INTRODUCTION TO REVISED APPLICATION

The application has been revised extensively and the changes are in regular font with the original grant in italics. We are grateful to the reviewers for their comments and criticisms. They have helped focus our studies and led to important advances in our knowledge of the mechanisms of cerebral vasospasm. The investigations now focus on the first section of the grant that was entitled Role of Nitric Oxide in Vasospasm.

The summary of the critique stated that there were concerns about “inadequate discussion of technical pitfalls, lack of discussion of alternative interpretations and conclusions and a diffuse approach to multiple apparently unrelated topics”. This led to a “superficial approach to important problems without investigating any in depth”. This has been addressed by eliminating the unrelated sections entitled Lipid Breakdown Products and Vasospasm and Extracellular Mechanisms of Vasospasm and by completion of work on Treatment of Vasospasm that has led us to conclude that magnesium (Mg2+) treatment does not prevent vasospasm. This allows us to focus on the role of nitric oxide (NO) in vasospasm. We have obtained important data that allow the hypothesis to be refined and now to be investigated in depth. Alterations in NO and the NO-cyclic guanosine monophosphate (cGMP)-protein kinase G (cGMP-dependent protein kinase or PKG) pathway are widely believed to be involved in vasospasm. Reviews of the literature written by some of the most respected basic scientists with expertise in cerebral circulation have cited a need to investigate this area and have suggested that vasospasm may be due in large part to loss of the vasodilator NO.5;12;32;71;72 The essential question remains, however, as to whether NO replacement can prevent vasospasm, which should be the case if the NO theory is correct. There is no convincing angiographic data to indicate that NO donors or, for example, intraventricular sodium nitroprusside, prevent or reverse vasospasm.80;81 At the International Conference on Cerebral Vasospasm in 2000, the investigator with the largest experience with this treatment indicated that he was unsure as to whether there was any effect on angiographic vasospasm (Jeff Thomas, personal communication, June, 2000). This is consistent with our data that suggest that deficiency of NO is not a sufficient cause and that other processes must be involved. Our preliminary data mandate a critical shift in thinking about the pathogenesis of vasospasm. We propose a series of experiments that we hope will provide the answers to the next set of questions about the mechanisms of vasospasm.

Critique 1 cites “insufficient discussion of experimental pitfalls” and that “alternative interpretations and conclusions are not realized”. We now discuss in more detail the possible interpretations of each of the experiments and what will be done based on different possible results. We discuss how experimental pitfalls will be overcome or circumvented. The electron paramagnetic resonance (EPR) spectroscopy results were criticized because of lack of quantitation and discussion of variability of results. This is now addressed. To determine specificity of formation of nitrosyl hemoglobin, we did EPR spectroscopy on blood clot placed in sites other than the subarachnoid space and found that nitrosyl hemoglobin is formed at these sites. There was “much concern with the use of sodium nitroprusside”. We completed this experiment and found that sodium nitroprusside increased cGMP in the cerebral arteries but did not prevent vasospasm. This suggests that mechanisms downstream or mediated separately from cGMP cause vasospasm or, as suggested by the reviewers, that sodium nitroprusside is toxic. Whether or not NO replacement prevents vasospasm is so fundamentally important to the advancement of knowledge in vasospasm that, as suggested by the reviewers, we began to examine the ability of a non-iron containing NO donor with a longer half-life, (Z)-1-[2-aminooethyl]-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (NOC-18 or DETA-NO), to prevent vasospasm. NO replacement with DETA-NO did not prevent vasospasm despite elevating cGMP in cerebral arteries. Our next key finding is that if a vasospastic artery is re-stimulated with blood clot 7 days after SAH at a time when it is vasospastic, then it contracts even further.95 Furthermore, the rate of reversal of vasospasm after removal of the blood clot is time-dependent. Vasospasm reverses more slowly the longer clot has been present. These results suggest that vasospasm does not fundamentally disrupt smooth muscle contraction but that there is rather a progressive disruption in the mechanism of vascular relaxation. It is possible that the
contractile apparatus in vasospasm behaves like a ratchet that allows contraction but not relaxation. It has been hypothesized that this resembles the rigor state which develops in skeletal muscle in response to depletion of adenosine triphosphate (ATP) but this is not supported by our data showing additional contraction is possible in response to repeated stimulation of vasospastic arteries. We recognize, however, that such a mechanism also could contribute to vasospasm.

The preliminary data suggest the effects of vasospasm must be due at least in part to events downstream of cGMP in the relaxation pathway and/or due to alterations in other contractile and/or relaxation pathways. Our findings contradict some previously published results but they are consistent with clinical facts about vasospasm. They explain the failure of sodium nitroprusside and papaverine to reverse vasospasm and are therefore validated by findings in humans and in our monkey model of vasospasm. Our preliminary data lead us to hypothesize that there is an alteration in the NO relaxation pathway downstream of cGMP and that potassium (K⁺) channels may mediate vasospasm. We propose investigations to work out the final common pathway.

The remainder of the critique 1 is of experiments in the sections on lipid breakdown products and vasospasm and extracellular mechanisms of vasospasm. These sections of the grant have been deleted so the concerns are not addressed.

Critique 2 also criticizes the “loosely organized” research plan that “addresses a number of important topics diffusely and superficially”. This and much of the subsequent critique has been addressed by deleting all of the experiments on lipid breakdown products, extracellular mechanisms of vasospasm and treatment of vasospasm. The reviewer recommends that we “stay focused on elucidating the mechanism of NO depletion and then explore extensively various therapeutic interventions aimed at preventing NO depletion”. We have taken this advice and will focus on NO depletion. NO depletion alone does not seem to be a sufficient explanation for vasospasm so we also focus our approach on what downstream mechanisms in vascular smooth muscle are involved in vasospasm.

The critique mentions lack of investigation of the role of inducible NO synthase (iNOS) in vasospasm. We have delineated the time course of changes in all 3 NOS isoforms after SAH in our monkey SAH model and measured NO in the subarachnoid space after SAH. These results show that eNOS and nNOS are reduced during vasospasm and that iNOS increases during this time. NO released into the perivascular space, however, remains below the limit of detection of the system that we employed, which was approximately 1 μM. We propose more sensitive methods to measure NO. As suggested by the reviewer, we measured nitrosyl hemoglobin formation in blood clots incubated in other sites such as in vitro and in the extradural space in vivo. Nitrosyl hemoglobin is formed in the subarachnoid space and extradural space in vivo, but not in vitro. In conjunction with our results described above, it seems that induction of iNOS may compensate for the reduction in eNOS and nNOS during vasospasm, but that these changes are not a fundamental cause of vasospasm because of the repeated failure of NO donors to prevent vasospasm.

The critique notes inconsistencies in the numbers of animals used. We clarified this and because of the difficulty in working out methods and mechanisms in monkeys, have proposed further experiments in a rat model.

In critique 3, the studies on Mg²⁺, while unrelated to the studies on NO, are supported. We therefore finished this work. Serum Mg²⁺ concentration can be elevated significantly into the accepted therapeutic range and yet does not reduce cerebral vasospasm. The recurring theme raised by the other critiques of the inclusion of unrelated areas of investigation, is addressed by deletion of the experiments on lipid breakdown products and extracellular mechanisms. The critique of these experiments is therefore not addressed. The reviewer raises the very interesting and important question of the relevance of experiments on the rat basilar artery in vitro to vasospasm in vivo. We are completely in agreement with the reviewer in this regard. On the other hand, it is extremely difficult to conduct extensive mechanistic studies in the monkey model. While prior versions of the rat model do not produce severe, delayed vasospasm like that occurring in monkeys or man, we have developed
and characterized a new model that does produce severe, prolonged vasospasm. We will test mechanisms in this model and confirm them in the monkey model. We suspect that the inability of prior models to cause severe, prolonged vasospasm is not because of a fundamental difference in the pathogenesis of vasospasm between species, but because the basilar artery in traditional mice, rat and dog models does not remain encased in blood clot for long enough and is thus not exposed long enough to the contents of erythrocytes released by hemolysate. In these models, blood injected into the subarachnoid space is washed away within 2 days, which primate experiments show is not enough time to produce severe, prolonged vasospasm.

Finally, the reviewer suggests using “chip technology to define regulatory mechanisms which are altered in the vascular wall during vasospasm”. We examined changes in gene expression during vasospasm in the monkey model of SAH. These results are described in our preliminary data. They demonstrate changes in a large number of genes such as those associated with inflammation and cell cycle and proliferation. We hypothesize that they reflect smooth muscle phenotype change during vasospasm. The recommendation of the reviewers of this grant to focus on one mechanism, however, has led us to not include investigations into this process in this grant.
A. LONG-TERM OBJECTIVES AND SPECIFIC AIMS

We are investigating cerebral vasospasm which is an important cause of cerebral ischemia after aneurysmal subarachnoid hemorrhage (SAH). The long-term objective of this grant is to determine the mechanism of vasospasm after SAH and to thereby develop treatments that will prevent and/or reverse it. It is known that vasospasm is caused by subarachnoid blood clot and that hemoglobin released from the clot is an important cause of vasospasm. The active portion of hemoglobin may be hemoglobin itself, breakdown products of hemoglobin, or substances produced by reactions stimulated by hemoglobin. The identification of these reactions remains uncertain. It is known, however, that these reactions cause smooth muscle contraction.11;46;69

Our clot removal and replacement studies in a monkey model of vasospasm have demonstrated that vasospastic arteries have impaired ability to relax. We have been unable to prevent vasospasm with NO donors despite the ability of NO donors to elevate cGMP in vasospastic arteries. These key findings suggest that vasospasm is at least in part due to a perturbation in vasodilatory mechanism(s) downstream of cGMP or in a separate relaxation pathway.95 Other investigators using our monkey model have suggested that NO donors can reduce vasospasm.61 We will therefore complete our studies to test the hypothesis that there is an NO-reversible component of vasospasm. In these studies, however, vasospasm was not completely prevented, supporting our data that there is a component of vasospasm that is not reversible with NO. We will thus conduct studies to test the hypothesis that there is an NO-independent component of vasospasm. These studies will investigate changes in second messenger pathways and effector mechanisms, in particular K+ channels, which mediate vasorelaxation downstream of NO and cGMP.74

1. Mechanisms of Impaired Vascular Relaxation in Vasospasm - NO-Reversible Vasospasm

A. Specific Aim 1: To define the extent to which vasospasm in monkeys is reversible with NO donors.
B. Specific Aim 2: To measure heme-NO adducts (nitrosyl hemoglobin) by EPR spectroscopy in clots removed from the subarachnoid space of monkeys at different times after SAH.
C. Specific Aim 3: To quantify NO in the perivascular space at different times after SAH in monkeys.
D. Specific Aim 4: To define the time course of changes in, and the immunohistochemical locations of, the 3 isoforms of NOS in cerebral arteries and perivascular blood clot after SAH in monkeys.

2. Mechanisms of Impaired Vascular Relaxation in Vasospasm - Vasospasm not Reversible with NO

A. Specific Aim 1: To measure PKG messenger ribonucleic acid (mRNA), protein and activity during the time course of vasospasm in monkeys.
B. Specific Aim 2: To assess K+ channel function during vasospasm in rats.
C. Specific Aim 3: To measure calcium (Ca2+) currents and smooth muscle Ca2+ handling during vasospasm in rats.
D. Specific Aim 4: To assess Ca2+ sensitivity of monkey cerebral arteries during the time course of vasospasm.

B. BACKGROUND AND SIGNIFICANCE

1. Vascular Relaxation is Impaired in Vasospasm

Vasospasm occurs when blood surrounds the outside of cerebral arteries (SAH) that normally are bathed by cerebrospinal fluid (CSF).42 The most common cause of SAH is rupture of a cerebral aneurysm. Vasospasm is contraction of the large conducting arteries of the circle of Willis that develops 3 to 4 days, is maximal at 7 days and resolves by 14 days after SAH. We demonstrated previously that hemoglobin in or released from subarachnoid erythrocytes contributes to the pathogenesis of vasospasm.45;46 This was based on experiments showing that injection of impure or pure hemoglobin solutions, in amounts found in the subarachnoid space after SAH, into the subarachnoid space of monkeys caused vasospasm that was angiographically and histologically similar to that occurring after subarachnoid placement of whole blood clot. There is abundant additional evidence that hemoglobin contributes to vasospasm.43 Mechanisms by which hemoglobin may cause vasospasm include binding and destruction of NO, generation of free radicals, increasing release of endothelin-
1 from endothelial cells, alteration of perivascular nerves, degradation to other vasoactive substances, metabolism of hemoglobin to bilirubin and/or bilirubin oxidation products and generation of other vasoactive lipid oxidation products and eicosanoids. Some of these processes are unlikely to be involved. There is at present no evidence that destruction of the nerves around the large cerebral arteries could cause severe vasospasm. Hemoglobin may increase release of endothelin-1 from endothelial cells but we reported that most of this increase is due to hemoglobin-mediated removal of NO. Furthermore, extraluminal hemoglobin probably does not penetrate into the arterial wall to the endothelial layer in significant enough amounts to do this. We have begun therefore investigating the remaining and likely mechanism that vasospasm might be due to hemoglobin binding and destruction of NO. This could be mediated by hemoglobin itself or by superoxide anion radical or other free radicals generated by hemoglobin. We have investigated some of these mechanisms using a monkey model of SAH and monkey cerebral arteries under isometric tension in vitro. Our preliminary data suggest that vasospasm is not completely reversible by NO replacement despite the ability of NO to elevate cGMP in vasospastic arteries. Events downstream of this or mediated by other relaxation pathways must be involved.

We considered that evidence supporting removal of NO by hemoglobin as a cause of cerebral vasospasm would be demonstration of heme-NO adduct formation (nitrosyl hemoglobin) in the perivascular space after SAH, prevention of vasospasm by replenishing NO and reproduction of vasospasm by administration of a NO scavenger distinct from hemoglobin. We provide here the first evidence for the formation of heme-NO adducts in the subarachnoid space after SAH in monkeys.

