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NITRIC OXIDE SYNTHASE INHIBITORS UNMASK ACETYLCHOLINE-MEDIATED CONSTRICTION OF CEREBRAL VESSELS IN THE *IN VITRO* ISOLATED GUINEA-PIG BRAIN

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Abstract—The control of arterial vascular tone by acetylcholine contributes to the regulation of cerebral blood flow. We analysed the effects of intraluminal application of acetylcholine (1 μM) on the cerebral vascular tone by measuring changes in resistance to perfusion pressure in an isolated guinea-pig brain preparation maintained *in vitro* by arterial perfusion under constant flow. Acetylcholine induced a reproducible, fast-onset dilation that was prevented by the nitric oxide scavenger Methylene Blue (10 μM) and by the muscarinic receptor antagonist atropine (0.1 μM). Prolonged arterial perfusion with the nitric oxide synthase inhibitors *N*-nitro-L-arginine (1 mM) and *N*-nitro-L-arginine methyl ester (30–100 μM) induced a slowly developing increase of 25.9 ± 13.44 mmHg in vascular tone and blocked the acetylcholine-induced vasodilation. In these experimental conditions, the dilation determined by the nitric oxide donor nitroprusside (0.1 μM) was unaffected. In five experiments, the blockade of dilation unmasked a slow acetylcholine-mediated vasoconstriction (14.40 ± 3.85 mmHg) that was antagonized by atropine.

The results demonstrate that acetylcholine exerts two simultaneous and opposite effects on guinea-pig cerebral vessels, characterized by a slow direct constriction concealed in physiological conditions by a fast vasodilation mediated through the release of nitric oxide by endothelial cells. Acetylcholine-mediated increase in vascular tone may play a role in aggravating cerebral perfusion when endothelial cell damage occurs during brain ischemia.

Key words: acetylcholine, guinea-pig, isolated brain preparation, nitric oxide, nitric oxide synthase.

Acetylcholine (ACh) contributes to the regulation of blood flow by promoting changes in vascular resistance. Several studies demonstrated that ACh increases cerebral blood flow[23,32,38] by inducing vasodilation of intracerebral arterioles[12] and pial arterioles[9] through the stimulation of nitric oxide (NO) release from endothelial cells.[3,20] The increase in cerebral blood flow mediated by ACh is indeed abolished by endothelial cell damage[39] and is prevented by nitric oxide synthase (NOS) inhibitors[26–29] that induce *per se* an increase in vascular tone.[20,40]

Cholinergic regulation of cerebrovascular activity is operated by local cholinergic interneurons[11] and via cholinergic fibers that originate from neurons in the basal forebrain and terminate either on the basal lamina of arterioles and capillaries[4,5,42] or on NOS-immunoreactive neurons.[44] As for the direct effect of ACh applied on brain vessels, the increase in cortical blood flow induced by stimulation of the cholinergic basal forebrain nuclei[8,13,29,43] has been shown to be mediated by NO release from both NOS-positive neurons and endothelial cells.[1,35,44,47]

We demonstrate that, in the intact vasculature of the isolated guinea-pig brain preparation,[14,16,34] a steady-state, NO-independent mechanism controls vessel tone; after prolonged exposure to NOS inhibitors, a slow, persistent increase in tone takes place, ACh-mediated vasodilation is abolished and a contraction due to muscarinic receptor activation is unmasked. Preliminary results have been published in abstract form.[30]

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Abbreviations: ACh, acetylcholine; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; L-NAME, *N*-nitro-L-arginine methyl ester; L-NNA, *N*-nitro-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; NP, nitroprusside.

