOC/oFQ, PKC activation, and O₂ generation in impaired excitatory amino acid acid-induced pial artery dilation after I/R and H₁I/R. NMDA and glutamate (both at 10⁻⁸ and 10⁻⁶ M) elicited reproducible pial small artery (120–160 μm) and arteriole (50–70 μm) vasodilation in sham control animals (data not shown). However, NMDA and glutamate-induced vasodilation was attenuated with 1 h of reperfusion after cerebral ischemia (Figs. 2 and 3). This postsult di...
minished excitatory amino acid dilation was partially prevented by pretreatment with \([F/G]\text{NOC/oFQ}(1–13)\text{-NH}_2\), staurosporine, and the free radical scavenger SODCAT (Figs. 2 and 3). In contrast, NMDA and glutamate-induced vasodilatation was reversed to vasoconstriction within 1 h of reperfusion after H+I/R (Figs. 4 and 5). This postsutinct excitatory amino acid-induced vasoconstriction was attenuated by \([F/G]\text{NOC/oFQ}(1–13)\text{-NH}_2\) (Figs. 4 and 5). Both staurosporine and SODCAT administration prevented this postsutinct NMDA and glutamate-induced vasoconstriction, although responses were only partially restored to control values (Figs. 4 and 5).

Effect of staurosporine, chelerythrine, \([F/G]\text{NOC/oFQ}(1–13)\text{-NH}_2\), SODCAT, and NOC/oFQ on pial artery diameter. Staurosporine, chelerythrine, \([F/G]\text{NOC/oFQ}(1–13)\text{-NH}_2\), SODCAT, and NOC/oFQ all had no effect on pial artery diameter.

Blood chemistry. Blood chemistry and mean arterial blood pressure values were obtained at the beginning and end of all experiments as well as during hypoxia. Hypoxia decreased PO\(_2\) to \(35 \pm 3\) mmHg, whereas the pH, PCO\(_2\), and mean arterial blood pressure values were unchanged. Values for pH, PCO\(_2\), PO\(_2\), and mean arterial blood pressure were \(7.45 \pm 0.02, 37 \pm 3\) mmHg, 92 \(\pm\) 5 mmHg, and 71 \(\pm\) 5 mmHg, respectively, at the start of experiments vs. \(7.44 \pm 0.02, 38 \pm 3\) mmHg, 91 \(\pm\) 6 mmHg, and 68 \(\pm\) 6 mmHg, respectively, at the end of experiments. There were no group differences in either blood pressure or blood chemistry values.

DISCUSSION

The results of the present study show that, under non-H-I conditions, topical administration of NOC/oFQ, in a concentration approximately that observed in cortical periarachnoid CSF after I/R or H+I/R (3), results in increased SOD-inhibitable NBT reduction by the newborn pig brain. These data indicate that O\(_2\)\(_2\) was generated. Because staurosporine and chelerythrine blunted this elevation in SOD-inhibitable NBT reduction by NOC/oFQ, these data indicate that PKC activation contributes to O\(_2\)\(_2\) generation by this opioid. Previously, staurosporine was observed to block the NBT reduction after topical application of the PKC activator phorbol 12,13-dibutyrate to the cerebral cortical surface of the piglet, indicating that staurosporine is an efficacious PKC inhibitor (2). Moreover, the putative NOC/oFQ antagonist \([F/G]\text{NOC/oFQ}(1–13)\text{-NH}_2\) (1, 16, 17) blocked NOC/oFQ-induced NBT reduction, indicating that this opioid generates O\(_2\)\(_2\) in a selective manner. Additionally, staurosporine, chelerythrine, and \([F/G]\text{NOC/oFQ}(1–13)\text{-NH}_2\) blunted I/R and H+I/R-induced elevated SOD-inhibitable NBT reduction. Previously, I/R was observed to be associated with generation of O\(_2\)\(_2\) on the piglet cerebral cortical surface (5). Results of the present study extend the latter
gen to generate \( O_2^- \), it is concluded that the radical being dealt with is \( O_2^- \).

The cerebrovascular consequences of free radical production are not fully understood. It has been suggested that \( O_2^- \) could be involved in irreversible vascular damage, delayed hypoperfusion, and edema produced by cerebral I/R (32). Topical application of a xanthine/xanthine oxidase-activated oxygen-generating system, severe hypertension, topical application of arachidonic acid, and fluid percussion brain injury cause morphological, functional, and biochemical cerebral artery abnormalities, which include reduced responsiveness to vasoconstrictor and vasodilator stimuli (2, 18, 20, 33–35). \( O_2^- \) and species derived from it, such as hydrogen peroxide and hydroxyl radical, appear to mediate these abnormalities (13, 18, 35). Intracellular generation of \( O_2^- \) or other species could alter structure and/or production of nucleotides, second messengers, receptors, and membranes, and the movement of \( O_2^- \) out of the cell through anion channels could result in high concentrations of activated oxygen species at cell surfaces, including the endothelium. More importantly, current concepts point toward the significant contribution to damage by the reaction of \( O_2^- \) with nitric oxide to form the highly reactive prooxidant peroxynitrite (8, 25). The latter species, and not \( O_2^- \), is currently thought to be the more direct mediator of damage. However, because oxygen free radical scavengers did not attenuate impairment of hypercapnic dilation after piglet cerebral I/R (23), posts ischemic loss of vasodilator responsiveness may not always involve \( O_2^- \) or a subsequent reduced form of oxygen.