Preliminary reports suggested that NO donors can reverse experimental vasospasm when administered intraarterially or reduce it when given by continuous intraarterial infusion. These studies are limited by the small numbers of animals studied (typically only 4 animals with no controls), lack of measurement of cGMP in the vasospastic arteries and the mild to moderate vasospasm that was studied and which is known to be more easily reversible. There is a preliminary report that intrathecal sodium nitroprusside reverses vasospasm in man although the management of these patients was complicated by the conduct of balloon angioplasty in some of them. Despite the limitations of these studies and our inability thus far to prevent vasospasm with NO donors, our preliminary data demonstrating nitrosyl hemoglobin and reductions in eNOS and nNOS during vasospasm strongly suggest that NO could be deficient after SAH and could contribute to vasospasm.

The second finding that is important to our hypothesis is that vasospasm is associated with failure of vascular relaxation. This is derived from our studies of the effect of clot removal on vasospasm. We use a monkey model in which SAH is created by placement of a blood clot into the subarachnoid space next to the right internal carotid, anterior cerebral and middle cerebral arteries (MCAs). The focal nature of the clot allows it to be removed and replaced at any time after the initial SAH or clot placement. We demonstrated that removal of the clot 1 to 7 days after SAH is associated with reversal of vasospasm. The rate of reversal of vasospasm depends on how long the clot has been present. The longer the clot has been present, the longer it takes for vasospasm to reverse. Second, there is no defect in the ability of the vasospastic artery to contract. Contraction can be maintained and even increased by a second application of blood 7 days after the first SAH. This is consistent with a study of 2 dogs in which injection of blood into the cisterna magna resulted in vasospasm lasting for several days. When the same amount of blood was injected 3 weeks later after the first vasospasm had resolved, there was immediate onset of even more severe vasospasm. This leads us to hypothesize that a defect in vascular relaxation contributes to vasospasm and that this is not due to a nonspecific disruption of the contractile apparatus.

There are other possibilities. First, the contractile apparatus could behave like a ratchet that allows contraction but not relaxation. A similar process has been suggested to underlie other types of tonic (prolonged) smooth muscle contraction although elucidation of its mechanism has been difficult and may in fact be a result of inhibition of relaxation processes or changes in Ca²⁺ sensitivity of the contractile apparatus.
We have chosen to focus on the latter 2 mechanisms in view of this and our preliminary data reviewed below. Second, delayed relaxation of vasospastic arteries following clot removal could be due to an alteration in the extracellular matrix of the arterial wall that maintains the artery in a narrowed state independent of the state of the smooth muscle cells. Indeed, numerous experiments demonstrated that vasospastic arteries studied under isometric tension in vitro are stiffer (less compliant) than normal arteries. These same studies, however, also demonstrate reduced ability of vasospastic arteries to contract to various agonists. It was suggested that these changes were due to fibrosis or some alteration in the extracellular matrix of the arterial wall. We were unable to demonstrate significant changes in the extracellular matrix of vasospastic arteries using immunohistochemistry and quantification of collagen by amino acid analysis although these methods may not be sensitive enough to detect subtle alterations. Reduced compliance and contractility could occur without detectable changes in the extracellular matrix as occurs, for example, during some types of arterial remodeling. It would have to be hypothesized that this stiffening impaired relaxation selectively, since the artery is able to contract further, at least to some degree. The other major problem with attributing lack of vasorelaxation to changes in arterial compliance and extracellular matrix composition is that every study thus far has demonstrated that such changes become progressively more severe over the second and third weeks after SAH, during which time vasospasm actually resolves.

2. Mechanisms of Vascular Relaxation that May be Altered in Vasospasm

Blood vessels relax when (1) the stimulus for contraction is removed, resulting in a “passive” fall in [Ca$^{2+}$], (2) there is active extrusion of Ca$^{2+}$ from the cytosol, resulting in reduction in [Ca$^{2+}$], and/or (3) there is reduced Ca$^{2+}$ sensitivity of the contractile apparatus.

With respect to the first mechanism, our clot removal studies have demonstrated the responses to removal of the stimulus. The effect of vasospasm on [Ca$^{2+}$] in vascular smooth muscle cells has been studied and [Ca$^{2+}$] has been reported to be increased or decreased. Most tonic (prolonged) smooth muscle contraction is not associated with a marked increase in [Ca$^{2+}$], and it seems unlikely that this would occur in vasospastic smooth muscle. We have repeatedly reported that hemoglobin and hemolysate, key candidates for the cause of vasospasm, cause only transient increases in [Ca$^{2+}$], in smooth muscle cells. Our proposed studies of K$^+$ channels will address Ca$^{2+}$ handling in vasospastic smooth muscle.

Regarding Ca$^{2+}$ extrusion, this may occur by multiple mechanisms (Fig. 1). Increased [Ca$^{2+}$] may result from Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ channels and possibly by receptor-operated Ca$^{2+}$ channels. It may also occur by release of Ca$^{2+}$ from intracellular stores via inositol 1,4,5-triphosphate (IP$_3$) or Ca$^{2+}$-induced Ca$^{2+}$ release. Extrusion or reduction in [Ca$^{2+}$] may occur by uptake into the intracellular stores or by extrusion out of the cell via Ca$^{2+}$ ATPases or the Ca$^{2+}$-Na$^+$ exchanger. Ca$^{2+}$-ATPases are located in the plasma membrane and the sarcoplasmic (or endoplasmic) reticulum. The plasma membrane Ca$^{2+}$-ATPase is inhibited by calmodulin and stimulated by PKG, and the sarcoplasmic reticulum Ca$^{2+}$-ATPase is regulated by phospholamban which is phosphorylated by cAMP-dependent protein kinase (PKA), PKG and Ca$^{2+}$/calmodulin-dependent protein kinase. Both types of Ca$^{2+}$-ATPase therefore are linked to NO-dependent relaxation downstream of cGMP and therefore need to be assessed in vasospastic smooth muscle. Some changes that have been noted to occur during vasospasm in dog basilar myocytes but whose molecular basis is unclear include impaired smooth muscle Ca$^{2+}$ regulation. There may be reduced function of the plasma membrane Ca$^{2+}$-ATPase after SAH in dogs, although a nonspecific assay was used so effects on the sarcoplasmic reticulum Ca$^{2+}$-ATPase are unknown.

The third mechanism of relaxation is alteration in Ca$^{2+}$ sensitivity of the contractile apparatus. The main regulatory mechanism in smooth muscle contraction is phosphorylation/dephosphorylation of the 20 kDa myosin light chain (MLC). MLC is phosphorylated by Ca$^{2+}$-calmodulin-activated MLC kinase and dephosphorylated by Ca$^{2+}$-independent MLC phosphatase. An increase in [Ca$^{2+}$], contracts smooth muscle by activating MLC kinase and phosphorylating MLC. However, the relationship between MLC phosphorylation and contraction versus [Ca$^{2+}$] is variable. MLC phosphorylation and tension can develop without changes in...
This would be an example of increased Ca$^{2+}$ sensitivity of the contractile apparatus. It is believed that Ca$^{2+}$ sensitization is mediated principally by activation of the GTPase RhoA that in turn activates Rho kinase. Rho kinase phosphorylates the regulatory subunit of MLC phosphatase and inhibits its activity. Relaxation can be associated with reduced Ca$^{2+}$ sensitivity. The mechanism involves NO activation of soluble guanylate cyclase in smooth muscle which elevates cGMP. Cyclic GMP activates PKG which relaxes by decreasing [Ca$^{2+}$], and by Ca$^{2+}$ desensitization. The intracellular pathways involved in NO-induced Ca$^{2+}$ desensitization are uncertain under physiological circumstances. We are interested primarily in whether or not vasospasm is associated with altered Ca$^{2+}$ sensitivity, which may be tested using permeabilized smooth muscle preparations suspended under isometric tension. There is evidence that the contractile apparatus may exhibit Ca$^{2+}$ sensitization during vasospasm.

**Figure 1:** Diagram of Ca$^{2+}$ regulation in vascular smooth muscle. PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; MLCK, myosin light chain kinase; ADP, adenosine diphosphate; ATP, adenosine triphosphate; IP$_3$, inositol 1,4,5-triphosphosphate.

Furthermore, there is evidence that alterations in K$^+$ channel function in vascular smooth muscle contribute to the prolonged cerebral vasospasm observed after SAH (Fig. 2). The key determinant of vascular tone is smooth muscle membrane potential, which is modulated primarily by K$^+$ conductance. Changes in vascular smooth muscle membrane potential of only a few millivolts cause large changes in vascular tone. The resting membrane potential in pressurized arterial smooth muscle cells lies approximately 30-45 mV positive to the equilibrium potential for K$^+$ (E$_K$), meaning that opening or closing of K$^+$ channels will lead to vasodilation or vasoconstriction, respectively. K$^+$ channels that have been characterized in cerebrovascular smooth muscle cells include inwardly rectifying K$^+$ channels (K$_{IR}$), adenosine triphosphate (ATP)-sensitive K$^+$ channels (K$_{ATP}$), voltage-dependent K$^+$ channels (K$_V$) and large conductance Ca$^{2+}$-activated K$^+$ channels (BK type of K$_{Ca2+}$ channel). K$_{Ca2+}$ and K$_V$ channels play a key role in regulation of membrane potential and vascular tone, and K$_{Ca2+}$ channels in particular have been shown to regulate membrane potential and vascular tone in human cerebral resistance arteries. These channels are uniquely situated to modulate vascular tone by virtue of their large conductance accompanied by sensitivity to [Ca$^{2+}$], as well as membrane potential, allowing these channels to function in synchrony with Ca$^{2+}$ channels and ryanodine receptors in controlling vascular smooth muscle membrane potential and arterial tone.
Figure 2: Pathways involved in NO-induced smooth muscle relaxation. SNP, sodium nitroprusside; PDE, phosphodiesterase; VOCC, voltage-operated Ca^{2+} channel.

Most evidence of involvement of K^{+} channels in vasospasm is indirect and based on pharmacological study of isolated arteries. Alterations in K^{+} channels detected directly by patch clamp recordings from isolated smooth muscle cells might be manifest as changes in current density, single channel conductance, open probability in relation to membrane potential and [Ca^{2+}], and sensitivity to pharmacological manipulation of the NO-cGMP-PKG-BK pathway. Electrophysiological recordings of vascular smooth muscle cells after experimental SAH demonstrated resting membrane potentials that were 10 to 20 mV depolarized with respect to controls. A single report in a rat single-hemorrhage model showed little difference in resting membrane potential between normal and vasospastic smooth muscle cells. Vasospasm in this model, however, was mild (< 7% reduction in basilar artery diameter) and less than that observed in other models including our rat model described below (> 20%). Pharmacological agonists of K_{ATP} channels partially reduce vasospasm after experimental SAH and NS1619, a pharmacological agonist of K_{Ca2+} channels, prevented contractions of rabbit basilar artery in response to hemolysate which is believed to be a key cause of vasospasm. Indirect electrophysiological evidence exists for decreased K^{+} conductance in vasospastic vascular smooth muscle and it is possible that the impaired relaxation in cerebral vasospasm is due to decreased availability of K^{+} channels as downstream targets of vasodilators such as NO and endothelial-derived hyperpolarizing factor (EDHF). The failure of endothelium-independent vasodilators such as papaverine to reverse vasospasm in our model suggest any potential role for EDHF is downstream of its endothelial release.

We have published the only direct evidence (ie. patch-clamp recordings of smooth muscle K^{+} currents) for alterations in K^{+} channels in vasospasm. Acute effects of hemolysate or hemoglobin on freshly isolated rat basilar artery smooth muscle cells were examined. Oxyhemoglobin enhanced outward K^{+} currents but these currents were not characterized further except to show that increased [Ca^{2+}] in the patch pipette or treatment with caffeine produced similar effects. Intracellular Ca^{2+} concentrations were not determined. The rapid decrease in input resistance and membrane blebbing that were observed suggest a direct toxic effect of the oxyhemoglobin solution used on the smooth muscle cells. This effect, however, has not been replicated when we used pure human hemoglobin A_{0} (Hemosol, Toronto, Canada) instead of impure hemoglobin (Sigma, St. Louis, MO). We next reported that hemolysate triggered Ca^{2+} release from IP_{3}-sensitive stores, leading to enhanced K_{Ca2+} current. Pure hemoglobin was not tested in these experiments. Since hemoglobin and hemolysate that contains hemoglobin are the principle causes of vasospasm, these findings suggest that alterations in K^{+} channels may occur during more prolonged exposure of cerebrovascular smooth muscle to these substances as occurs after SAH during vasospasm.

3. Choice of Animal Models

Determining the mechanisms of vasospasm may be very difficult to do in our monkey (Macaca fascicularis) model of SAH and vasospasm. The model produces vasospasm that is identical to that occurring in man. This accurate replication of the disease is an unequaled advantage of this model over all other models of SAH but the numbers of animals required and time that would need to be spent to work out mechanisms make it impractical. Therefore, we have developed and will use a rat model of SAH and vasospasm. We will use this...
model to define changes in vasodilatory mechanisms affected by vasospasm and then determine specifically whether or not these mechanisms occur in the monkey model. Rat models of SAH traditionally have been very poor models of human vasospasm because there is no significant delayed vasospasm. Our model has solved this limitation, suggesting that the inability to produce severe vasospasm in prior studies in the rat was not due to a fundamental difference in the mechanism of vasospasm in rats but due to the inability to keep high enough concentrations of blood around the cerebral arteries for a long enough time to cause severe, prolonged vasospasm.

4. Significance

The work done under this grant has defined thinking about cerebral vasospasm. We definitely demonstrated that hemoglobin causes vasospasm. Endothelin antagonists can reduce vasospasm but the role of endothelin in vasospasm probably is secondary. The role of ATP, one of the next most abundant substances in erythrocytes, was investigated and found to be minimal. Thrombin in clot probably contributes to the delay in onset of vasospasm. The experiments completed since the last review of this grant necessitate a fundamental change in thinking about the mechanism of vasospasm. We demonstrate that extracellular binding of NO by hemoglobin occurs and that there are alterations in the isoforms of NOS after SAH but that these phenomena do not completely explain vasospasm. NO replacement does not completely prevent vasospasm. Other processes must be altered in vasospasm.