EXPERIMENTAL PROCEDURES

The experiments were carried out on brains isolated from young adult guinea-pigs (150–200 g) maintained *in vitro* by arterial perfusion. The procedure for brain isolation has been described extensively elsewhere.[14,16,31,34] In brief, during anesthesia with pentobarbital (20 mg/kg, i.p.), the brain was extracted under hypothermic conditions and transferred to an incubation chamber, where a micro-cannula was inserted in the basilar artery to ensure arterial perfusion with a complex saline solution (composition, in mM: NaCl, 126; KCl, 3; KH_2PO_4, 1.2; $MgSO_4$, 1.3; $CaCl_2$, 2.4; $NaHCO_3$, 26; glucose, 15; HEPES, 2.1; 3% dextran, mol. wt 70,000) oxygenated with a 95% O_2–5% CO_2 gas mixture. Arterial perfusion at 5.5 ml/min was performed via a peristaltic pump. Experiments were performed at 32°C. All efforts were made to minimize the suffering and the number of animals used. The experimental procedure was approved by the Ethical Committee of the Istituto Nazionale Neurologico in accordance with the regulations on animal research. A pressure transducer (Model 800, Bentley) was inserted between the peristaltic pump and the micro-cannula to monitor the arterial pressure. The changes in vascular tone were measured as increases or decreases in the resistance to flow opposed by the entire cerebral arterial system. The voltage signal recorded at the pressure transducer was amplified and digitized via a National AD board (AT-MIO32) for off-line analysis with Labview software developed in our laboratory by Dr Gerardo Biella. The isolated guinea-pig brain has been utilized in the last decade for electrophysiological studies of brain activity.[7,17] More recently, we demonstrated that in this preparation the vascular system is functionally and structurally preserved.[15,16]

Drugs diluted in the perfusate were applied intraluminally at the following concentrations: *N*-nitro-L-arginine methyl ester (L-NAME), 30–100 μM; *N*-nitro-L-arginine (L-NNA), 1 mM; ACh, 1 μM; nitroprusside (NP), 0.1 μM; U46619, 30–100 nM; atropine, 0.1 μM; Methylene Blue, 10 μM. With the exception of U46619 (provided by Cayman Chemical Company, USA), all drugs were obtained from Sigma. The results are expressed as mean values ± S.D. of *n* replications.

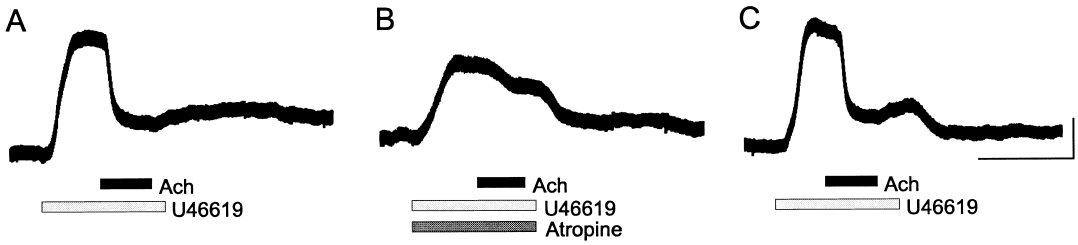

Fig. 1. Intraluminal application of ACh induces a muscarinic receptor-dependent vasodilation. A rapid vasodilation is determined by arterial perfusion of 1 μM ACh during the constriction exerted by U46619 (0.1 μM; A). (B) The ACh-induced dilation is prevented by co-perfusion with atropine (0.1 μM; B). The effect of atropine is reversible (C). Upward and downward deflections correspond to constriction and dilation of the cerebral vessels, respectively. In this and in the following figures, the timing of the drug perfusions is indicated by the bars below the traces. Calibration bars: 10 min and 10 mmHg.

