Acta Neurochir Suppl (2008) 104: 139–147

Printed in Austria

RETRACTED CHAPTER: Dysfunction of nitric oxide synthases as a cause and therapeutic target in delayed cerebral vasospasm after SAH

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Summary

Nitric oxide (NO), also known as endothelium-derived relaxing factor, is produced by endothelial nitric oxide synthase (eNOS) in the intima and by neuronal nitric oxide synthase (nNOS) in the adventitia of cerebral vessels. It dilates the arteries in response to shear stress, metabolic demands, pterygopalatine ganglion stimulation, and chemoregulation. Subarachnoid haemorrhage (SAH) interrupts this regulation of cerebral blood flow. Hemoglobin, gradually released from erythrocytes in the subarachnoid space destroys nNOS-containing neurons in the conductive arteries. This deprives the arteries of NO, leading to the initiation of delayed vasospasm. But such vessel narrowing increases shear stress, which stimulates eNOS. This mechanism normally would lead to increased production of NO and dilation of arteries. However, transient eNOS dysfunction evoked by an increase of the endogenous competitive nitric oxide synthase (NOS) inhibitor, asymmetric dimethyl-arginine (ADMA), prevents this vasodilation. eNOS dysfunction has been recently shown to be evoked by increased levels of ADMA in CSF in response to the presence of bilirubin-oxidized fragments (BOXes). A direct cause of the increased ADMA CSF level is most likely decreased ADMA elimination due to the disappearance of ADMA-hydrolyzing enzyme (DDAH II) immunoreactivity in the arteries in spasm. This eNOS dysfunction sustains vasospasm. CSF ADMA levels are closely associated with the degree and time-course of vasospasm; when CSF ADMA levels decrease, vasospasm resolves. Thus, the exogenous delivery of NO, inhibiting the L-arginine-methylating enzyme (IPRMT3) or stimulating DDAH II, may provide new therapeutic modalities to prevent and treat vasospasm. This paper will present results of preclinical studies supporting the NO-based hypothesis of delayed cerebral vasospasm development and its prevention by increased NO availability.

Keywords: Nitric oxide; NO donors; SAH; vasospasm; PDE; ADMA; nitrite.

Introduction

Annually as many as 28,000 Americans suffer subarachnoid haemorrhage (SAH) from a ruptured intracranial aneurysm. About one week after the SAH, a severe narrowing of the cerebral arteries develops in up to 70% of them [42, 86, 92, 107] and results in delayed ischemic neurological deficits (DIND) in about 25% of these patients. Half of the post-SAH patients suffer severe permanent neurological dysfunction or death due to DIND [42, 86, 92]. Despite intensive worldwide research, the fact that the first report of DIND was published in the mid-nineteenth century [26] and that cerebral vasospasm was diagnosed for the first time more than 50 years ago [18, 81], its pathomechanism remains unclear [70].

In spite of some controversies, hemoglobin has been accepted as a cause of vasospasm [53, 54]. Since the discovery that nitric oxide, an endothelium-derived relaxing factor [22], has 1000 times higher affinity for hemoglobin than oxygen [52], neurosurgeons and neuroscientists have been interested in its role in cerebral vasospasm after SAH [2, 8, 16, 44, 45, 55, 64–66, 70, 87, 90, 92, 94, 95, 103]. NO influence on blood flow [11, 15, 99, 106, 113], disappearance of neuronal immunoreactivity from the arteries in spasm [75], endothelial nitric oxide synthase dysfunction in cerebral vessels after SAH [37], decreased levels of nitrite in the CSF during vasospasm development [40, 70, 76], as well NO affinity for the heme moiety [52] together strongly suggest that decreased availability of NO in the cerebral arterial wall after SAH is responsible for delayed cerebral vasospasm [70]. Recent research has significantly advanced our understanding of the NO-related pathophysiological changes in the cerebral arteries leading to vasospasm and introduced new possibilities for NO-based therapy for vasospasm [23, 76, 98].

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The original version of this chapter was retracted.
The retraction note to this chapter can be found at
https://doi.org/10.1007/978-3-211-75718-5_93.