Second, we demonstrate that vasospasm is not associated with a substantial defect in smooth muscle contraction but with a defect in relaxation. This is in spite of a large body of literature showing that vasospastic arteries have reduced contractility and compliance. This has led to the assumption that they are less able to contract. The basis for this was thought to be depletion of high energy phosphates in the smooth muscle cells. While this may occur, it may not be of fundamental importance in vasospasm. We previously have suggested that impaired relaxation contributes to vasospasm but this has always focused on impaired function of the NO system. We have advanced this theory with data showing that the extracellular removal of NO cannot account for vasospasm and that other processes in the smooth muscle cells themselves must be involved.

C. PROGRESS REPORT AND PRELIMINARY DATA

We published 20 peer-reviewed scientific articles, a textbook on cerebral vasospasm and 29 book chapters and invited review articles in the last funding cycle (listed below). Of 9 specific aims included in the last application, work was completed on 4 and begun on 2. Work was not done on the remaining 3 aims because of lack of support for the original hypothesis on the role of ATP and endothelins in vasospasm. Additional experiments were conducted on an additional 4 aims (5 years of support were requested, 4 were granted). The last application listed 8 specific aims. 1 was completed, 5 were not investigated further based on recommendations of the reviewers and work continues and is to be expanded on the remaining 2. Dr. Macdonald assumed the role of Principal Investigator, taking over from Dr. Weir. Dr. Weir will remain actively involved as co-Principal Investigator until his retirement in 2002.

Peer-reviewed manuscripts


**CHAPTERS**


1. Evidence of Failure of Relaxation Mechanisms in Vasospasm

Our clot removal studies in the monkey model of vasospasm suggest that there is impaired relaxation of vasospastic cerebral arteries. Initially these studies were conducted to determine whether or not the factors responsible for vasospasm after SAH cause the cerebral arteries to be narrowed independent of subarachnoid blood clot or whether the continued presence of clot is required for the entire time of vasospasm. The 3 key findings of this study were that vasospasm always is dependent on the presence of subarachnoid blood clot; ie. it reverses more quickly at any time if the clot is removed compared to if the clot is left in. Second, the rate of reversal becomes slower the longer the time from SAH to clot removal. Third, we removed the clots and made extracts of them and found that they still possessed significant contractile activity in vitro (Table 1) despite the

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fact that when they were in the monkey’s head, vasospasm was resolving. We suggested that there is an adaptive response in the cerebral arteries that may contribute to resolution of vasospasm. Other possible explanations were that clot removal has no effect on day 5 because the existing clot does not release spasmogens (the spasmogens are encased in the clot or removed by cells such as macrophages prior to exerting their effect on the arterial wall), the spasmogens cannot reach the arterial wall because of periarterial fibrosis or that the spasmogen is in the arterial wall by day 5 so that clot removal has no effect.

Table 1: Concentrations of ATP and Hemoglobins in Clot Extracts From the Subarachnoid Space of Monkeys. 

<table>
<thead>
<tr>
<th>Day after SAH</th>
<th>N</th>
<th>Total Hemoglobin (μmol/g)</th>
<th>% Ferrous Hemoglobin</th>
<th>ATP (μM)</th>
<th>Maximal Contraction (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8</td>
<td>3.5 ± 0.3</td>
<td>99 ± 0.3</td>
<td>9.9 ± 4.5$</td>
<td>0.154 ± 0.048</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>2.7 ± 1.4</td>
<td>97 ± 1.2</td>
<td>0.080 ± 0.016</td>
<td>0.161 ± 0.042</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>3.6 ± 0.4</td>
<td>89 ± 2†</td>
<td>0.050 ± 0.010</td>
<td>0.127 ± 0.034</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>3.3 ± 0.2</td>
<td>68 ± 8†</td>
<td>0.033 ± 0.008</td>
<td>0.118 ± 0.036</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
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<td>67 ± 10†</td>
<td>0.10 ± 0.06</td>
<td>0.092 ± 0.014$</td>
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</tbody>
</table>

Values are means ± standard deviation. $ p < 0.05 versus days 1, 3, 5 and 7. † p < 0.05 versus day 0 and 1. ‡ p < 0.05 versus days 0, 3 and 5. ¶ p < 0.05 versus days 0, 1, 3 and 5.

These hypotheses were tested in 17 monkeys that underwent baseline angiography and unilateral SAH. Angiography was repeated 7 days later and the clot then was removed and replaced with fresh clot (n = 7) or not replaced (n = 5). The removed clot was placed on the left side (n = 12). Control animals did not have clot removal on day 7 (n = 5) and/or had fresh clot placed on the left side on day 7 (n = 5). Angiography was repeated every 2 days until euthanasia on day 14. This study showed that if a new clot was placed on the right artery that had already been exposed to clot for 7 days, then even more severe vasospasm developed (Fig. 3). If the clot removed from the right was placed on the left, this clot also caused substantial vasospasm (Fig. 3).

We weighed clots at times of manipulations and note that a low weight of 7 day old clot (0.85 ± 0.47 g) produced as much spasm as a higher weight of fresh clot (3.81 ± 0.45 g). Histological examination of clots 7 days after SAH showed they were surrounded by CD68-positive cells (macrophages) that contained iron and that may sequester spasmogens. The clot itself contains spasmogens that are not released until the clot is removed, broken up and placed on the other side of the head. There is no barrier in the artery itself since placing a new clot causes vasospasm on the previously spastic artery.

These results suggest that failure of vascular relaxation is an important process in vasospasm. This is confirmed by the inability of papaverine to reverse established vasospasm in monkeys and in at least some cases in humans, as reviewed extensively in our book.42 The main action of papaverine is to inhibit phosphodiesterases and thereby elevate concentrations of cAMP and cGMP.52 Other reported actions include inhibition of voltage-gated Ca$^{2+}$ channels and inhibition of intracellular Ca$^{2+}$ release. Infusion of sodium nitroprusside or isoproterenol into vasospastic dog cerebral arteries caused statistically-insignificant 8 ± 1% and 10 ± 2% increases in arterial diameter, respectively, whereas cGMP and cAMP were increased 28% and 35% respectively.52 We tested the ability of papaverine (up to 200 mg) infused into the basilar artery to reverse vasospasm in dogs.49 The percent of vasospasm that could be reversed decreased with time, with 63 ± 11% reversal on day 7 and 20 ± 12% reversal on day 14.

We also assessed the ability of papaverine to reverse established vasospasm 7 days after SAH in the monkey model. Angiography and creation of SAH was performed on day 0. Angiography was repeated 7 days later and papaverine was infused into the internal carotid artery at its site of entrance into the cranium. Infusion of 20 to 130 mg papaverine had no effect on angiographic vasospasm (Fig. 4).
In summary, these data establish that vasospasm is associated with impaired relaxation and that there is a component of vasospasm that is not reversible by drugs that elevate cAMP and cGMP in smooth muscle cells.

**Figure 3:** Graphs of middle cerebral artery (MCA) diameter versus day after clot placement for arteries undergoing no manipulation (no clot removal, n = 5), clot removal (n = 5) or placement of new clot (clot-new clot, n = 7) (left graph). Placement of a new clot produces persistent and progressively more severe vasospasm in contrast to the resolution of vasospasm that occurs when the clot is removed or left in place. If the clot removed from the right is placed on the left (7 day old clot, right graph, n = 12), this clot causes the same degree of spasm as a fresh clot placed on day 0 (n = 16) or placed on the left 7 days after SAH on the right (n = 5). Values are means ± standard deviation.

**Figure 4:** Effect of selective infusion of papaverine into the internal carotid artery of monkeys 7 days after SAH. There is no significant effect on vasospasm. Values are means ± standard deviation (n = 4).

2. NO-Dependent Vasospasm

The first part of the last submission of this grant hypothesized that NO binding by hemoglobin and/or a reaction generated by hemoglobin, such as superoxide anion radical, removed the vasodilator NO and contributed to vasospasm. Our preliminary data confirm that this occurs although whether or not it contributes to vasospasm will be based on the ability of NO donors to prevent vasospasm. Other mechanisms, however, must be involved since NO donors tested thus far elevate cGMP in vasospastic arteries but do not prevent vasospasm. We first asked whether there was evidence that hemoglobin itself bound NO after SAH. Monkeys had baseline angiography followed by creation of SAH by clot placement in the subarachnoid space. They were euthanized 3, 7 or 14 days after SAH and the clots removed. Clots were subjected to EPR spectroscopy to measure heme-NO adducts (nitrosyl hemoglobin). Nitrosyl hemoglobin was not detected in fresh blood clot or when blood clot was incubated in vitro whereas it was detected in clots incubated in vivo in the extradural or the subarachnoid space (Fig. 5).
Figure 5: EPR spectra of fresh monkey blood clot (A) or blood clot incubated in vitro for 7 days (B) showing no nitrosyl hemoglobin. Clot removed from the extradural (C) or subarachnoid space (D) of monkeys 7 days after SAH demonstrates the characteristic triplet signal of heme-NO (nitrosyl hemoglobin). DPPH; diphenylpicrylhydrazyl.

Quantification of these results shows that nitrosyl hemoglobin is present 3, 7 and 14 days after SAH and that there is no significant difference between these times in the amount present in subarachnoid clot or in the amount present compared to extradural clot (Fig. 6). We will study 7 more animals per group in order to increase n to 10.

Figure 6: Nitrosyl hemoglobin concentration in clots removed from the extradural space 7 days after SAH or from the subarachnoid space 3, 7 or 14 days after SAH in monkeys. There were no significant differences between groups. Values are mean ± standard deviation (n = 3 per measurement).

These results are interesting because there is nitrosyl hemoglobin present 14 days after SAH which is when vasospasm has resolved. One explanation is that other processes mediate relaxation at this time. Another is that because there is little clot left at this time, what is present may not be able to bind more NO and this mechanism of producing vasospasm may be inactive. Another is that encasement of the clot limits access of hemoglobin in the clot to the vascular wall. We can test these theories by removing the clots and comparing EPR results from native clots with that from portions of the same clots that are bubbled with NO gas for increasing times up to 25 minutes. We demonstrated the ability to detect increasing nitrosyl hemoglobin by doing this with fresh blood clot (Fig. 7).

Figure 7: EPR detection of nitrosyl hemoglobin in fresh blood clot bubbled for increasing times with pure NO gas.

This would give us the value for maximal NO binding of the clot at least acutely and allow us to determine whether or not there is maximal nitrosyl hemoglobin formation in a particular clot.

Since there was evidence that hemoglobin binds NO after SAH and this reaction could thus contribute to vasospasm, we next tested the hypothesis that replenishing NO into the subarachnoid space after SAH could prevent vasospasm. A randomized, blinded study was conducted in which 13
monkeys underwent cerebral angiography and creation of right SAH. Subcutaneous osmotic pumps were implanted to deliver sodium nitroprusside (n = 7) or vehicle (0.9% NaCl, n = 6) via catheters directly into the area of the right MCA and adjacent subarachnoid clot. Angiography was repeated 7 days later and animals were euthanized.

Angiography showed that sodium nitroprusside did not prevent vasospasm (Fig. 8). Delivery of sodium nitroprusside was not the problem because there was a significant increase in cGMP in the MCAs of treated animals compared to vehicle (p < 0.05, unpaired t-test, Fig. 9). Furthermore, the group treated with nitroprusside had elevated concentrations of nitrosyl hemoglobin, measured by EPR spectroscopy and cyanomethemoglobin, measured by spectrophotometry, in CSF on day 7. A nitrosyl hemoglobin signal was detected in 4 of 6 placebo animals and all animals treated with sodium nitroprusside. Mean concentration of nitrosyl hemoglobin in the sodium nitroprusside group (94 ± 83) was higher than in the placebo group (17 ± 21, p > 0.05, unpaired t-test), representing a greater than 5-fold increase.

**Figure 8:** Bar graph of percent change between days 0 and 7 in angiographic diameters for right (A) and left (B) cerebral arteries. Comparisons within groups over time (paired t-tests) showed significant reductions in diameter as indicated. There were no significant differences between groups for any artery (values are means ± standard deviation, n = 5 - 7 per group, C3, extradural internal carotid artery; C4, intradural internal carotid artery; ACA, anterior cerebral artery).

**Figure 9:** Bar graphs of concentrations of cGMP (top row) and cAMP (bottom row) in right (left column) and left (right column) MCA and cortex from animals in the sodium nitroprusside and placebo groups. There was a significant increase in cGMP in the right MCA of animals given sodium nitroprusside compared to placebo animals (p < 0.05, unpaired t-test). There also was a trend for cGMP levels to be higher in right cortex and left MCA of animals treated with sodium nitroprusside compared to placebo (values are means ± standard deviation, n = 5 - 7 per group).

We concluded that the lack of effect of sodium nitroprusside was not due to inadequate drug delivery because cGMP levels were significantly increased in vasospastic arteries. Vasospasm may not have been prevented because of a toxic effect of sodium nitroprusside metabolites and/or involvement of
smooth muscle relaxation or contraction processes downstream of cGMP or involving different pathways.

An important piece of data is the level of elevation of cGMP that is required to induce relaxation. We tested this by suspending rings of monkey basilar artery under isometric tension in vitro. Concentration-contraction curves in response to addition of prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) were constructed. Rings were washed and then precontracted with a concentration of PGF$_{2\alpha}$ that caused a 50% maximal contraction and then relaxed with DETA-NO (1 mM) or treated with phosphate buffer as controls. They were frozen in liquid N$_2$ and assayed for cAMP and cGMP. A 3-fold increase in cGMP was associated with a 100% relaxation of precontraction tension (Table 2). We express the values as a fold change because the absolute values are difficult to interpret due to differences in baseline cGMP concentration that may be secondary to changes occurring when the arterial ring is not innervated or perfused.