RESULTS

The experiments were performed on 21 isolated guinea-pig brains. The arterial basal resistance to perfusion pressure was 34.26 ± 8.15 mmHg ($n = 7$) and 33.36 ± 10.80 mmHg ($n = 5$) at 15 and 32°C, respectively. No changes in vascular resistance were observed in untreated brains up to 6–7 h ($n = 5$). At the onset of the experiment, the functional viability of the vessels was tested by intraluminal perfusion with U46619 (30–100 nM), a potent vasoconstrictor that activates the thromboxane A2 receptors on cerebral arterioles.[2] The choice of U46619 was also justified by the fact that this agonist mimics a platelet-dependent vasoconstriction, and therefore better reflects a cerebrovascular pathogenic event. At the plateau of the constriction (21.89 ± 8.7 mmHg above basal; $n = 13$), ACh (1 μM) was added to the perfusion buffer, in the presence of U46619, to test the ability of the endothelial cells to release NO and induce vasodilation (Fig. 1A). ACh caused a fast-onset dilation that reduced by 16.62 ± 4.59 mmHg (~76%) the U46619-dependent increase vessel tone. The ACh-induced dilation was antagonized by simultaneous arterial perfusion with the muscarinic receptor antagonist atropine (0.1 μM; $n = 11$; Fig. 1B). The effect of atropine was fully reverted upon washing of the preparation with control solution (Fig. 1C). The fall in vascular resistance to perfusion pressure determined by ACh was functionally antagonized by the NO scavenger Methylene Blue (10 μM; $n = 3$; Fig. 2B), suggesting that NO release mediates the ACh effect in the isolated brain vasculature of the guinea-pig. The inhibitory effect of Methylene Blue was not reversible, even after repeated washings of the preparation with control buffer. To confirm, in our experimental conditions, the involvement of NO in the ACh-induced vasodilation, the preparation was intraluminally perfused with either one of the NOS inhibitors, L-NNA ($n = 3$) and L-NAME ($n = 11$). In eight of 14 experiments, treatment with the NOS inhibitors increased vascular tone (25.9 ± 13.44 mmHg above basal values). In six of 14 experiments, no changes in the perfusion pressure occurred even after long-lasting perfusion (approximately 3 h) with the NOS inhibitors. In seven of eight experiments, we observed an impairment of the ACh-dependent vasodilation, which required at least 3.5 h of continuous perfusion with the NOS inhibitor to be revealed. In Fig. 3A and B, the effect of ACh before and during L-NNA is shown. The NO donor NP (0.1 μM) was briefly perfused after abolishing the ACh-induced vasodilation, in order to test the vasodilating capacity of the brain vasculature. In seven tests with NP during NOS inhibitors, a prompt vasodilation was obtained (Fig. 3B).

The ACh-dependent vasodilation observed during the early stages of perfusion with NOS inhibitors was followed by a slowly increasing constriction (Figs 4, 5A). In five experiments in which NOS inhibitors increased vessel tone, the disappearance of the vasodilation unmasked a 14.40 ± 3.85 mmHg vasoconstriction upon ACh perfusion (Fig. 4Ad). As illustrated in Fig. 5, the full development of vasoconstrictor efficacy occurred according to a slower time-course (190.2 ± 115.34 s; $n = 5$) in comparison with the pre-NOS inhibitor ACh dilation (18.4 ± 15.88 s; $n = 5$). The constriction was abolished by co-administration of 0.1 μM atropine (Fig. 5C; $n = 2$). Atropine alone perfused for 3–4 h did not change the vascular tone ($n = 2$; not shown).

DISCUSSION

The present study demonstrates that ACh influences cerebral vessel tone via two, possibly simultaneous, effects, both mediated by muscarinic receptors: a fast NO-dependent dilation and a slower vasoconstriction. The vasodilatory effect of ACh is dependent on the release of NO from the endothelium, as demonstrated by its abolition following arterial perfusion with NOS inhibitors and the NO scavenger Methylene Blue. Recently, Methylene Blue has been shown to possess additional pharmacological activities.[37] However, previous studies by one of the authors demonstrated the efficacy of Methylene Blue as an NO scavenger by comparison with

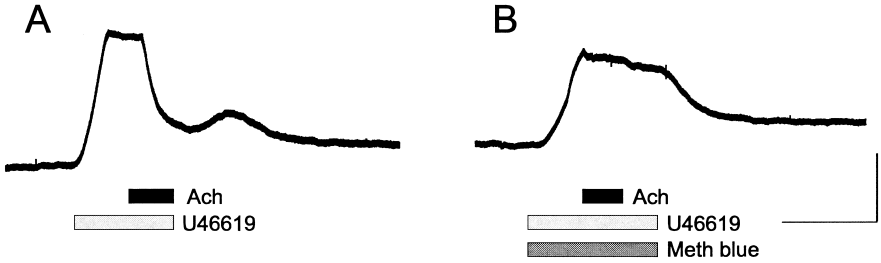

Fig. 2. The NO scavenger Methylene Blue (10 μM; B) blocks the vasodilation induced by ACh (1 μM) on the U46619-induced constriction. Calibration bars: 10 min and 20 mmHg.