NO and pathomechanism(s) of delayed cerebral vasospasm

There is little doubt that ferrous hemoglobins (oxyhemoglobin and deoxyhemoglobin) slowly released from erythrocytes in the subarachnoid space oxidized and metabolized are directly and/or indirectly responsible for the development of cerebral vasospasm [13, 54, 71]. At the time of vasospasm, the nNOS-expressing (nitroxic, neuronal NOS-containing) neurons disappear from the arterial adventitia [75], diminishing NO availability and resulting in vasoconstriction [70]. However, this initial narrowing of the artery stimulates eNOS by increased shear stress [35]. Thus, increased NO production should counteract decreased NO availability and lead to vasodilation. But, the persistence of delayed cerebral vasospasm, lowered cyclic GMP levels in the arterial wall [43], and decreased nitrites in the CSF [39, 70, 73, 76] with preserved expression of eNOS [75] suggest the existence of an endothelial dysfunction that affects eNOS and decreases NO production [37]. This eNOS dysfunction may result from an increased activity of phosphodiesterase (PDE) leading to a quicker elimination of 3′, 5′ cGMP [90] or as recently has been shown, it may be evoked by the endogenous inhibition of eNOS by asymmetric dimethylarginine, an endogenous inhibitor of NOS [39], probably in response to the presence of oxidized degradation fragments of bilirubin in haemorrhagic CSF [13]. Recently, the presence of ADMA (Jung *et al*., in press) and BOXes in the CSF and their association with the degree and time course of vasospasm have been reported in patients with SAH [13, 78]. This mechanism sustains vasospasm. Then, in the last phase of vasospasm, oxidation and elimination of BOXes reduce ADMA levels in the CSF [39] (Jung *et al.*, in press), resulting in increased NO production by eNOS and recovery of endothelial dilatory activity [70].

Decreased nitrite levels and their close correlation with development and degree of vasospasm after SAH [39, 70, 73, 76] further supports the hypothesis that decreased NO availability is responsible or at least significantly contributes to cerebral vasospasm [70]. Reversal and prevention of cerebral vasospasm by NO/NO donors support this hypothesis [2, 72].

Thus, decreased NO availability in the cerebral conductive arteries responsible for development of vasospasm is evoked by the initial elimination of nNOS (first hit) by oxyhemoglobin followed by the inhibition of eNOS by ADMA in the perivascular space (second hit).

These observations suggest that the NO-based mechanism of delayed cerebral vasospasm remains not only multifactorial or affecting different structures of the arterial wall but also longitudinal (i.e., dependent on the time-related change in hemoglobin released from the subarachnoid clot) [70] and as such should be addressed accordingly.

NO-based prevention and treatment of vasospasm

Incomplete understanding of the etiology of vasospasm has hindered developing successful treatment [53, 109, 110]. Although the pathogenesis of vasospasm after SAH is probably multifactorial, imbalance between vasoconstricting (endothelin-1, endothelium-derived constricting factor) and vasodilating influences on vascular tone in response to the presence of blood in the subarachnoid space almost certainly play an integrating role [54, 71]. The above-mentioned mechanisms of initiation, sustenance, and resolution of delayed cerebral vasospasm open the possibility to develop vasospasm-preventing treatment with NO replacement and sequential, targeted therapy, which may yield novel treatment for this life-threatening complication of SAH.

Neuronal NOS protection

The initial treatment directly after SAH was proposed many years ago [20, 27] and fortunately was recently rediscovered [47]. It is to remove the clot and bloody CSF, thereby decreasing levels of neurotoxic oxyhemoglobin in the vicinity of conductive vessels. Recombinant tissue plasminogen activator (rt-PA) was used to enhance the effect of CSF drainage [20]. The removal of blood and its degradation products from the vicinity of the cerebral arteries should prevent the death of noxinergic neurons in the adventitia of the arteries, and block the initial spasm of the arteries as well as decrease the availability of oxyhemoglobin that can be metabolized to BOXes. However, it is unlikely that all the blood can be removed. Since in this phase of vasospasm, the dominant effect that has to be blocked is oxyhemoglobin neurotoxicity, the chelation of ferrous iron of oxyhemoglobin by an intracellular Fe^{+2} iron chelator such as dipyridyl has been proposed [33]. Eliminating ferrous hemoglobin by either or both of these methods may prevent neuronal apoptosis in the adventitia, protecting a basic mechanism of the neuronal vasodilatory response [75, 101]. Another beneficial effect of both these therapies may be to reduce oxidative stress in the subarachnoid space and in the vicinity of the conductive arteries that should then decrease the levels of vasoac-

tive heme metabolites, especially BOXes [13]. This should successfully block the deactivation of DDAH thus limiting ADMA increase in the CSF and dysfunction of eNOS.

NO delivery: systemic

During the initial phase of vasospasm, NO replacement may be a helpful adjunct because it should quench oxyhemoglobin as has been proposed by Doyle [17] leading to its oxidation (methemoglobin) and/or nitrosylation/nitration (SNO-hemoglobin, Fe(II)HbNO). This NO-based quenching effect on ferrous hemoglobins (''the reversed sink effect'') should enhance the effectiveness of CSF drainage and iron chelation resulting in further protecting nNOS and eNOS activities.