Table 2: Concentrations of cGMP and cAMP in Arterial Rings Tested in Vitro After Exposure to DETA-NO or in Vivo After Exposure to Sodium Nitroprusside*

<table>
<thead>
<tr>
<th>Compound</th>
<th>In Vitro Control</th>
<th>DETA-NO</th>
<th>Fold Increase</th>
<th>Right MCA In Vivo Control</th>
<th>Nitroprusside</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP</td>
<td>2.18</td>
<td>1.91</td>
<td>0.88</td>
<td>15.5 ± 9.4</td>
<td>13.0 ± 4.9</td>
<td>0.8</td>
</tr>
<tr>
<td>cGMP</td>
<td>1.58</td>
<td>4.70</td>
<td>2.97</td>
<td>0.22 ± 0.18</td>
<td>0.63 ± 0.29</td>
<td>2.9</td>
</tr>
</tbody>
</table>

* Values are pmol/mg protein and are mean ± standard deviation (n = 1 - 7 per group).

The reviewers raised concerns over the use of sodium nitroprusside and recommended the use of a non-iron containing NO donor with a longer half life. Of these donors, DETA-NO is an NO donor with a half life of > 500 minutes in 0.1 M phosphate buffer at pH 7.4 and 22°C and therefore might be optimal. It was determined based on subarachnoid space volume CSF production rates and known amount of clot placed that it would be necessary to infuse up to 12 ml per day of DETA-NO, 1 mM. It was decided to infuse substances via a silicone elastomer catheter in the subarachnoid space next to the right MCA. The catheter was tunneled subcutaneously to an exit site near the midline in the interscapular area where it was connected to a pump for continuous infusion. The stability of DETA-NO under infusion conditions was tested by incubating solutions of DETA-NO at 22°C (the temperature in the reservoir on the back of the monkey) for days and testing their ability to relax the rat aorta precontracted with norepinephrine, 10 $\mu$M. It was determined that 30% of the activity was retained at 24 hours, suggesting that 70% of the NO was released in 24 hours or that the half life was under 24 hours. No activity remained after 7 days, suggesting that DETA-NO incubated for this time could be used as a control solution. Experiments in vitro demonstrated maximal relaxation of monkey basilar artery under isometric tension to 30-100 $\mu$M DETA-NO, which would allow 10-30 fold dilution in vivo. We randomly allocated monkeys to 3 groups (n = 2-4 per group thus far). All had baseline angiography and SAH on day 0 and repeat angiography and sacrifice on day 7 in addition to: 1. Infusion of DETA-NO, 1 mM at 12 ml/day, with fresh solution infused each day, 2. Infusion of DETA-NO that had been allowed to decompose for 7 days in vitro, 1 mM at 12 ml/day or 3. Controls with no other manipulations.

Endpoints were angiographic vasospasm and levels of cGMP and cAMP in the cerebral arteries. Vasospasm occurred in the 2 control groups (infusion of decomposed DETA-NO and no infusion, data for decomposed DETA-NO are shown, Fig. 10) and was associated with no difference between right and left side artery cGMP levels. Infusion of DETA-NO did not prevent vasospasm but did increase cGMP concentrations 5.0 ± 5.0 fold (not significant) in the right compared to the left MCA. There was some variability in cGMP levels so that more animals need to be done, nevertheless mild to moderate vasospasm was observed even in those with the highest increases in cGMP in the MCA. This suggests vasospasm is not going to be preventable by NO replacement, but that at there is at least a component that may be reversible with NO donors because animals
with high cGMP had mild to moderate but not severe vasospasm.

**Figure 10:** Bar graph of percent change between day 0 and day 7 in angiographic arterial diameters for right (A) and left (B) cerebral arteries. Comparisons within groups over time (paired t-tests) showed significant reductions in MCA diameter ($p < 0.05$) in animals treated with DETA-NO or vehicle (decayed DETA-NO). There were no significant differences between groups (Values are means ± standard deviation).

![Bar graph of percent change between day 0 and day 7 in angiographic arterial diameters for right and left cerebral arteries.](image)

We plan to complete these experiments. It seems unlikely that vasospasm will ever be prevented completely by NO donors, supporting our hypothesis that there is an NO-independent component of vasospasm. We will therefore, investigate the mechanism of this. If there is no effect of DETA-NO on vasospasm, we plan to administer NO donors by continuous intraarterial infusion into the internal carotid artery in order to determine if intraluminal NO replacement can prevent or reduce vasospasm, thus supporting our data suggesting that there is an NO-dependent component to vasospasm. We will pursue NO-dependent vasospasm because our preliminary data demonstrate plausible mechanisms such as presence of nitrosyl hemoglobin and, as reviewed below, reductions in NOS isoforms in vasospasm and because others have suggested that NO donors can at least partially prevent vasospasm.

An important question raised in the critique of the last grant submission is where the NO arises that binds to the heme and whether or not inducible NO synthase (iNOS) increases NO concentrations to levels that may exert deleterious effects or that would compensate for NO deficiency after SAH. We measured eNOS, nNOS and iNOS mRNA and protein 3, 7 and 14 days after SAH in MCAs in our monkey model using methods we have described. Angiography showed a 45 ± 13% (mean ± standard deviation, $p < 0.05$) decrease in MCA diameter 3 days, a 41 ± 23% ($p < 0.05$) decrease 7 days and an insignificant 6 ± 14% decrease 14 days after SAH. The RNA for eNOS was significantly reduced (1.7 ± 0.5-fold) 7 days after SAH. There was a significant, 1.7 ± 0.2-fold reduction in eNOS protein on days 3 and 7 after SAH that returned to normal by day 14. There was a statistically insignificant reduction in nNOS on days 3 and 7 followed by return to normal on day 14. There were no significant changes in iNOS mRNA whereas iNOS protein increased on days 3 and 7 (7 ± 9 and 2.7 ± 2.8-fold, respectively, $p > 0.05$) and significantly decreased (2.7 ± 1.1-fold, $p < 0.05$) on day 14. Immunohistochemistry localized eNOS to endothelium, nNOS to brain and perivascular adventitia and iNOS to inflammatory cells in the subarachnoid space.

The reciprocal changes in NOS isoforms suggest that it will be important to know the NO concentration in the perivascular space at different times after SAH in monkeys. We determined the ability to spin trap NO in the subarachnoid space after SAH. 1 mm diameter NO-permeable teflon tubing was shaped into a coil 5 mm in diameter. The ends were placed in a vial of solution containing the nitronyl nitroxide, D8 (provided by Dr. Halpern). These tubes were placed into the left (control side) and right (clot-side) subarachnoid space next to the MCAs of monkeys that were 3, 7 or 14 days after SAH. Multiple measurements could be conducted, and we also able to assess the partial pressure of oxygen in the perivascular space and the blood clot with a fiber-
optic oxygen sensor (Oxylite™ multichannel fibre-optic pO2 monitoring system, Oxford Optronics, Oxford, UK). With animals ventilated on room air in order to maintain normal arterial blood PO2 (P\textsubscript{a}O\textsubscript{2}), measurements in the subarachnoid space demonstrated normal PO2 in the subarachnoid space next to the MCA but progressive hypoxia within the subarachnoid blood clot (Table 3). We were unable to detect NO by this method, which was sensitive in vitro to solutions containing 1 µM NO. We will test 2 more sensitive assays for NO; amperometric NO sensors and microdialysis with hemoglobin which are methods that can be used in vivo and may be sensitive to nanomolar concentrations of NO. The clot PO2 measurements are consistent with our findings above suggesting that the clot becomes an encased, avascular, hypoxic mass after SAH and that this mechanism limits vasospasm by containing spasmogens such as hemoglobin. This may be critically important because the hemoglobin in the interior of the clot is ferrous hemoglobin (Table 1) that likely is mostly deoxyhemoglobin. It will only slowly oxidize to methemoglobin because of the lack of oxygen. The reactions of hemoglobin with NO include binding to heme, the β-cysteines of the globin chains, and oxidation reactions. Our PO2 measurements suggest which reactions will predominate in the hypoxic clot. Deoxyhemoglobin reacts most rapidly with NO but its encasement probably limits NO binding and therefore vasospasm.

**Figure 11:** Immunoblotting of eNOS and nNOS at different times after right (R) SAH in monkeys.

<table>
<thead>
<tr>
<th>Day</th>
<th>P\textsubscript{o}2</th>
<th>Left Subarachnoid Space PO2</th>
<th>Right Subarachnoid Space PO2</th>
<th>Right Subarachnoid Clot</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>94 ± 9</td>
<td>26 ± 2</td>
<td>23 ± 3</td>
<td>35 ± 6</td>
</tr>
<tr>
<td>7</td>
<td>91 ± 13</td>
<td>20 ± 6</td>
<td>23 ± 9</td>
<td>8 ± 5</td>
</tr>
<tr>
<td>14</td>
<td>94 ± 9</td>
<td>26 ± 2</td>
<td>21 ± 8</td>
<td>6 ± 4</td>
</tr>
</tbody>
</table>

* Values are means ± standard deviation, n = 3 per measurement.

### 3. NO-Independent Vasospasm

One reviewer recommended use of chip technology to try to gain understanding of molecular mechanisms of vasospasm. We used complementary deoxyribonucleic acid (cDNA) array analysis to define changes in gene expression occurring during vasospasm after subarachnoid hemorrhage (SAH). Right SAH was created in 3 monkeys and the right and left MCAs were collected 3, 7 or 14 days later. Vasospasm was assessed by angiography performed on day 0 and at tissue harvest. We used cDNA arrays containing 5184 genes to screen for changes in gene expression by comparing the right and left MCAs. There was significant (> 5-fold expression of mRNA compared to internal standard control) expression of 537 genes (10%) in the MCAs.
genes (31%) did not change significantly and 373 (69%) were differentially expressed at 3, 7 or 14 days after SAH. These 373 genes changed from 1.2 to 7-fold compared to control arteries. The most common pattern was a progressive increase with increased time after SAH. Functions of differentially-expressed genes included regulation of gene expression, cell proliferation, inflammation, membrane proteins and receptors, kinases and phosphatases. There was a marked increase in parathyroid hormone and parathyroid hormone receptor with time after SAH. Immunoblotting demonstrated a significant increase in parathyroid hormone receptor protein. The upregulation of these proteins involved in vascular relaxation suggests that they may play a role in vasospasm. The progressive increase in mRNAs involved in the functions noted above suggests that the pathogenesis of cerebral vasospasm involves cell proliferation, inflammation and possibly smooth muscle phenotype change.

In order to determine changes in smooth muscle cell K⁺ currents after chronic vasospasm, we have developed a reliable rat SAH model in our laboratory using clot placement against the basilar artery, mimicking the disease process in humans. This model recapitulates both angiographic vasospasm and histopathological changes observed in human and experimental vasospasm (Fig. 12). It produces more severe, prolonged vasospasm than injection models because a greater amount of blood is placed and remains in contact with the artery for a longer time (Fig. 13).

The relative availability and ease of use of the rat model will permit extensive investigations into the biophysical properties of K⁺ channels and of Ca²⁺ handling in dissociated normal and vasospastic smooth muscle cells in vitro. We will use this model to first define what specific changes occur in vasospasm. After we have defined alterations in these cells, we will determine if similar changes occur in the monkey model of SAH by comparing smooth muscle cells from monkey vasospastic (right) and control (left) MCAs.

We have acutely dissociated smooth muscle cells from the rat basilar artery and monkey MCA and have recorded whole-cell K⁺ currents from these cells using both conventional and nystatin-perforated patch-clamp techniques (Fig. 14). Single channel records demonstrate BK channels in these cells (Fig. 14), which contribute predominantly to recorded whole-cell currents as evidenced by sensitivity to tetraethylammonium (TEA), a specific blocker BK channels at concentrations of 1-10 μM (Fig. 14). Investigation of BK channel function in smooth muscle is incomplete without knowledge of Ca²⁺ influx and handling. We have thus investigated [Ca²⁺], dynamics and Ca²⁺ channels in smooth muscle cells using Ca²⁺ imaging (Fig. 15) and patch-clamp (Fig. 15) techniques. The aforementioned studies will be expanded to allow comparison of K⁺ channel function and Ca²⁺ handling in normal versus vasospastic smooth muscle cells.
**Figure 13:** Bar graph of per cent reduction in basilar artery diameter versus time after autologous clot placement against the basilar artery of rats (n = 6 per time, values are means ± standard deviation, p < 0.05 by unpaired t-test versus day 0).

**Figure 14:** Whole cell currents in response to ramp (A) and step (B) voltage protocols in a typical rat basilar smooth muscle cell are shown. TEA blocks these currents (C) with apparent $k_d = 404 \mu M$ (n=8). **D.** Single channel currents from cell-attached patches (held at indicated membrane potentials, mV) from rat basilar artery myocytes in isotonic K⁺ saline. **E.** Conductances of $166 \pm 8$ pS and $132 \pm 14$ pS (for inward and outward currents, respectively; n=2-5 per data point) were observed, showing typical mild inward rectification in cell-attached patches.
Figure 15: A. Small arteriolar branch isolated from rat basilar artery. B. Ca$^{2+}$ transients from arteriole pictured in C, in response to brief applications of KCl, 50 mM and Br A23187, 1 µM. Each different color trace represents the fura-2 ratio from a different region of interest measured across the arteriole. C. Whole-cell L-type Ca$^{2+}$ currents recorded from isolated rat basilar myocyte. D. Current-voltage relationship of same cell as in C, in response to 1 µM nicardipine and 1 µM Bay K 8644.

4. Lipid Breakdown Products and Vasospasm and 5. Extracellular Mechanisms of Vasospasm

We have not investigated these processes further based on the recommendations of the reviewers.

6. Treatment of Vasospasm

**Specific Aim:** To assess the efficacy of Mg$^{2+}$ in the prophylaxis of vasospasm in a primate model.

We completed work on this experiment. The appropriate dosing regimen for continuous intravenous administration of MgSO$_4$ was determined in control monkeys and found to be similar to that used in human stroke trials. Next, we began a blinded, placebo-controlled trial of MgSO$_4$. 7 animals were randomized and the results analyzed by an independent statistician (Dr. Karrison). It was noted that significant vasospasm developed in the 5 animals treated with MgSO$_4$ despite doubling of serum Mg$^{2+}$ (the goal is to double serum levels) after day 2 (Fig. 16, Table 4). When these results were compared to the controls and a cohort of previous untreated historical controls, there was no significant difference in vasospasm and the 95% confidence interval for the difference in means suggested that the maximal possible effect of Mg$^{2+}$ was to reduce MCA vasospasm by 8%. We considered it not worth pursuing because of the minimal potential therapeutic effect and because cerebral ischemia is not an endpoint in our model which is important because Mg$^{2+}$ is being studied in clinical trials of cerebral ischemia. While our results suggest that Mg$^{2+}$ will not significantly affect angiographic vasospasm, if a clinical trial demonstrated benefit in cerebral ischemia, trials in SAH would still be indicated.

