Review Article

Vascular plasticity in cerebrovascular disorders

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Cerebral ischemia remains a major cause of morbidity and mortality with little advancement in subacute treatment options. This review aims to cover and discuss novel insight obtained during the last decade into plastic changes in the vasoconstrictor receptor profiles of cerebral arteries and microvessels that takes place after different types of stroke. Receptors like the endothelin type B, angiotensin type 1, and 5-hydroxytryptamine type 1B/1D receptors are upregulated in the smooth muscle layer of cerebral arteries after different types of ischemic stroke as well as after subarachnoid hemorrhage, yielding rather dramatic changes in the contractility of the vessels. Some of the signal transduction processes mediating this receptor upregulation have been elucidated. In particular the extracellular regulated kinase 1/2 pathway, which is activated early in the process, has proven to be a promising therapeutic target for prevention of vasoconstrictor receptor upregulation after stroke. Together, those findings provide new perspectives on the pathophysiology of ischemic stroke and point toward a novel way of reducing vasoconstriction, neuronal cell death, and thus neurologic deficits after stroke.

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Introduction

Stroke is the third leading cause of death worldwide after ischemic heart disease and all types of cancer combined (Warlow *et al*, 2003). Recent statistics of the American Heart Association reveals that stroke accounts for one of every 17 deaths in the United States (Lloyd-Jones *et al*, 2009). With the growing elderly population, stroke prevalence and stroke-related costs steadily increase.

There are two major types of stroke: (1) ischemic stroke, which occurs when the blood supply to the entire brain (e.g., during a cardiac arrest) or a circumscribed part of the brain (e.g., by thromboembolic occlusion of a cerebral artery) is disrupted and (2) hemorrhagic stroke, which occurs when a cerebral artery leaks blood into the brain tissue

(intracerebral hemorrhage) or the subarachnoid space surrounding the brain (subarachnoid hemorrhage, SAH). Despite intense basic and translational research for the last four decades, the treatments for both types of stroke are still mainly focused on prevention of new attacks by minimizing the risk factors involved and on acute relief of the cause of the stroke, such as dissolution of the thrombotic clot or closure of the bleeding cerebral artery. With regards to the longer-term pathophysiology and development of cerebral damage in the subacute period following a stroke, treatment options are few and ineffective.

Ischemic Stroke

The brain area affected by a focal ischemic stroke consists of two parts: the central ischemic core where the neurons have no chance of surviving without rapid reperfusion, and the penumbra, a perifocal area with less severe ischemia, which is potentially salvageable. Cell death in the core is caused primarily by a severe reduction in blood flow below the threshold of energy failure (Hossmann, 2009). In the subacute period following the stroke, an expansion of the ischemic core into the penumbral area is

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often seen. Mechanisms underlying the growth of an infarct into the periinfarct penumbra are not well understood, but it is thought to involve a gradual time-dependent increase in the threshold of energy failure and a delayed cell death caused by molecular disturbances in the neurons initiated by the acute ischemic insult (Hossmann, 2009).

With regard to treating focal ischemic stroke, two major approaches have been developed; reperfusion of the occluded artery by dissolution of the clot with a thrombolytic drug (recombinant tissue-type plasminogen activator), and administration of neuroprotective agents. Use of recombinant tissue-type plasminogen activator is limited by time constraints; it must be administered within 4.5 hours after stroke onset to reduce the risk of hemorrhagic transformation (Shobha *et al*, 2011; Steiner and Lyden, 2011). As regards to the neuroprotective approach, there is no lack of targets identified. A major focus has been on free radicals and oxidative damage, which are important players in excitotoxicity, apoptosis, inflammation, and neuronal cell death after ischemia and reperfusion (McCulloch and Dewar, 2001). However, at present >1,026 neuroprotective treatments have been tried, all with poor outcome in major clinical trials (O'Collins *et al*, 2006). With the latest failure of the free radical-trapping agent NXY-059 in acute ischemic stroke (Lees *et al*, 2006), the era of large stroke trials with neuroprotective agents may have come to a halt.

Subarachnoid Hemorrhage

Hemorrhagic strokes offer yet another challenge. One type of cerebral hemorrhage is SAH due to the rupture of an arterial aneurysm. The cerebral ischemia associated with SAH follows a biphasic course: first, there is an acute phase with a rapid and transient drop in cerebral blood flow (CBF) associated with the extravasation of blood and the resulting increased intracranial pressure. The acute phase carries a mortality of about 15%, which has been reduced due to aneurysmal clipping/coiling operations to prevent rebleeding. However, in surviving subjects, the acute phase is often followed by a late phase of more prolonged cerebral ischemia often associated with pathological constriction of cerebral arteries known as cerebral vasospasm (CVS). This delayed cerebral ischemia is a progressive and sometimes reversible failure of cerebral perfusion that can progress to infarction and is associated with poor outcome (Hijdra *et al*, 1987).