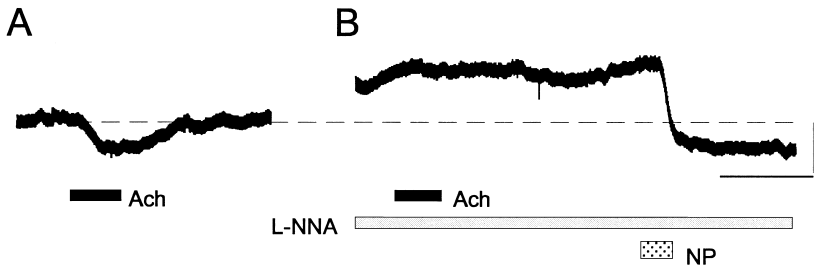

Fig. 3. The ACh-induced vasodilation (A) is blocked by arterial perfusion with 1 mM L-NNA (B). In these conditions, perfusion with the NO donor NP (0.1 μM) induced a dramatic vasodilation, indicating that the ability of the endothelium to react to NO was functionally preserved. The baseline difference between the two traces reflects the tonic vasoconstriction determined by L-NNA. Resting arterial resistance to perfusion pressure = 28 mmHg (dotted line). Calibration bars: 10 min and 5 mmHg.

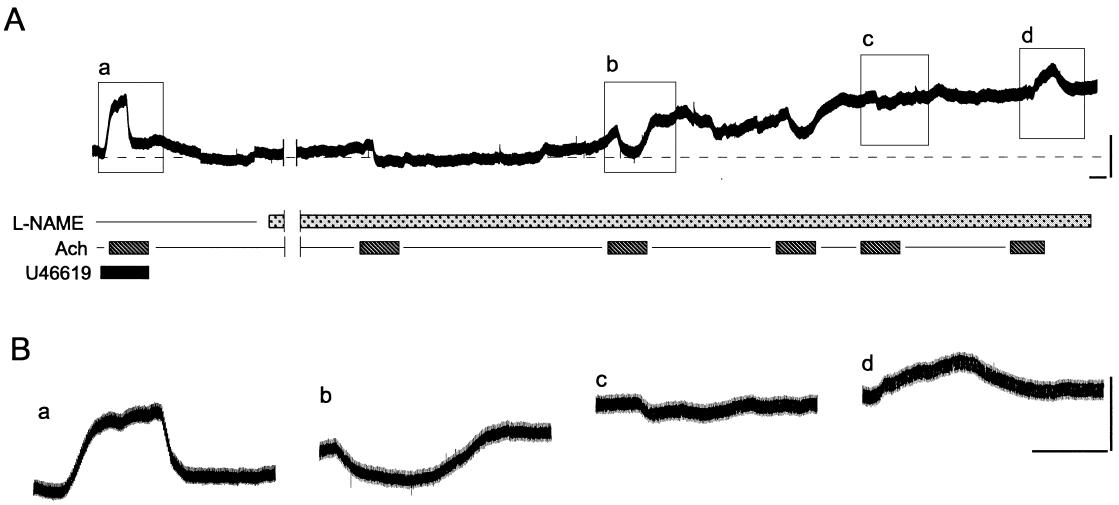

Fig. 4. Effect of perfusion with L-NAME (30 μM) at slow time resolution (A). The events outlined in A are shown on a faster time-scale in B. In control conditions, a brief ACh (1 μM) application induced vasodilation on the U46619 constriction (a). After 3 h of perfusion with L-NAME, the ACh-induced dilation progressively vanished (b, c) and a constrictory response was isolated (d). Calibration bars: 5 min and 20 mmHg.

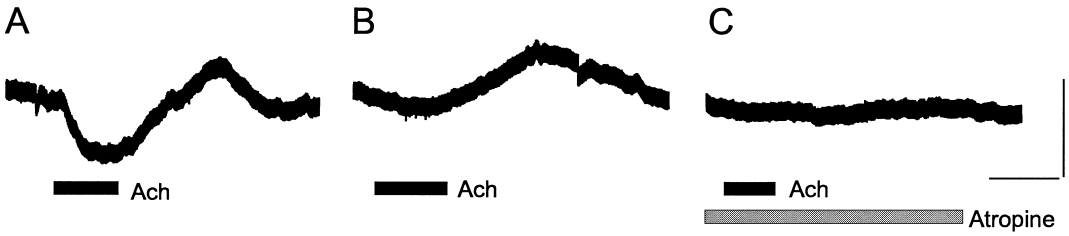

Fig. 5. Responses to 1 μM ACh before (A) and during perfusion with L-NAME (30 μM; B). The ACh-induced constriction observed during L-NAME was prevented by simultaneous atropine perfusion (C). Resting arterial resistance to perfusion pressure = 31.5 mmHg (dotted line). Calibration bars: 10 min and 10 mmHg.