Figure 16: Bar graph showing angiographic MCA diameters in 5 monkeys with SAH treated for 7 days with intravenous MgSO$_4$ compared to 24 historical controls ± 2 concurrent placebo-treated animals. There is significant vasospasm of all of the shown right side arteries except the control group ACA and there are no differences in degree of vasospasm between groups. Values are means ± standard deviation.
Table 4: Magnesium Levels in Monkeys With SAH Given Intravenous MgSO₄

<table>
<thead>
<tr>
<th>Day</th>
<th>Mg²⁺ Dose (g/kg)</th>
<th>Mg²⁺ Infusion (g/kg/d)</th>
<th>CSF Total</th>
<th>CSF Ionized</th>
<th>Serum Total</th>
<th>Serum Ionized</th>
<th>Serum % Ionized</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.086 ± 0.000</td>
<td>0.032 ± 0.004</td>
<td>1.69</td>
<td>1.27</td>
<td>0.73 ± 0.08</td>
<td>0.48 ± 0.05</td>
<td>66 ± 5</td>
</tr>
<tr>
<td>2</td>
<td>0.029 ± 0.041</td>
<td>0.032 ± 0.004</td>
<td>1.26</td>
<td>1.27</td>
<td>0.64 ± 0.24</td>
<td>0.64 ± 0.24</td>
<td>64 ± 4</td>
</tr>
<tr>
<td>4</td>
<td>0.022 ± 0.043</td>
<td>0.032 ± 0.004</td>
<td>1.47</td>
<td>1.47</td>
<td>0.88 ± 0.37</td>
<td>0.88 ± 0.37</td>
<td>61 ± 6</td>
</tr>
<tr>
<td>6</td>
<td>none</td>
<td>0.033 ± 0.004</td>
<td>2.04</td>
<td>2.04</td>
<td>0.96 ± 0.05</td>
<td>0.96 ± 0.05</td>
<td>59 ± 1</td>
</tr>
<tr>
<td>7</td>
<td>none</td>
<td>Stopped</td>
<td>1.58</td>
<td>1.58</td>
<td>2.39 ± 0.85</td>
<td>1.53 ± 0.32</td>
<td>55 ± 3</td>
</tr>
</tbody>
</table>

* Values are in mM except % and as indicated and are means ± standard deviations. N = 1 - 5 per measurement.

D. RESEARCH DESIGN AND METHODS

1. Mechanisms of Impaired Vascular Relaxation in Vasospasm - NO-Reversible Vasospasm

   A. Specific Aim 1: To define the extent to which vasospasm in monkeys is reversible with NO donors.

   **Hypothesis, Endpoints, Rationale**

   The hypothesis is that a component of vasospasm is due to hemoglobin-mediated abrogation of the tonic relaxant effect of basal NO release from cerebral arteries. Proof that this mechanism contributes to vasospasm would include demonstration of prevention of vasospasm by NO donors. We will test this using DETA-NO administered intrathecally. We will measure cGMP and cAMP in the cerebral arteries to determine if NO donors elevate cGMP in the cerebral arteries. This will ensure that adequate drug delivery occurred which will be important particularly if vasospasm is not completely prevented. EPR spectroscopy of removed clots will determine whether or not nitrosyl hemoglobin is elevated in clots removed from animals treated with NO donors which should be the case. It has been suggested that the mechanism by which hemoglobin causes vasospasm is by removing NO.12;43;72 These experiments will advance the field by determining for the first time whether such a mechanism is important.

   **Experiment**

   The trial of intrathecal DETA-NO will be completed. Monkeys will undergo baseline cerebral angiography and creation of right SAH. They will be randomly allocated to receive (1) DETA-NO, 1 mM at 12 ml/day intrathecal, with fresh solution infused each day, (2) infusion of DETA-NO that had been allowed to decompose for 7 days in vitro, 1 mM at 12 ml/day, or (3) controls with no other manipulations. After infusion for 7 days, angiography will be repeated and animals euthanized. The cGMP and cAMP content of the right clot side and left control side MCAs and nitrosyl hemoglobin in the clots will be measured. Tissues from control untreated animals can be used for other studies described below.

   **Methods**

   1. **Monkey Model:** This model is extensively described in prior publications.22;77 On day 0, monkeys will be placed under general anesthesia. Physiological parameters that affect cerebral blood flow and arterial diameters and that might change during the anesthetic will be monitored (heart rate, temperature, PaO₂, PaCO₂, blood pressure). Fresh arterial blood will be withdrawn and allowed to clot. A portion will be stored in liquid N₂. Cerebral angiography will be performed. A right craniectomy and exposure of the right internal carotid, anterior cerebral and MCAs will be performed and clotted arterial blood will be weighed and placed next to the cerebral arteries. Wounds will be closed. For infusion of DETA-NO, a silicone elastomer catheter will be placed in the subarachnoid space along the MCA and tunneled subcutaneously to an exit site over the thoracic spine. It will be connected to a pump (CADD Legacy 1 ambulatory infusion pump model 6400, Sims Deltec, Inc., St. Paul, MN) carried in a vest (Lomir Biomedical Malone, NY) worn by the monkey. The pump will be filled with DETA-NO or DETA-NO incubated in vitro for 7 days. Control animals have no catheter or DETA-
NO exposure.

2. Measurement of cGMP and cAMP: Cyclic nucleotides will be measured by enzyme immunoassay. Artery segments will be removed at autopsy and rapidly frozen in liquid N₂. They will be homogenized in ice cold 6% trichloroacetic acid using a glass homogenizer. The homogenate will be centrifuged at 2000 x g for 10 minutes at 4°C to remove precipitated protein. The supernatant fluid will be extracted three times with 3 volumes of diethyl ether to remove the trichloroacetic acid and then heated to 70°C for 5 minutes to remove residual ether. The samples will be assayed for cAMP and cGMP using an enzyme immunoassay (Cayman Chemical, Ann Arbor, MI). The protein concentration in the pellet will be determined by BCA Protein Assay Reagent (Pierce, Rockford, IL). The cyclic nucleotide content of the artery segments will be expressed as pmol/mg of protein.

3. EPR Spectroscopy: 0.5 ml of frozen blood clot will be thawed, reduced with sodium dithionate (50 mg Na₂S₂O₄, Sigma, St. Louis, MO) and refrozen in liquid N₂ in 4 mm glass tubes. In order to test the specificity of formation of nitrosyl hemoglobin in the subarachnoid space, 0.5 ml of fresh blood clot as well as 0.5 ml portions of blood clot incubated for 1 to 7 days in the dark under sterile conditions at 37°C also will be assessed for formation of nitrosyl hemoglobin using the same method. EPR spectroscopy will be performed on a Varian E-12 X-band spectrometer operating at the X-band. Samples will be irradiated with a microwave power of 20 mW subjected to Zeeman modulation with amplitude 10 G and frequency 100 kHz. The scan range will be 400 G and sample temperature 77 K. The nitrosyl hemoglobin EPR triplet signal amplitude will be measured and quantified based on the height of the second line of the signal at g = 2.003 using a single sample diphenylpicrylhydrazyl as the internal standard.

4. Statistical Analysis: We work with Dr. Karrison, a statistician, beginning with the planning stages of each animal experiment to determine appropriate design and group sizes based on predicted sizes of differences to be observed. We randomize animals into groups and use blinding as much as possible at all stages of data collection, interpretation of results and data analysis. All statistical analyses will be carried out with advice from our statistician. The mortality rate in our last 189 monkeys was 12.5% but this includes complex clot removal and replacement experiments. We estimate a 10% mortality rate for these and all experiments using monkeys described below. For sample-size determination we focused on the primary comparison of the change from day 0 to 7 in the MCA diameter. A sample size of n = 8 animals per group will provide 80% power to detect a 1.4 standard deviation difference in group means where standard deviation refers to the standard deviation of within-animal differences. Based on previous data in 9 control monkeys, the observed mean change in MCA diameter from day 0 to 7 was -0.79 mm with a standard deviation of 0.27 mm. The proposed study therefore be able to detect a true reduction by DETA-NO in the magnitude of change of about 1.4 x 0.27 = 0.38 mm, which is less than half of that observed in the previous data. To account for a 10% mortality, we will study 10 animals in each group.

Arterial diameters from cerebral angiograms will be measured using an optical micrometer by a blinded investigator. Comparisons between groups will be performed by one way ANOVA or ANOVA for repeated measurements, as appropriate, followed by Tukey multiple comparison tests. Comparisons between 2 measurements will be by paired t-tests within groups and unpaired t-tests between different groups. These statistical methods are used for most of our experiments comparing endpoints between groups at each time and within groups over time, unless mentioned otherwise, and will not be detailed further.

Anticipated Results, Experimental Problems

We will complete experiments using DETA-NO intrathecally (24 more monkeys). We anticipate that a component of vasospasm will be reversible with DETA-NO. If cGMP levels are elevated in the treated arteries compared to controls, this would support our hypothesis. If DETA-NO fails to prevent vasospasm and to increase cGMP then the dose used may have been inadequate. We would then consider 2 possible experiments. One would be to do a dose-response trial of increasing doses of intrathecal DETA-NO with measurement of angiographic vasospasm and cGMP in the cerebral arteries to determine if there is any dose that can elevate cGMP in these arteries. Alternatively, we will use intraarterial infusion of an NO donor with a short half-life,
such as proli-NO (half life of 1.8 seconds at pH 7.4). It was reported that proli-NO prevented vasospasm in our model although measurement of cGMP was not done in that study.\textsuperscript{61} If NO donors do not prevent vasospasm but do elevate cGMP, this would suggest that NO removal does not contribute to vasospasm and would demonstrate even greater importance of NO-independent mechanisms that we are investigating as described below. If NO donors do not prevent vasospasm or elevate cGMP, this would suggest a defect at or before soluble guanylate cyclase (see Fig. 2). This could be due to defective function of this enzyme or inadequate substrate GTP. Our preliminary data show the smooth muscle can elevate cGMP, suggesting that this is unlikely. It may be found that NO donors elevate cGMP submaximally compared to control measurements (e.g. the elevations we detect in arteries relaxed with NO donors \textit{in vitro} under isometric tension) which would suggest a combined deficit in cGMP generation and in downstream components. This might be tested by administering stable analogues of cGMP but this would be difficult at this point due to lack of specific agents that would enter the smooth muscle cells efficiently \textit{in vivo}.

\textbf{B. Specific Aim 2: To measure heme-NO adducts by EPR spectroscopy in clots removed from the subarachnoid space of monkeys at different times after SAH.}

\textit{Hypothesis, Endpoints, Rationale}

The hypothesis is that if hemoglobin causes vasospasm by removing NO, then subarachnoid clots containing hemoglobin should contain heme-NO adducts. There should be a correlation between the degree of vasospasm and nitrosyl hemoglobin formation although this would be complicated by potential variations in the rate of NO formation and if hemoglobin in clot reached maximal NO binding capacity. The endpoint is heme-NO adduct formation measured by EPR in subarachnoid clots removed from monkeys at different times after SAH.

\textit{Experiment}

Monkeys will have baseline angiography followed by creation of SAH by clot placement in the right subarachnoid space using our previously-described model. A portion of clot will be stored in liquid N\textsubscript{2} on day 0 to serve as a control. We also will analyze clot placed in the extradural space at the time of each surgery. Angiography will be repeated and clots removed rapidly and frozen in liquid N\textsubscript{2} at euthanasia 3, 7 or 14 days after SAH. Clots will be subjected to EPR and correlated with angiographic vasospasm. Portions of each clot will be bubbled with pure NO gas to determine the maximal amount of nitrosyl hemoglobin that can form in the clot. We will use 7-day old clots from the control group of the experiment above. We require an additional 7 animals per time for 3 and 14 days plus 10% mortality (16 animals).

\textit{Methods}

1. \textbf{Monkey Model:} This will be carried out using our standard model of SAH. On days 3, 7 or 14, animals will undergo a cerebral angiogram to document the degree of cerebral vasospasm. Animals eventually euthanized on day 14 will have angiography on day 7 to document vasospasm. After repeat angiography, animals will be euthanized and the subarachnoid clots rapidly removed and frozen in liquid N\textsubscript{2} and then analyzed by EPR spectroscopy.

2. \textbf{EPR Spectroscopy:} This will be conducted as described above. For determining maximal nitrosyl hemoglobin formation, we will expose known volumes of clot (and therefore weights) after thawing and suspending in phosphate buffer under anaerobic conditions under argon gas to NO gas for increasing times. Nitrosyl hemoglobin formation will be quantified as described above at each time up to 25 minutes which was the time of maximal formation of nitrosyl hemoglobin in our preliminary experiments.

3. \textbf{Statistical Analysis:} There is a larger standard deviation for these measurements than for other endpoints, such as angiographic arterial diameters. Furthermore, 3 groups will be examined rather than 2 groups. We estimate that a sample size of n = 10 animals per group will provide 80% power to detect a 1 standard deviation difference in group means. For subsequent experiments, we have estimated that the biochemical and other non-angiographic endpoints will have similar variability and therefore, have estimated group sizes of 10 animals.