Because it had been previously observed that NOC/oFQ interacts with NMDA and glutamate in studies unrelated to vascular activity (12, 29, 36), additional studies were designed to investigate the relationship among NOC/oFQ, \( O_2^- \), PKC activation, and excitatory amino acid-induced vascular activity after I/R and H+I/R. The results of those studies show that NMDA-induced pial artery dilation was attenuated after I/R, consistent with previous studies (9). After H+I/R, however, dilator responses to NMDA and glutamate were reversed to vasoconstriction. Results of this study extend those of others (9) in that the present study shows that glutamate as well as NMDA-induced pial artery dilation is altered after I/R. Additionally, others did not note a reversal of NMDA-induced dilation to vasoconstriction after global cerebral ischemia (9). Such post-insult excitatory amino acid-induced impaired vasodilation or vasoconstriction was attenuated by [F/G]NOC/oFQ(1–13)-NH₂, indicating NOC/oFQ involvement in this altered vascular activity. However, both staurosporine and SODCAT administration prevented the post-H+I/R excitatory amino acid vasoconstriction, although responses were only partially restored to control values. Together, these data suggest that PKC-dependent \( O_2^- \) generation links NOC/oFQ release to impaired NMDA and glutamate-induced pial artery dilation after H-I. However, because both staurosporine and SODCAT prevented impairment of excitatory amino acid dilation to a greater extent than...
[F/G]NOC/oFQ(1–13)-NH2 in I/R animals, those data further suggest that other as yet to be determined factors also contribute to activation of PKC, subsequent O2 generation, and final impairment of excitatory amino acid-induced vasodilation after H+I/R.

The mechanism by which NMDA-induced pial artery dilation is altered after global cerebral I/R or combined H+I/R is unclear at this time. Recent work by others suggests a role for oxygen free radicals and protein synthesis (6, 9, 31). In that proposed scenario, increased cyclooxygenase synthesis might account for the previously observed role for oxygen free radicals in I/R-associated cerebrovascular derangement (31). Alternatively, the observed beneficial action of protein synthase inhibitors might relate to the block of the production of an unidentified regulatory protein that is rapidly overexpressed after ischemia (31). Interestingly, adenosine, which is released during hypoxia, has been observed to inhibit NMDA-induced pial artery dilation when coadministered with this excitatory amino acid (7), very similarly to that observed with NOC/oFQ. In those studies it was suggested that adenosine might reduce calcium entry into nerve cells and activation of nitric oxide synthase by promoting hyperpolarization or by blocking N- and Q-type channels (7). It was further suggested that adenosine might reduce presynaptic glutamate release and thus suppress autoamplification of glutamate effects (7). Equally interesting, then, is the observation that NOC/oFQ can inhibit the release of glutamate from rat cerebrocortical slices and can inhibit glutamatergic transmission in the rat spinal cord (12, 29). NOC/oFQ signaling can also be modulated by NMDA (36). More distal mechanisms by which NOC/oFQ-induced O2 generation might alter NMDA-induced pial artery dilation, as observed in the present study, are currently uncertain.

The experimental design of the present study did not allow for the identification of the cellular site of origin for NOC/oFQ detected in cortical periarachnoid CSF. Potential cellular sites of origin include neurons, glia, vascular smooth muscle, and endothelial cells.

Although glutamate is an excitatory neurotransmitter thought to be a predominant contributor to neurotoxicity associated with H-I (10, 24), little attention has been paid to the functional implications of vascular abnormalities to NMDA and glutamate after such an insult. In the present study, endogenous NOC/oFQ could either function to limit vascular responses to abnormally high glutamate levels after fluid percussion injury or, alternatively, exacerbate them. It is speculated that the latter is more plausible. Recent data show that, at concentrations higher than those studied presently, NOC/oFQ-induced vasodilation is reversed to vasoconstriction after I/R and H+I/R (3). The preadministration of the NOC/oFQ antagonist [F/G]NOC/oFQ(1–13)-NH2 attenuated reductions in cerebral blood flow observed after H-I, thereby acting in a neuroprotective or vasoprotective manner (3). Therefore, it is hypothesized that the abnormal vascular responses to glutamate and NMDA are deleterious and that H-I-accentuated release of NOC/oFQ contributes to impaired cerebral hemodynamics via modulation of vasodilation by excitatory neurotransmitters.

Opioids are important contributors to the regulation of the piglet cerebral circulation (18). Results of the present study extend such studies by characterizing the contribution of the newly described opioid NOC/oFQ to altered cerebrovascular regulation observed after I/R and H+I/R.

In conclusion, the results of the present study show that NOC/oFQ, in concentrations present in CSF after H-I, increases O2 production in a PKC-dependent manner and contributes to this production after H-I. These data also show that NOC/oFQ contributes to impaired NMDA and glutamate-induced pial artery dilation after H-I. These data suggest, therefore, that PKC-dependent O2 generation links NOC/oFQ release to impaired NMDA-induced cerebrovasodilation after H-I.

The author thanks Miriam Kulkarni for technical assistance in the performance of the experiments.

This research was supported by grants from the National Institutes of Health, the American Heart Association, Pennsylvania-Delaware Affiliate, and the University of Pennsylvania Research Foundation.

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