The only pharmacological treatment for delayed cerebral ischemia after SAH is the calcium channel antagonist nimodipine, which is part of the standard care (Dorhout Mees *et al*, 2007). However, the effect is very modest, preventing bad outcome in only one of 13 treated patients (Feigin *et al*, 1998). Nimodipine does not affect angiographic vasospasm at the clinically used dose (Allen *et al*, 1983; Espinosa *et al*, 1984), suggesting that clinical effects are due to

either direct neuroprotection or other factors, such as prevention of spreading ischemia (see below). In contrast, one of the experimental treatments tried against delayed cerebral ischemia in SAH, the endothelin receptor antagonist clazosentan, reduced angiographic vasospasm, but had no significant effect on long-term clinical outcome (Macdonald *et al*, 2008a). These findings prompted the search for other contributing mechanisms in delayed cerebral ischemia in addition to proximal vasospasms (Hansen-Schwartz *et al*, 2007; Pluta *et al*, 2009). Interestingly, a number of recent studies suggest that subacute changes in the vasoconstrictive machinery of the cerebral microcirculation after SAH may result in deleterious changes in the hemodynamic response to cortical spreading depolarizations occurring after SAH (Dreier *et al*, 2006, 2009). While coupled to vasodilatation under normal conditions, spreading depolarization can be coupled to microcirculatory vasoconstriction and ischemia after SAH (Iadecola, 2009).

Plasticity of Cerebrovascular Receptors: A Novel Aspect of the Pathophysiology of Stroke

In recent years, a novel aspect of the pathophysiology of stroke has been revealed, namely the upregulation of vasoconstrictor receptors in the smooth muscle of cerebral arteries after a stroke. These changes are detrimental since they imply a decreased perfusion of the ischemic brain area(s). Thus, whereas much stroke research has focused on the molecular mechanisms of neuronal cell death, molecular changes in the vasculature of ischemic brain areas may be equally important for the development of brain damage after a stroke. This paper reviews our current knowledge on the field of cerebrovascular receptor changes after stroke, the molecular mechanisms mediating these changes, and their potential as pharmacological targets in stroke treatment.

Receptor Changes in Experimental Stroke

The following section will discuss cerebrovascular receptor plasticity in animal stroke models: (1) permanent focal cerebral ischemia using the Tamura method (Tamura *et al*, 1981a, b), (2) transient focal cerebral ischemia induced by middle cerebral artery occlusion (MCAO) followed by reperfusion (Memezawa *et al*, 1992a), (3) transient forebrain ischemia induced by two-artery occlusion and hypovolemia (Smith *et al*, 1984), and (4) SAH induced by injection of blood into the ophthalmic cistern (Prunell *et al*, 2002).

Table 1 gives an overview of vasoconstrictor receptor changes demonstrated in various stroke models and in patients.

Table 1 Overview of cerebrovascular receptor changes demonstrated in various stroke models