\textbf{Anticipated Results, Experimental Problems}

We anticipate confirming our preliminary data showing that nitrosyl hemoglobin is present in subarachnoid...
clots at all 3 times after SAH. There may be no significant differences over time which would be interesting in view of the resolution of vasospasm by 14 days after SAH. As discussed above, this could be due to (1) NO binding becoming an insignificant mechanism 14 days after SAH because of the minimal amount of clot left, (2) the clot having bound the maximal amount of NO that it can and/or (3) because the clot is encased and the hemoglobin is inaccessible to NO. This can be determined based on the measurement of maximal NO binding capacity of each clot. We anticipate that day 14 clots will demonstrate marked increases in nitrosyl hemoglobin after bubbling with NO \textit{in vitro}. Second, NO formation may vary after SAH and contribute to variation in formation of nitrosyl hemoglobin. High rates of formation of NO could result in higher amounts of nitrosyl hemoglobin but in no increase in the amount of vasospasm. We therefore propose in the next specific aim to continue our studies measuring NO in the perivascular space after SAH and in a subsequent aim to assess NOS isoforms.

C. Specific Aim 3: To quantify NO in the perivascular space at different times after SAH in monkeys.

Hypothesis, Rationale, Endpoints

We will specifically determine the concentrations of NO in the perivascular space after SAH in monkeys. These data, in combination with measurements of nitrosyl hemoglobin and of NOS isoforms, will allow us to gain an understanding of NO formation after SAH, of its scavenging by hemoglobin and of the potential contribution of this process to vasospasm. The endpoint is measurement of NO in the periadventitial subarachnoid space at different times after SAH. We will delineate the source of detected NO by administering specific inhibitors of the different NOS isoforms and assessing the effects on NO detected in the perivascular space.

Experiments

1. We will perform baseline angiography and right SAH on monkeys (n = 10 per group). 3, 7 or 14 days later, animals will be placed under anesthesia and have left craniotomy performed with opening of the arachnoid over the proximal MCA as described above under preliminary data. An NO-sensitive electrode and a microdialysis probe will be inserted into the subarachnoid space immediately adjacent to the MCA and held in place with sutures. Similar probes will be placed and measurements made on the right side after reopening the incision on that side and dissectingatraumatically along the MCA to its proximal portion.

2. After baseline measurements, we will administer specific inhibitors of iNOS (1400W) and nNOS (N^G- propyl-L-arginine). By adding one inhibitor first then the next in different order in different animals, we can potentially determine the contribution of each NOS isoform to total perivascular NO concentration.

3. The arteries and clots removed at sacrifice will be used for determination of NOS isoforms in the next specific aim and measurement of NOS activities so that it can be determined if the above inhibitors accomplished their inhibition \textit{in vivo}.

Methods

1. Monkey Model: Methods have been described above.

2. Measurement of NO: We will use 2 methods to detect NO in the perivascular space after SAH. Spin trapping with a nitronyl nitroxide (D8) did not detect NO, suggesting based on our tests \textit{in vitro} that the concentration of NO is less than 1 \mu M in the perivascular space. More sensitive methods that can be used \textit{in vivo} in the presence of proteins include electrochemical methods and oxyhemoglobin assay. Because of the difficulties in conducting monkey studies, we will use both methods simultaneously. We will place World Precision Instruments ISO-NOP200 (200 \mu m) sensor in the subarachnoid space and connect them to ISO-NO Mark 2 detectors (World Precision Instruments, Sarasota, FL). These probes are sensitive and specific amperometric NO detectors that have been used for similar \textit{in vivo} experiments.

Second, we will use pure human ferrous hemoglobin A_0 as an NO detector. This assay is based on the principle reaction of oxyhemoglobin with NO to form methemoglobin and nitrate. Microdialysis probes (CMA/20, North Chelmsford, MA) will be placed in the subarachnoid space next to the MCA. On the right side the probe will be advanced along the artery after exposing the more superficial portion of the artery.
will ensure minimal disruption of the deeper area of contact between the artery and clot. Probes will be perfused with pure oxyhemoglobin (Hemosol, Inc., letter attached) at 2 μl per minute. 50 μl samples will be collected on ice and anaerobic conditions to minimize autooxidation of oxyhemoglobin. Conversion of oxy- to methemoglobin is monitored spectrophotometrically and is a measure of NO production.13 Standardization will be by microdialysis of standard solutions of known amounts of NO in bicarbonate buffer.

3. Hemoglobin Spectrophotometry: The conversion of oxy- to methemoglobin will be monitored by multiple wavelength spectrophotometric measurement (Spectronic Genesys 5, Milton Roy Inc., Ivyland, PA) of absorbances at isobestic points and maximal absorbance wavelengths as we have done before.13,41,77

4. Administration of NOS Inhibitors: We will attempt to define contributions of each isoform of NOS to total NO production by administering a selective iNOS inhibitor (1400W, Calbiochem, San Diego, CA)14 or a selective nNOS inhibitor (N⁶-propyl-L-arginine, Calbiochem).93 We will administer these drugs intravenously to monkeys under anesthesia during monitoring for NO production in the subarachnoid space. Doses will be determined based on known blood volumes and inhibitory concentrations.

5. Measurement of NOS Isoform Activities: We previously assayed NOS activity by assessing conversion of radioactive L-arginine to L-citrulline in dog basilar artery.76 We plan to use this assay (NOSdetect™ Assay kit, Stratagene, La Jolla, CA) to quantify NOS isoforms by assessing the overall activity in homogenized MCAs and adjacent clot and of in the presence of the inhibitors added in vivo in concurrently run samples of the same tissues. There should be no change in NOS activity if the inhibitors added in vivo were active.

Anticipated Results, Experimental Problems

We anticipate being able to detect NO using these systems. We will conduct preliminary experiments in vitro to determine the sensitivity of these methods and to generate standard curves for both methods. Detectors and microdialysis probes will be placed in solutions bubbled with known concentrations of NO in order to determine the sensitivity of the detectors. Factors that can alter results of hemoglobin spectrophotometry, such as solution pH, will be monitored. We will begin experiments in limited numbers of animals in vivo in order to assess whether NO can be detected and whether or not adequate doses of NOS inhibitors are administered. If NO cannot be detected, we will administer NO donors such as proli-NO intraarterially into the carotid artery to determine if NO can be detected then in the perivascular space under any circumstances. If this does not allow detection of NO, we will harvest arteries and assay NOS activity in the presence and absence of selective inhibitors in order to determine relative contributions of each isoform to any potential NO production in vivo and measure NO metabolites in perivascular clot by Griess reaction.

D. Specific Aim 4: To define the time course of changes in and the immunohistochemical locations of the 3 isoforms of NOS in cerebral arteries and perivascular blood clot after SAH in monkeys.

Hypothesis, Rationale, Endpoints

Knowledge of NOS isoform changes and distribution are required in order to determine if there is a decrease in NO production after SAH that could contribute to vasospasm. Endpoints are amounts of mRNA and protein for each NOS isoform in the cerebral arteries and remaining perivascular clot in monkeys 3, 7 and 14 days after SAH and immunohistochemical location of NOS isoforms in these tissues at these times.

Experiments

This will be carried out using our standard model of SAH with the arteries being obtained from 30 animals used above under Specific Aim 3. Animals will undergo baseline angiography and creation of right SAH. On days 3, 7 or 14, animals will undergo a cerebral angiogram to document the degree of cerebral vasospasm. Animals eventually euthanized on day 14 will have angiography on day 7 to document vasospasm. After repeat angiography, animals will be euthanized and the subarachnoid clots rapidly removed and frozen in liquid N₂ and then analyzed by EPR spectroscopy. We have previously obtained tissues for multiple measurements in such experiments by removing the cerebral arteries and adjacent brain tissue. A portion of right and left MCA is removed and frozen in liquid N₂ to be used for immunoblotting and reverse transcriptase polymerase chain reaction (RT-PCR). The remaining portion is left attached to the brain and a block cut out, fixed and used for
immunohistochemistry.

**Methods**

1. **Immunoblotting** for NOS isoforms will be performed using methods we have reported previously.\(^{41;44;50;58;94}\) We extract total RNA and protein from MCA and brain tissue using Trizol reagent (Gibco, Gaithersburg, MD). Protein concentration will be determined by BCA Protein Assay Reagent (Pierce, Rockford, IL). Protein will be denatured by boiling in 1% 2-mercaptoethanol and then stored at -20°C until use. The RNA fraction will be used as described below.

   Protein extracts will be separated by sodium dodecyl sulphate - polyacrylamide gel electrophoresis followed by electrotransfer onto nitrocellulose membranes. Membranes will be blocked for 2 hours with 5% milk in Tris-NaCl buffer containing 0.1% Tween 20 and then incubated overnight at 4°C with primary mouse monoclonal antibodies to human eNOS, rabbit polyclonal antibodies to mouse iNOS or mouse monoclonal antibodies to human nNOS (generally 1:2500, BD Transduction Laboratories, San Diego, CA). The membranes will be washed and incubated with a peroxidase-conjugated secondary antibody diluted in 2% milk for 2 hours at 25°C. Labeling of specific bands on membranes will be visualized using ECL detection reagents (NEN Life Science Products, Boston, MA). The density of specific bands will be analyzed using the ImageQuant computer program and standardized as the ratio of density of the NOS band to the density of \(\beta\)-actin.

2. **Immunohistochemistry:** We fix tissues for 24 hours in 4% paraformaldehyde in phosphate buffered saline (PBS). They will then sunk in 20% sucrose and then embedded in OCT compound (Tissue-Tek, Miles Pharmaceuticals, Ekhart, Indiana) and rapidly frozen on dry ice. Cross-sections 10 μm thick will be cut in a cryostat (Leica Cryocut 1800, Nussloch, West Germany) and mounted on clean glass slides. Endogenous peroxidase activity will be blocked by exposure of sections to H\(_2\)O\(_2\), 0.6%, for 30 minutes followed by washing 3 times in 0.1 M PBS, pH 7.4. They will be permeabilized with 0.2% Triton X-100 in PBS containing 1% bovine serum albumin for 30 minutes and then incubated in 0.5% bovine serum albumin and 0.5% goat serum in PBS. They will be incubated for 24 hours at 4°C with the above NOS antibodies. Secondary antibodies will be biotinylated goat, anti-rabbit or mouse secondary antibody (1:200, Vector Laboratories, Burlingame, CA). Detection will be by ABC reagent (Vector Laboratories, Burlingame, CA). Negative controls will be processed using bovine serum albumin instead of the primary antibody.

3. **PCR:** We previously used competitive RT-PCR to quantify mRNA in monkey cerebral arteries.\(^{20;21}\) We now use quantitative RT-PCR with real time Taqman technology, a method that Dr. Aihara, one of our research associates, is familiar with and has published before.\(^3\) RNA is extracted as described.\(^{20;21}\) Specific primers and Taqman probes for PCR for each target cDNA were selected using human (for eNOS) and M. fascicularis (for iNOS and nNOS) cDNA sequences published in the Genbank (Table 5).

### Table 5: Sequences for Real Time RT-PCR for NOS Isoforms

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Taqman Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>GCTGCATGACATTGAGAGCAA</td>
<td>TGAGGACTCGGCAAAACAC</td>
<td>CGATGCTCCAACCTCTCATCTCTACC</td>
</tr>
<tr>
<td>nNOS</td>
<td>GATGACAACCGATACATGAGGATT</td>
<td>CAAATGGAAGCCATGGACTCAGA</td>
<td>CCTGCGGAGCTGAGTTGAGCAAA</td>
</tr>
<tr>
<td>iNOS</td>
<td>CCGACAGCATGAGGATGCAA</td>
<td>GCAAGATTTGGACTCTGAAGTC</td>
<td>CAGGACACATTTCAACAAAGGCCA</td>
</tr>
<tr>
<td>(\beta)-actin</td>
<td>CCTGAGGCTCTCTTCCAACCT</td>
<td>ACGGATGCACAGTCACACCTT</td>
<td>ATGGAGTCCTGTGCAAGAAACTAC</td>
</tr>
</tbody>
</table>

4. **Data and Statistical Analysis:** Immunoblotting will be assessed semiquantitatively by densitometry. Interpretation of immunohistochemistry will be carried out in conjunction with Drs. Wollmann and Utset who are experienced neuropathologists. We generally have 2 observers grade changes observed on 3-point scales and analyze group differences using statistical analysis appropriate for categorical variables.\(^{47;58}\) Interobserver
variability will be calculated for each variable and assessed by Kendall's coefficient of concordance. A Kruskal-Wallis test will be used to assess differences between multiple groups and a Wilcoxon rank-sum test for 2 groups. Significance will be taken at p < 0.05.

Anticipated Results, Experimental Problems

Our preliminary results show a decrease in eNOS and nNOS during vasospasm with a return to normal by day 14 when vasospasm resolves. Inducible NOS is increased during the time of vasospasm. It is anticipated that this pattern will be observed in the subsequent work. We anticipate being able to correlate these changes with function of the NOS isoforms as determined from Specific Aim 3 above and with NO production in the subarachnoid space and to determine the location of iNOS expression.

2. Mechanisms of Impaired Vascular Relaxation in Vasospasm - Vasospasm not Reversible with NO

The experiments will be conducted on 30 monkeys (n = 10 for times 3, 7 and 14 days after SAH) for Specific Aims 1 and 4. Specific Aims 2 and 3 will be conducted in a rat model of vasospasm because a more frequent supply of tissue is required to carry out patch clamp and Ca\(^{2+}\) microfluorimetry. The hypothesis common to these experiments is that there is a component of vasospasm that is not reversible by NO donors despite elevation of cGMP in the vasospastic arteries. We hypothesize dysfunction in the NO relaxation pathway (Fig. 2) downstream of cGMP, components of which include PKG and as an important final common mediator, K\(^{+}\) channels. The rationale is to determine at what site(s) in this pathway the abnormality is.

A. Specific Aim 1: To measure PKG messenger ribonucleic acid (mRNA), protein and activity during the time course of vasospasm in monkeys.

Experiment

The same time course protocol as described above will be used. Monkeys will undergo baseline angiography followed by right SAH. They will be euthanized on days 3, 7 and 14 (n = 10 per time) and right and left MCAs removed and divided into 2 portions. One will be used to assess PKG activity and the second for Ca\(^{2+}\) sensitivity measurements under Specific Aim 4. Protein and mRNA will be obtained from the arteries of monkeys used in the time course studies above for measurement of NOS isoforms. We previously have obtained enough protein and mRNA to assess multiple substances.