In the past, NO was administered systemically in the form of nitrates as nitroglycerin (NTG) and sodium nitroprusside (SNAP) [21, 31, 45]. Intravenous delivery of NTG/SNAP was efficacious in preventing cerebral vasospasm in animal models [19, 36, 60]. However, using NTG/SNAP in animals and patients was limited by its strong hypotensive effect [19, 45]. Therefore it was proposed that NTG/SNAP be combined with hypotensive agents [3]. Furthermore, a non-discriminative dilation of the cerebral vasculature led to the development of the ''steal syndrome'' [4, 36, 68] increased ICP [19], and lower perfusion pressure. Thus, this technique of NO delivery did not spark clinical interest because of the high risk of potential ischemic complications (especially in hemodynamically unstable patients with cerebral vasospasm) and the difficulty to predict pharamacokinetics because nitrates require an enzymatic step to release NO [5, 29, 93].

Recently, small-dose nitroglycerin delivery via a transdermal patch was shown to prevent cerebral vasospasm in a rabbit model of SAH, thus avoiding the undesirable decrease in blood pressure [36]. But its effectiveness needs to be confirmed clinically. Furthemore, the long-term therapy (2–3 weeks) with SNAP resulted in cyanide toxicity [80]. We have also tried intravenous delivery of a newly developed NO donor [82], which spontaneously releases NO and has an extremely short half-life (1.8 sec). However, we saw no effect on delayed cerebral vasospasm before decreased arterial blood pressure was observed (Pluta, unpublished data).

Despite yielding positive results in experimental settings and in some preliminary pilot clinical studies, nitrates as NO donors had limited effectiveness because of their significant vasodilatory peripheral effect, which led to decreased blood pressure (with possible disasterous decrease of CBF or cyanide toxicity). However, all these obstacles can be overcome by the systemic use of nitrite.

Nitrite, on demand, local but systemically administered NO donor

Recently it has been reported that, in the blood, nitrite is an endogenous NO donor [14, 108] representing a major bioavailable pool of NO with deoxyhemoglobin acting as a nitrite reductase during hypoxic conditions in the acidic environment [10, 14, 59]. Similar conditions (i.e., presence of deoxyhemoglobin [71] and low pH [83]) exist in the subarachnoid space after SAH. Therefore, the lower CSF nitrite levels after SAH and during development of vasospasm may be caused not only by a decreased NO production by neuronal and endothelial NOS, but also by an increased consumption of nitrite. Therefore, the intravenous delivery of nitrite should overcome diminished NO production in the arterial wall after SAH.

Nitrite has unique properties as an endogenous NO-donor. Under physiologic pH, nitrite forms nitrous acid, which can react with nitrite to form N_2O_3 [25]. These reactive nitrogen species can nitrosate thiols (which can also be vasoactive) or, in the presence of an electron donor, produce NO [14, 25]. Recently, this mechanism was confirmed both *in vitro* [59] and *in vivo* [14, 76, 108], showing that deoxyhemoglobin and presumably other deoxyheme proteins reduce nitrite to NO. We tested the hypothesis that nitrite releases NO locally in the subarachnoid space in a primate model of SAH [76] and demonstrated that the intravenous continuous infusion of sodium nitrite for 14 days prevents the development of vasospasm without any effect on blood pressure and with only clinically insignificant increases of methemoglobin levels in blood.

Despite these good safety records and the fact that nitrite has been used for centuries in the meat, poultry, and fish industries because of its antibacterial action, especially against botulinum spores [89], there are potential problems with its use. An FDA-supported study reported that nitrite doubles the risk of lymphomas in rat [62] and suggested that it had increased cancer incidence and tumor growth rate in animal studies [91]. Nevertheless, the human studies did not clarify this issue. Some of them confirmed the association between nitrite in food and neoplasm development, especially in the brain [34]; others were inconclusive [57]; and some complete-

ly rejected the association, at least in adults in Eastern Nebraska [12]. Furthermore, one study has shown that inhalant nitrite increased angiogenesis which results in accelerated tumor growth [102], while another demonstrated that the increased nitrite levels correlated positively with vasculo- and angiogenesis [24]. But the opposite effect was also reported, showing that NO inhibited angiogenesis and tumor growth [69]. These controversial and unclear results [34], the fact that nitrite is still used in the meat industry [89], and the recently reported presence of nitrite and nitrosamines in many organs including brain, aorta, liver, kidney, and the heart [10] suggest that: 1) nitrite may not be as dangerous as previously thought, and 2) carefully designed epidemiological studies of the biological role of nitrites are necessary. Additionally, well-planned studies of dosing and adverse effects of sodium nitrite should elucidate the pharmacokinetics of sodium nitrite in humans, establish the proper dosage and safety profile, and hopefully offer a new therapeutic modality for patients surviving aneurysmal SAH.