oxyhaemoglobin at equimolar concentrations.[21] The dilatory action of ACh mediated by NO has been demonstrated previously in cats, rats and other vertebrates.[3,18,19,25,26,28,39,45] Cerebral cholinergic vasodilation is mediated by intracerebral arterioles[12] and pial arterioles,[9] as well as by isolated capacitance arteries.[20] Accordingly, we propose that the changes in vascular resistance to constant flow observed in our experimental conditions reflect the modification in the vascular district of higher resistance represented by small arterioles and capillaries. Further experiments that allow direct measurements of vessel diameter will be necessary to elucidate the issue.

A constricting effect of ACh cerebral arteries was reported previously on isolated cat pial arteries.[19] In this study, constriction was induced by very high concentrations of ACh (10–1000 μM), whereas more physiological dosages, similar to those utilized in our experiments (1 μM), induced dilation exclusively. The vasoconstriction revealed by the blockade of the ACh-mediated relaxation may represent the effect of the activation of muscarinic receptors located on arteriolar smooth muscle cells, postsynaptic to the perivascular cholinergic terminals.[4,5,43] The slower onset of vasoconstriction in comparison to the fast NO-dependent vasodilation could be accounted for by the time required for ACh to cross the blood–brain barrier and attain the necessary bioavailability to explicate its direct effect on muscle cells. An ACh-mediated increase in vascular tone has also been demonstrated when the endothelium is damaged in peripheral vessels[22] and in isolated cerebral arterioles.[39] In line with these observations, Rosenblum[36] recently showed that NO-mediated vasodilation was selectively impaired during the period that follows ischemia and reperfusion, probably

because of the reduced ability of the endothelium to synthesize NO after the ischemic insult. According to our results and to the findings discussed above, it can be speculated that the ACh-mediated vasoconstriction unmasked by the functional impairment of the endothelium could worsen the ischemic damage by decreasing blood flow in a condition that requires an enhanced oxygen supply.

L-NAME, but not other NOS inhibitors such as L-NNA, has been demonstrated to exert an anti-cholinergic action by blocking the muscarinic receptors in pulmonary vessels,[33] hindlimb[46] and coronary arteries.[10] The anti-muscarinic action of L-NAME has never been demonstrated in the cerebral vascular system, and has recently been questioned both by binding studies in the rat brain[24] and by functional studies in the small intestine.[6] The slow time-course of the L-NAME effect observed in our experiments (onset after 3 h of direct arterial perfusion) is compatible with an enzymatic inhibition rather than a receptor antagonism. The demonstrations that (i) L-NNA and L-NAME block ACh-mediated dilation with a similar time-course, (ii) atropine-sensitive, ACh-induced vasoconstriction is still observed after prolonged perfusions with L-NAME (up to 4 h), and (iii) intraluminal perfusion with atropine does not lead to vasoconstriction, suggest that the increase in vascular tone mediated by L-NAME should not be ascribed to receptor antagonism of ACh on cerebral vessels, i.e. to the release of a cholinergic dilatory tone.

In a recent study, cholinergic denervation of cortical microvessels in Alzheimer's desease was found to be coupled with an increase in the caliber of the cortical capillaries, interpreted as a compromised ability to adapt cortical perfusion via cholinergic modulation.[41] These results might represent indirect evidence that cholinergic activation exerts a tonic vasoconstriction on the cerebral vascular system. Our observation of an ACh-mediated vasoconstriction could speculatively be called upon to explain these results.

CONCLUSIONS

The present study demonstrates, for the first time, a direct constrictory effect of physiological concentrations of ACh on undamaged cerebral vessels in which the NO-dependent dilation has been functionally impaired. The experiments confirm that the *in vitro* isolated guinea-pig brain is a suitable preparation to study the brain vascular system in a condition of preservation of the neuronal activity, vascular responsiveness and endothelial function. The combined evaluation of the interactions between the neuronal and vascular compartments in this preparation could be useful to understand the complex mechanisms that regulate brain excitability in physiological and pathological conditions.

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