Methods

Methods for immunoblotting and real-time PCR are described above. There are 2 isoforms of PKG although type 1 predominates in smooth muscle. Antibodies to PKG type 1 will be obtained from Calbiochem (San Diego, CA). PKG and PKA activities will be assayed by phosphotransferase activities that measure transfer of \([\gamma^{32}\text{P}]\) phosphoryl group transfer from ATP to specific peptide substrates of PKG and PKA.60

Anticipated Results, Experimental Problems

We speculate that there may be a decrease in PKG in vasospastic arteries. It has been demonstrated that phenotypic change of vascular smooth muscle from a contractile to a synthetic phenotype, which is known to accompany vascular injury, is associated with a reduction in PKG.4,70 Our preliminary data with cDNA arrays, for example, strongly suggest that there is a phenotype change in at least some smooth muscle cells in vasospastic arteries. The PKG assays are difficult due to the low expression of these kinases in most cells.70 If activity cannot be measured, we will rely on changes in protein and mRNA. We will have abundant basilar artery tissue available from the above monkeys which we can use to develop assay conditions and select PCR primers and immunoblotting antibodies.

B. Specific Aim 2: To assess K\(^{+}\) channel function during vasospasm in rats.

Experiments

Groups of rats will undergo baseline angiography followed by transclival exposure of the basilar artery and placement of clot from 0.3 ml autologous blood against the artery. They will be awakened and undergo repeat angiography and euthanasia 1, 2, 4, 7 or 10 (n = 12 to 20 per group) days later. These times will be selected because they span the entire time course of vasospasm in this model. Basilar artery smooth muscle cells will be
harvested and studied by patch clamp and Ca\textsuperscript{2+} microfluorimetry as described below. Results from vasospastic smooth muscle cells will be compared with smooth muscle cells obtained from sham-operated animals.

**Methods**

1. **Smooth muscle cell dissociation:** Experiments will be carried out on freshly-isolated rat basilar artery smooth muscle cells as previously described\textsuperscript{91,92}. Briefly, rats will be anesthetized and decapitated. The basilar arteries will be removed and placed in buffer (mM: NaCl 130, KCl 5, CaCl\textsubscript{2} 0.8, MgCl\textsubscript{2} 1.3, glucose 5, N-2-hydroxyethylpiperazine-N‘-2-ethane-sulfonic acid [HEPES], penicillin 100 units/ml, streptomycin 0.1 g/l). The arteries will be cut into small pieces and incubated for 1 hour in collagenase (type II, 0.5 g/l), elastase (0.5 g/l), hyaluronidase (type IV-S, 0.5 g/l), and deoxyribonuclease I (0.1 g/l). They will be washed in fresh solution containing CaCl\textsubscript{2} (0.2 mM), trypsin inhibitor (0.5 g/l) and deoxyribonuclease I (0.1 g/l) and then triturated gently. They will be plated on glass cover slips, stored at 4\textdegree C, and used within 12 h. The cells stain positively for \textit{\alpha}-actin and contract to agonists such as KCl and serotonin, confirming that they are smooth muscle cells.

2. **Electrophysiology:** Electrodes will be prepared from glass capillary tubing using a horizontal electrode puller (Model P-97, Sutter Instrument Co., Novado, CA), with pipette resistances in the range of 3-4 M\textOmega. Electrodes will be positioned using a 3-dimensional vernier-type hydraulic micromanipulator (MW-3, Narishige Co., Tokyo, Japan). Seals will be formed by applying gentle negative pressure. Voltage steps will be applied with pulse protocols driven by a Dell pentium computer equipped with A-D and D-A converters (DigiData 1200, Axon Instruments Inc., Foster City, CA). Whole-cell and single channel currents will be amplified on a patch clamp amplifier (Axopatch 1D, Axon Instruments) and acquired using pCLAMP 6.0.4 software. Specific K\textsuperscript{+} currents will be isolated using a combination of voltage protocols and antagonists (see below).

3. **Drugs and Solutions:** Bath solution will contain (in mM) NaCl 140, KCl 5, MgCl\textsubscript{2} 2, HEPES 10, glucose 10, pH 7.4 (NaCl 80, KCl 60 for some experiments investigating K\textsubscript{IR} currents, NaCl 0, KCl 150 for some experiments recording single channel currents in cell-attached mode). For recording conventional whole-cell K\textsubscript{Ca2+} currents, the pipette will contain (in mM) KCl 145, MgCl\textsubscript{2} 2, CaCl\textsubscript{2} 3.66, K-ATP 2, EGTA 5, HEPES 10, glucose 10, pH 7.2. For recording whole-cell K\textsubscript{Ca2+} currents using nystatin-perforated patch, the pipette will contain KCl 55, K\textsubscript{2}SO\textsubscript{4} 75, MgCl\textsubscript{2} 8, HEPES 10, nystatin 200 \mu g/ml. For recording K\textsubscript{ATP} currents, pipette will contain (in mM) KCl 145, MgCl\textsubscript{2} 1.2, EGTA 1, HEPES 10, pH 7.2. For recording K\textsubscript{v} or K\textsubscript{IR} currents, the pipette will contain KCl 145, MgCl\textsubscript{2} 2, CaCl\textsubscript{2} 0.1, K-ATP 2, EGTA 10, HEPES 10, glucose 10, pH 7.2. Selective block of each current will be accomplished by addition to the bath of 100 nM iberiotoxin for K\textsubscript{Ca2+} channels, 10 \mu M glibenclamide for K\textsubscript{ATP} channels, 1 mM Ba\textsuperscript{2+} for K\textsubscript{IR} channels, and 5 mM 4-aminopyridine for K\textsubscript{v} channels. Single channel recordings in cell-attached mode will use a bath and pipette solution similar to conventional whole-cell experiments except that Na\textsuperscript{+} will be replaced with K\textsuperscript{+} (isotonic K\textsuperscript{+} solution). When investigating K\textsubscript{Ca2+} channel Ca\textsuperscript{2+} sensitivity using excised inside-out patches, symmetrical K\textsuperscript{+} gradients will be used and the bath solution will be the same as the intracellular solution employed for whole-cell K\textsubscript{Ca2+} studies except that free [Ca\textsuperscript{2+}] will be adjusted by adding varying amounts of total Ca\textsuperscript{2+} chosen using the public-domain WinMaxC computer program. BK channel sensitivity to manipulations of the PKG second messenger pathway will be tested using the membrane-permeant cGMP analog 8-bromo-cGMP (100 \mu M) and the NO donors DETA-NO and sodium nitroprusside.

4. **Statistical Analysis:** Whole-cell currents will be analyzed using pClamp software (version 6.0.4). I-V curves will be constructed and the current-voltage relationships of whole-cell pharmacologically identified currents will be examined. Cell capacitance will be determined from capacitance compensation settings on the Axopatch 1-D amplifier. Current density will be defined as pA/pF, and compared between normal and vasospastic smooth muscle. Similarly, single channel records will be analyzed using Fetchan and Pstat components of the same software suite. Single channel current will be determined by fitting all-point-histograms to multiple Gaussian distributions and open probability investigated using maximum-likelihood fits of idealized open and closed times. Estimates of channel number and conductance will also be derived using
noise analysis of whole-cell current families to corroborate current density and single channel conductances as determined above.

Anticipated Results, Experimental Problems

We hypothesize that there is diminished BK channel function in vasospastic smooth muscle in comparison to normal myocytes, thus leading to increased smooth muscle cell excitability. This may be manifest as diminished BK whole-cell currents when normalized across cells as pA/pF. Our intracellular solution contains approximately 300-400 nM [Ca\(^{2+}\)], enough to maximally activate all BK channels when depolarized to +80 mV (test pulse potential when examining current density). However, altered BK channel function may be masked by such maximal Ca\(^{2+}\) and voltage stimulus. Use of nystatin-perforated patch, which leaves the intracellular milieu (and thus any alterations in second-messenger regulation of BK channels) relatively intact, may be more sensitive to the hypothesized changes which may follow vasospasm. Diminished BK channel function may also manifest itself as (1) altered channel sensitivity to Ca\(^{2+}\), (2) shifts in current-voltage relationship, (3) changes in channel gating properties and/or (4) altered BK channel sensitivity to the PKG second-messenger pathway. Each aspect of BK channel function will thus require evaluation in vasospastic versus normal smooth muscle cells. Furthermore, analysis of BK currents is incomplete without investigation of the underlying Ca\(^{2+}\) dynamics which serve to activate these currents, and so Ca\(^{2+}\) channels and [Ca\(^{2+}\)], dynamics will need to be examined (see Specific Aim 3). For example, a finding of diminished BK channel function may be less significant if Ca\(^{2+}\) currents and/or [Ca\(^{2+}\)], levels are observed to also be diminished.

Experimental problems include possible confounding effects of the enzymatic acute dissociation process required to study single vascular smooth muscle cells, lack of smooth muscle-endothelium interaction which determines much of arterial resting tone, and possible altered basal conductances due to the absence of pressurization and thus of myogenic tone in a single cell preparation. It is hoped that given the same (necessary) procedures will be applied to both vasospastic and control arteries, this compensate for the potential pitfalls discussed, but they must nevertheless be acknowledged.

We plan in subsequent experiments to determine if changes found in the rat model also occur in vasospastic smooth muscle cells isolated from our monkey model. It is possible that we will not find changes in K\(^+\) channel function in vasospastic smooth muscle and that there is dysfunction proximal to this final common mediator of relaxation. This in itself would be a key finding. We would then investigate changes occurring in the intermediary steps (Fig. 2).

C. Specific Aim 3: To measure calcium (Ca\(^{2+}\)) currents and smooth muscle Ca\(^{2+}\) handling during vasospasm in rats.

Experiments

Groups of rats will undergo experimental SAH and subsequent isolation of basilar artery smooth muscle cells and recording of Ca\(^{2+}\) currents and [Ca\(^{2+}\)], dynamics in a fashion similar to Specific Aim 2 (see above). Only changes from the previously discussed protocol are noted below.

Methods

1. Electrophysiology: Patch pipettes will be prepared and electrophysiological data recorded as detailed in Specific Aim 2, except that the bath solution will contain (in mM) TEA-Cl 125, 4-AP 5, MgCl\(_2\) 1, BaCl\(_2\) 10, HEPES 10, glucose 12.5, pH=7.2. The patch pipette will contain (in mM) NaCl 120, CsCl 10, MgCl\(_2\) 8, HEPES 10, nystatin 200 µg/ml, pH=7.35.

2. Ca\(^{2+}\) Imaging: Cells will be placed in a standard extracellular buffer (mM: NaCl 145, KCl 3, CaCl\(_2\) 2, MgCl\(_2\) 1, glucose 10, HEPES 10, pH 7.4). They will be loaded with fura-2/AM for 30 minutes at room temperature in the extracellular buffer solution, rinsed and placed in a plexiglass perfusion chamber with openings for perfusion and aspiration. Cells will be perfused for 10 minutes to allow de-esterification of the fura-2.

Digital [Ca\(^{2+}\)], imaging will be performed by video microfluorimetry with an intensified CCD camera (Hamamatsu, Bridgewater, NJ) coupled to a Nikon Diaphot microscope (40X Fluor objective, Nikon, Inc., New
York) and software (Universal Imaging Corp., West Chester, PA) on a personal computer. Sample illumination will be supplied by a 150-W Xenon arc lamp, and excitation wavelengths will be selected by computer control of the filter wheel. Fluorescence imaging will be obtained with alternating excitation wavelengths of 340 and 380 nm, and an emission wavelength of 510 nm through the CCD camera. Data will be displayed in real time and stored on hard disk. Background fluorescence will be obtained from a cell-free position of the same slip and will be subtracted from all recording prior to calculation of the 340/380 ratio. EGTA (0.1 mM) will be included in all experiments using Ca\(^{2+}\)-free extracellular buffer.

In some experiments, a high-speed photomultiplier system (Photon Technologies Inc. Model 814) will be employed instead for simultaneous recording of intracellular Ca\(^{2+}\) transients and electrophysiological currents.

**D. Specific Aim 4:** To assess Ca\(^{2+}\) sensitivity of monkey cerebral arteries during the time course of vasospasm.

**Experiments**

Right and left MCAs will be obtained from Specific aim 1 at 3, 7 and 14 days after SAH. They will be suspended under isometric tension and Ca\(^{2+}\) sensitivity will be assessed by permeabilizing randomly selected rings without endothelium with α-toxin in order to leave regulatory pathways in the smooth muscle as intact as possible. Control measurements will be taken in nonpermeabilized rings with and without endothelium.

**Methods**

The monkey basilar artery will be removed from the brain and placed in Krebs-Henseleit buffer and cut into 3 mm rings. These are suspended in 5 ml tissue baths at 37°C and studied under isometric tension using standard methods and as we have reported previously. Some rings will be permeabilized after equilibration and measurement of contraction to KCl, 60 mM. In these rings, endothelium will be removed by inserting a tungsten rod into the ring lumen. Permeabilization will be by incubation in relaxing solution (in mM: 85 KCl, 5 MgCl\(_2\), 5 Na\(_2\)ATP, 5 creatine phosphate, 2 EGTA and 20 Tris maleate, pH 7.1) containing *Staphylococcus α-toxin*, 20 μg/ml (List, Campbell, CA) for 30 minutes. Ca\(^{2+}\)-dependent contractions will be induced by addition of increasing bath Ca\(^{2+}\) concentration.

**Anticipated Results, Experimental Problems**

We anticipate that there will be increased Ca\(^{2+}\) sensitivity of contraction in vasospastic smooth muscle because of loss of NO-pathway-mediated Ca\(^{2+}\) desensitization that is normally present. The main experimental problem that must be addressed is that regulatory pathways that modulate Ca\(^{2+}\) dependence of contraction can exert their effects by altering the dependence of MLC phosphorylation on [Ca\(^{2+}\)], or the dependence of force on phosphorylation. We will primarily measure the former effect but alterations in the NO pathway may affect the latter relationship as well. Furthermore, multiple factors may influence the relation between MLC phosphorylation and [Ca\(^{2+}\)], including protein kinase C, Rho kinase and mitogen-activated kinases. If we do not find changes in this relationship, it may be necessary to examine the relationship between MLC phosphorylation and force by measuring the former at varying levels of force generation by rings of normal and vasospastic arteries in vitro. If this relationship was altered, a compare in these arteries of possible regulators of this relationship such as calponin and caldesmon phosphorylation, activation of Rho kinase and protein kinase C may be indicated. While we must acknowledge that these systems may be involved, we cannot propose to investigate them at this stage but this may be necessary in future studies.