NO delivery: regional

The isolation of brain vasculature for regional drug delivery was developed many decades ago with the first cerebral arteriography performed by Moniz in 1935 [58]. The development of cerebral arteriography followed by a nonselective opening of the blood–brain barrier with the intracarotid infusion of mannitol for chemotherapy [61] and the development of endovascular treatment for vascular CNS diseases by Serbinienko [88] have proven the therapeutic possibility of isolating the brain vasculature using endovascular access. They were further followed by intraarterial angioplasty and the delivery of papaverine against vasospasm [41, 51], as well as intraarterial administration of rt-PA to treat thrombotic stroke [9, 84]. Thus, to avoid the peripheral vasodilatory effect of systemic NO donor administration, two changes have been made: the route of administration was changed to direct intracarotid/intracerebral arteries infusion [2, 41, 51, 74] and nitrates were replaced by nonenzymatic NO donors. These donors included: NO gas solution [2], 3-morpholinosydnonimine (SIN-1), S-ntroso–N-acetylpenicillamine (SNAP), S-nitrosoglutathione (GSNO) [111], NONOates [74, 82], and recently nitrite [76]. Among all NO donors, NONOates have received the most attention, due to the release of NO with predictable pharmacokinetics (half-life ranging from a second to several hours). ProliNO with a $T_{1/2} = 1.7$ sec was an obvious favorite for studying the NO effect on cerebral vasculature [72, 74, 108]. However, because of the obvious disadvantages of intracarotid/intracerebral arterial drug administration that include the increased risk of severe complications, patient and family anxiety, and the necessity of around-the-clock accessibility of the neurointerventional team, this treatment was not clinically attractive.

NO delivery: local

Traditionally, local delivery means that a drug is administered directly in the vicinity of a targeted area. Until recently, with regard to NO delivery, this meant that NO gas, NO donors, or nitrate had to be delivered to the affected organ, the lung (inhalation) or topically on the surface of the exposed tissue.

In the case of aneurysmal SAH, local administration delivers NO donors intrathecally or intraventricularly [19, 79, 97, 98, 112]. Such a route can avoid many disadvantages of systemic administration. However, drug distribution through intrathecal delivery in the SAH setting is poorly understood. It is difficult to accept that any compound delivered intrathecally and/or intraventricularly with the thick clot enveloping the conductive arteries in the subarachnoid space can penetrate the clot to reach the arterial wall to exert its effect directly on this artery. Moreover, intrathecal and/or intraventricular delivery of a strong vasodilator can cause vessels that are more easily accessible (i.e., those that are not covered by the clot) to further dilate, resulting in the ''steal syndrome'' [4, 68]. None of these issues has been properly addressed, either experimentally or clinically and both a beneficial effect [19, 97, 98, 112] and failure to improve cerebral vasospasm were reported with NO donors administrated via these routes [79].

It appears that all of the abovementioned drawbacks of NO delivery can be avoided by the newly proposed delivery of the NO donor directly into the vicinity of the artery by placing a controlled-release polymer loaded with the NO donor at the time the aneurysm is surgically repaired. This method was reported to prevent vasospasm with the NO donor and ibuprofen in a primate model of SAH but needs further clinical confirmation [23, 77]. The obvious disadvantage of this method is that it requires surgical access to the region of interest and with the rapidly increasing popularity of endovascular therapy [28, 63] instead of surgical treatment for intracranial aneurysm, its use may be limited.

Another NO addressing therapies for cerebral vasospasm

Inhibition of ADMA production

NO production is tightly controlled by multilevel mechanisms requiring the presence of oxygen and L-arginine as substrates for enzymatic cleavage of NO by NOS, the enzyme, which for proper action requires the presence of several co-enzymes (heme, flavin adenine mono- and dinucleotides, NADPH, and tetrahydrobiopterin) as well as co-factors (calcium and calmodulin) [7]. Furthermore, NOS activity is modulated by an ''internal'' negative feedback between NO and a heme moiety of NOS [35], as well as by the competitive inhibition of NOS by ADMA produced by double methylation of L-arginine by a type I protein-arginine methyl transferase (PRMT I) and degraded by dimethylarginine dimethylamonihydrolase (DDAH) [104].