**5. TIME LINE OF EXPERIMENTS**

We will complete NO donor experiments and EPR in monkeys in the first year. NO quantification will be done in the second year and the assessment of NOS isoforms, PKG and Ca\(^{2+}\) sensitivity in the third and fourth years. Rat experiments will continue each year.

**E. HUMAN SUBJECTS**
None proposed.

F. VERTEBRATE ANIMALS

(1) Experiments using a total of 100 monkeys (Macaca fascicularis) have been proposed. These are adult monkeys (2.5 - 5 kg) of either sex. A general outline for the conduct and care of animals under these experiments follows. All experiments will be conducted under protocols approved by the Animal Care and Use Committee of the University of Chicago. Monkeys always will be randomly allocated to the groups within experiment to remove time and practice as variables in the experimental design. Triple blinding is employed in all experiments as necessary. Animals undergo baseline cerebral angiography followed by unilateral SAH. There is then some different manipulation or not over the next 3, 7 or 14 days or no intervention depending on the protocol. All animals then undergo cerebral angiography and are euthanized after the second angiogram.

In all experiments, animals will be sedated with ketamine, 10 mg/kg intramuscularly. Animals will be weighed and the right axilla, the head and the back in some cases will be shaved in the preparation room outside of the operating room. An intravenous catheter will be inserted in a calf vein and they will be intubated and ventilated on oxygen and 1 to 3% isoflurane using a small animal respirator. End-tidal and arterial PCO₂, body temperature, P₅₀, blood pressure and heart rate will be monitored and maintained in physiological ranges. The right axilla will be prepared sterily with povidone-iodine solution that will be allowed to dry for 3 minutes. The area will be draped in sterile fashion. A linear incision will be made over the axillary artery and it will be exposed using microsurgical technique. It will be cannulated with an 18 or 20 gauge polypropylene catheter. 15 mls blood will be withdrawn and allowed to clot. This volume of blood drawn once does not cause side effects in monkeys and will be replaced with adequate intravenous fluids that are infused through the intravenous catheter during the surgery. A single, anteroposterior midarterial phase angiogram will be obtained by manual injection of 8 mls iohexol meglumine, 60%. Following the angiogram, the catheter will be removed and the axillary artery will be ligated. The wound will be sutured closed with interrupted, monofilament nylon sutures. Infection, limb ischemia, wound hematoma, or any other complications developed in 0.5% following angiography performed as above. The risk of these complications, therefore, is very small. If limb ischemia develops, as indicated by paralysis, swelling, or pain, the animal will be euthanized. Signs of pain include but would not necessarily be limited to decreased activity, paralysis, decreased food and water intake, or other abnormal behavior. Infection has not occurred but if infection develops, as judged by redness, swelling, or discharge from the wound, the perioperative antibiotics will be continued. The antibiotic will be enrofloxacin, 2.5 mg/kg intramuscularly, bid. All medications for treatment of infections or other surgical complications are reviewed with the veterinary and investigator staff and agreed upon before initiation of use.

Following the angiogram, the scalp will be prepared and draped in sterile fashion with povidone-iodine solution. A standard right or left frontotemporal craniectomy will be performed with the head fixed in 3-point pin fixation. A microscope and microsurgical technique will be used to open the basal cisterns and place 5 ml clotted autologous blood against the arteries of the right side of the circle of Willis. The wound will be closed in layers with interrupted absorbable sutures for muscle and fascia and interrupted monofilament nylon sutures for skin. In some animals, an intravenous catheter will be inserted into the femoral vein and tunneled subcutaneously to the back where it will exit and be connected to a pump for continuous infusion. In other animals, drugs will be infused into the cisterns with osmotic pumps or the clot will be separated from the cerebral arteries by a dialysis membrane. In our previous experiments with uni- and bilateral SAH in 189 monkeys, the postoperative mortality rate was 12.5%. The usual postoperative mortality rate in prior experiments with unilateral SAH in monkeys at the University of Chicago was 10%.

All animals will then be allowed to awaken and recover from anesthesia. Depending on the protocol, each animal will have been randomized then to drug injections for 7 days or to no intervention and/or to euthanasia 3, 7 or 14 days post-SAH. Drugs may be delivered intracisternally through osmotic pumps implanted at the site
of surgery as previously described in numerous publications from our laboratory using the monkey model. On day 3, 7 or 14, all animals will be sedated and anesthetized as on day 0. Angiography will then be repeated through an arteriotomy proximal to the arteriotomy performed on day 0. Blood pressure, endtidal and arterial PCO$_2$, PO$_2$, heart rate, and temperature will be monitored as on day 0. After angiography, CSF will be aspirated from the cisterna magna. Animals will be euthanized by intravenous injection of sodium pentobarbital, 100 mg/kg, or exsanguination under general anesthesia. They will be fixed by perfusion or samples removed immediately as indicated for future studies.

The complication rate following creation of SAH in monkeys is approximately 13% morbidity and mortality. If animals develop focal neurological deficit, manifest by signs such as hemiparesis or hemisensory loss, this will be assessed by the investigators and the veterinary staff. If it is believed that the animal could survive and is not suffering and is able to eat and drink and does not appear to be in distress then they will not be euthanized immediately. If the animal is unable to take adequate fluid and nutrition or appears to be in pain, manifest by but not necessarily exclusively by decreased activity, lethargy, malaise, poor food and water intake, or picking at incisions, then they will be euthanized immediately. An angiogram will be obtained prior to euthanasia with the animal under general anesthesia as described above. If infection develops at the craniotomy site, arteriotomy site, over a drug injection site through an indwelling intravenous catheter or over an osmotic pump, animals will be treated with antibiotics. This will be enrofloxacin, 2.5 mg/kg intramuscularly bid. As with all complications, management will be discussed frequently as indicated clinically with the veterinary staff and plans made accordingly. If infection is serious and not able to be controlled with antibiotics, then animals will be placed under general anesthesia, subjected to angiography, and euthanized.

The degree of neurological deficit that will mandate immediate euthanasia will be determined in consultation with the veterinary staff. For example, a monkey with a minor head tilt, mild lethargy or slight decrease in appetite, but still eating and taking oral fluids will not be immediately euthanized. This situation will be open to interpretation based on consultation with the investigator staff and veterinarians, who both have experience with approximately 189 monkeys in this model. If seizures develop, which has been the case in approximately 5% of animals, animals will be treated with lorazepam acutely, 0.1 mg/kg intramuscularly followed by dilantin, 6 mg/kg intramuscularly tid. If seizures cannot be controlled adequately, then angiography will be repeated with the animals under general anesthesia and they will be euthanized.

Additional experiments are proposed on Sprague-Dawley rats. Approximately 100 will be used per year as part of our rat model of clot placement against the basilar artery. They will be anesthetized with ketamine, 90 mg/kg and xylazine, 10 mg/kg, intramuscularly. For angiography the C-arm available in Carlson will be used and has proved adequate for angiography in rats in previous experiments. The surgical procedure requires approximately 60 minutes. Following induction of anesthesia, the right axillary area and the anterior neck will be shaved and prepared sterily with alcohol. The animal will be positioned prone and the right axillary artery will be exposed using microsurgical techniques and sterile conditions. The artery will be catheterized with a 24-gauge polyethylene catheter and 0.3 ml of blood will be withdrawn from the axillary artery. A single mid arterial phase anteroposterior angiogram will be taken of the basilar artery by injection of 1 ml iohexol. The catheter will be removed and the artery will be ligated proximally and distally to the arteriotomy with 6-0 monofilament nylon suture. This wound will be sutured closed with interrupted 5-0 nylon sutures. Among over 30 previous rats subjected to this angiographic procedure, limb ischemia, infection, or other complications of angiography were not observed. Next, the anterior neck will be prepared sterily and the clivus will be exposed through a midline incision using sterile microsurgical technique. The clivus will be drilled away to expose the dura over the brainstem. The dura and arachnoid will be opened and the basilar artery exposed. The trough created in the clivus will be filled with 0.1 ml autologous blood clot. The wound will be closed with interrupted 5-0 nylon sutures and the animals will be allowed to awaken. 7 days following this procedure, rats will be subjected to angiography as described above followed by sacrificed under anesthesia induced as described above by decapitation. The basilar artery will be removed and smooth muscle cells isolated for patch
clamp and calcium imaging studies.

(2) A model of SAH and chronic cerebral vasospasm has been developed in Macaca fascicularis and used over the past 17 years. It results in routine angiographic vasospasm, and sometimes delayed ischemic deficit and cerebral infarction. The use of the model has permitted a series of reliable observations regarding the pathophysiology and histology of the condition. The time course of the angiographic spasm (peaking on days 3 to 7 and substantially gone by day 14) is identical to that which occurs in humans and is not replicated in any other model except by the artificial use of multiple injections of blood into the CSF of dogs. The incidence of cerebral infarction in monkeys also is similar in some human series (about 5 - 10% of patients die from infarction due to vasospasm alone). We have accumulated a great deal of data which has been published in numerous publications using this model and this species and to our knowledge this species is not endangered. We attempt to use the very minimal number of animals possible while still arriving at defensible conclusions and hypothesis testing. We feel that our experiments are a good example of animal research which has directly stimulated human clinical trials such as our published work on nimodipine, 21-aminosteroids (tirilizad) and intrathecal tissue plasminogen activator for the prophylaxis of vasospasm.

We believe that it is impossible to study the disease process of delayed ischemia and chronic cerebral vasospasm in its complete sense except by using animals, and the model that most closely approximates the condition in man was developed by our laboratory using primates. Delayed neurologic deficits and infarction have not been demonstrated to occur reliably without including iatrogenic arterial occlusions in any other model. While primates are relatively costly we feel that the results of the experiments can be directly applicable to human investigations and treatment. Statistical considerations in the selection of experimental groups have been detailed in the description of the research plan. Based on our previous series of experiments we feel that if the individual groupings get down much below 8 to 10 we have difficulty in establishing significant differences between groups. It is planned to use approximately 100 monkeys over 4 years.

We are very cognizant of the responsibility of using primates and substitute less phylogenetically evolved animals wherever possible. We use rats to study basic pathophysiological and molecular biological aspects of vasospasm. We know more about the isolated rat basilar artery smooth muscle cell and about the rat genome than about other species used in vasospasm research such as monkeys, dogs, and rabbits. Hypotheses supported in the rat studies are further tested in monkeys. To solve the problem of vasospasm we prefer to use arteries that are as close to human - structurally, biochemically and pharmacologically - as is possible. It is vital to establish a data base which will be sufficiently detailed to permit the construction of a rational hypothesis to guide medical therapy. The studies so far performed by us have made contributions to contemporary clinical care - the use of calcium antagonist, clot removal, hypertension and hypervolemia, intrathecal tissue-plasminogen activator, 21-aminosteroids, and the inefficacy of kanamycin - reserpine and nitroprusside - phenylephrine.

Additional experiments are proposed using Sprague-Dawley rats of either sex. They will weigh between 150 and 300 g. Approximately 100 per year will be used in a rat model of clot placement against the basilar artery that is designed to mimic vasospasm as it occurs in man. We believe this more closely approximates vasospasm as it occurs in man than injections of blood into the cisterna magna because a solid, thick clot, which is required for vasospasm to develop in man, is used.

(3) The animals are cared for in the Carlson Animal Care Facility that is fully staffed with animal care technicians and fulltime veterinarians. Both technicians and veterinarians perform daily rounds on all animals and assess monkeys and rats for several factors including but not necessarily limited to activity, incisions, food and water intake, weight, vital signs and neurological deficits. We work very closely with the veterinarians on protocols involving primates.
(4) All surgical procedures are performed under general anesthesia using sterile technique - in a manner the same as our patients are treated. For monkeys, no restraining devices are used. For infusion of DETA-NO, the monkey will wear a vest containing a pump connected to an intrathecal catheter. This will allow the animal to move freely about in its cage. They are sedated 2 days before any surgical interventions and are placed in the vest which they wear for 2 days in order to become acclimatized to it. At baseline animals receive ketamine 6-10 mg/kg intramuscularly to prevent distress and pain. Once they are sedated the animals have an intravenous line established and are intubated and ventilated with oxygen and 0.5 - 2% isoflurane administered by variable phase small animal respirator. Physiological variables are monitored as in human surgery (heart rate, blood pressure, PaCO₂, Pao₂, temperature) and used to help determine adequate general anesthesia. If any manipulations are required during the week prior to euthanasia the animals are sedated with ketamine 6-10 mg/kg intramuscularly no more frequently than every 12 hours. Animals are monitored after they have been sedated or given an anesthesia until they are awake. If the animals are considered to have discomfort in the postoperative period, buprenorphine 0.03 - 0.05 mg/kg will be administered intramuscularly as the long acting analgesic of choice for monkeys.

Rats are euthanized by decapitation under general anesthesia with ketamine, 90 mg/kg and xylazine, 10 mg/kg intramuscularly.

(5) Monkeys will be euthanized under a surgical plane of general anesthesia by exsanguination or by intravenous injection of an overdose of sodium pentobarbital, 100 mg/kg. A surgical plane of anesthesia will be determined as that from which there is decreased muscle tone, no palpebral reflex, no movement in response to surgical manipulations, and no alteration in heart rate or blood pressure in response to surgical manipulations. Rats will be euthanized by decapitation when under general anesthesia induced by injection of ketamine, 90 mg/kg and xylazine, 10 mg/kg intramuscularly. A surgical plane of anesthesia in rats will be determined as that from which there is decreased muscle tone, no palpebral reflex, and no response to heel and tail pinch. These methods of euthanasia are consistent with the recommendations of the American Veterinary Medical Association.

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H. CONSORTIUM/CONTRACTUAL ARRANGEMENTS

None

I. CONSULTANTS

None