We have shown that in a primate model of SAH and in patients following a ruptured aneurysm, AMDA CSF levels significantly increased concurrently with the development of vasospasm and gradually decreased with vasospasm resolution [39] (Jung *et al.*, in press). The degree of arteriographic vasospasm and the concentration of ADMA in the CSF were tightly correlated and CSF ADMA levels followed the time course of vasospasm [39] (Jung *et al.*, in press). These results suggest that the endogenous inhibition of NOS by ADMA may be a source of endothelial dysfunction facilitating and supporting development of cerebral vasospasm following SAH especially since DDAH2 activity disappears from the arteries in spasm after SAH [39].

The regulation of PRMT and DDAH activities has recently been carefully studied. It has been reported that the second end-product of NOS activity, L-citrulline, inhibits DDAH [104] and that S-nitrosylation of DDAH also inhibited its action [50]. Moreover, it has been shown that LDL-cholesterol upregulates ADMA synthesis by the activation of PRMT [6] suggesting that statins, drugs lowering plasma cholesterol levels, may at least indirectly affect DDAH activity. Statins are known to correct endothelial dysfunction [30, 85] and recently simvastatin was shown to increase eNOS activity and ameliorate vasospasm [56]. Yet, another cholesterol-lowering drug, probucol, was shown to promote the functional re-endothelization of the stripped aorta [48] and to preserve the endothelial vasodilatory functions by reducing ADMA levels [38]. Thus, we used probucol, a drug a with high octanol/water partition coefficient (logP 10.91) [85], which assures a significant penetration of the blood–brain barrier, to inhibit increased ADMA levels in the CSF after SAH and to prevent the development of vasospasm in a primate model of SAH [73].

In the *in vitro* experiments, probucol confirmed [38] its potency to decrease ADMA production by endothelial cells. It also increased nitrite levels *in vitro*, suggesting that it stimulated NO production [49] by eNOS [46, 96, 114] in response to increased DDAH activity [1].

These results encouraged the preclinical trial of probucol in a double-blinded, placebo-controlled experiment to investigate its effectiveness to inhibit increased ADMA levels in the CSF and to prevent vasospasm. Unfortunately, probucol administered orally, despite achieving therapeutic levels in serum, failed to inhibit ADMA increase in the CSF or to prevent vasospasm after SAH.

Despite the clear failure of probucol, the results of this study do not exclude the possibility that pharmacologically lowering CSF ADMA levels by a proper agent [1] may prevent development of post-haemorrhagic delayed cerebral vasospasm. At this moment, at least two more drugs are of interest because they were shown to increase DDAH activity. Both estrogen [32] and all-trans-retinoic acid stimulated DDAH activity leading to increased NO production by eNOS [1].

Prevention of vasospasm by PDE selective inhibitor

NO relaxes smooth muscle cells and dilates blood vessels stimulating soluble guanylate cyclase (sGC), which produces 3′-5′cGMP. The latter sequestrates intracellular Ca^{2+}, which relaxes vascular smooth muscles. Intracellular cGMP is inactivated by cyclic nucleotide phosphodiesterases (PDEs). There are several isoforms of PDEs (Types 1–6); however, only PDE5 is abundant in vascular smooth muscle cells. PDE5 inhibitors (such as Viagra) have been used to increase blood flow and dilate blood vessels. However, their use is limited due to their non-selective activity. Recently, a group of highly selective intracellular PDE5 inhibitors (E4021, SCH 51866) was introduced in clinical trials to control hypertension, pulmonary hypertension, respiratory distress, platelet aggregation, and erectile dysfunction [67, 100, 105]. Thus, there is a possibility that increased 3′, 5′ cGMP in the cerebral arterial wall by selective inhibition of PDE5 can prevent development of delayed vasospasm after SAH. Nevertheless, to elucidate the role of cGMP and PDE5 inhibitor in development of delayed vasospasm, well-designed experimental and clinical studies need to be carried out.

Conclusion

Despite significant progress on the pathophysiology of delayed cerebral vasospasm following ruptured intracranial aneurysm, there is no treatment for this dreadful complication of SAH. However, recent advances in understanding the roles of NO, NO donors, NOS, and nitrite in physiological and pathophysiological conditions, suggest the possible development of a therapy which will address decreased NO availability in cerebral arteries, thereby avoiding the undesirable side effects of nitrates.

Acknowledgment

This research was supported by the Intramural Research Program of the NIH, National Institute of Neurological Disorders and Stroke.

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