<i>Stroke model</i>	<i>Cerebrovascular receptors demonstrated to be upregulated</i>	<i>References</i>
<i>Rat focal ischemic stroke</i> 2 hours proximal MCAO with reperfusion (Memezawa <i>et al</i> , 1992a)	ET _B : mRNA, protein levels, and contractile function increased in MCA at 24 and 48 hours after MCAO. Protein levels increased in microvessels at 48 hours after MCAO 5-HT _{1B} : protein levels increased in MCA and microvessels at 48 hours after MCAO AT ₁ : contractile function and protein levels, but not mRNA levels, increased in MCA at 48 hours after MCAO. Protein levels increased in microvessels at 48 hours after MCAO	Stenman <i>et al</i> (2002, 2007); Stenman and Edvinsson (2004); Henriksson <i>et al</i> (2007a, b); Maddahi and Edvinsson (2008)
2 hours distal MCAO with reperfusion (Bonfils <i>et al</i> , 2006)	ET _B : contractile function increased in MCA at 1, 24, and 48 hours after MCAO distally to the occlusion. Protein levels increased at 24 and 48 hours after MCAO distally to the occlusion	Kristiansen <i>et al</i> , 2011
Permanent distal MCAO (Tamura <i>et al</i> , 1981a, b)	ET _B , 5-HT _{1B} , and AT ₁ : contractile function and protein levels increased in MCA at 24 and 48 hours after MCAO distally, but not proximally, to the occlusion	Rasmussen <i>et al</i> (2011)
<i>Rat forebrain ischemia</i> 15 minutes carotid artery occlusion combined with hypovolemia (Smith <i>et al</i> , 1984)	ET _B and 5-HT _{1B} : contractile function and protein levels increased in ACA and MCA, but not in BA at 24 hours after the ischemic insult	Johansson <i>et al</i> (2011)
<i>Rat SAH</i> Prechiasmatic intracisternal injection of autologous blood (Prunell <i>et al</i> , 2002)	ET _B , 5-HT _{1B} , and AT ₁ : contractile function increased in MCA and BA at 12 hours (only ET _B and 5-HT _{1B}), 24, and 48 hours after SAH. mRNA levels increased in MCA and BA at 1 hour (only ET _B), 24, and 48 hours after SAH. Protein levels increased in BA and MCA at 3 hours (only ET _B), 6 hours (only AT ₁), 12, 24, and 48 hours after SAH TX _{A2} : contractile function, mRNA levels, and protein levels increased in MCA and BA at 48 hours after SAH	Hansen-Schwartz <i>et al</i> (2003a, b); Vikman and Edvinsson (2006); Ansar <i>et al</i> , 2008; Ansar <i>et al</i> (2010); Ansar and Edvinsson (2009)
<i>Organ culture of isolated cerebral arteries</i> Organ culture of rat cerebral arteries	ET _B : contractile function increased after 6, 24, and 48 hours of organ culture. mRNA and protein levels increased after 24 and 48 hours of organ culture 5-HT _{1B} : contractile function increased after 24 and 48 hours of organ culture mRNA levels increased after 24 hours of organ culture	Nilsson <i>et al</i> (1997); Leseth <i>et al</i> (1999); Hansen-Schwartz and Edvinsson (2000); Hoel <i>et al</i> (2001); Hansen-Schwartz <i>et al</i> (2002a); Henriksson <i>et al</i> (2003); Kristiansen <i>et al</i> (2011)
Organ culture of human cerebral arteries	ET _B : mRNA levels increased after 48 hours of organ culture ET _A , 5-HT _{1B} and AT ₁ : contractile function, mRNA, and protein levels increased after 48 hours of organ culture	Hansen-Schwartz <i>et al</i> (2002a); Ansar <i>et al</i> (2009); Ahnstedt <i>et al</i> (2011)
<i>Cerebral arteries from stroke patients</i> Human cerebral arteries from stroke patients	ET _B , ET _A , 5-HT _{1B} , and AT ₁ : mRNA and protein levels increased in stroke patients	Hansen-Schwartz <i>et al</i> (2002c); Vikman and Edvinsson (2006)

ACA, anterior cerebral artery; BA, basilar artery; ET_A, endothelin type A; ET_B, endothelin type B; MCAO, middle cerebral artery occlusion; SAH, subarachnoid hemorrhage; TX_{A2}, thromboxane A₂.

Ischemic Stroke

Some of the most prominent receptors showing plasticity in cerebral arteries are the endothelin receptors. Endothelial cells produce endothelin 1 (ET-1), which mediates its vasoactive effects through

two different G-protein-coupled receptors (GPCRs), the endothelin type A (ET_A) receptor, and the endothelin type B (ET_B) receptor (Masaki, 1995). The ET_A receptors are situated on the vascular smooth muscle cells, where their activation gives rise to strong vasoconstriction. Normally, ET_B recep-

tors are located on the endothelium of cerebral vessels, where their activation results in dilatation via the release of nitric oxide (Szok *et al*, 2001). Thus, in freshly isolated cerebral arteries from healthy rats, ET-1 induces a strong contractile effect, while the ET_B receptor specific agonist sarafotoxin 6c (S6c) does not; in fact S6c induces a dilatatory response if the artery is precontracted with another vasoconstrictor, such as thromboxane. This shows that only the ET_A receptor mediates vasoconstriction in normal rat cerebral arteries (Hansen-Schwartz and Edvinsson, 2000; Leseth *et al*, 1999).

However, ET_B receptor function is altered in a rat model of focal ischemic stroke consisting of 2 hours of unilateral MCAO at a point close to the Circle of Willis, followed by reperfusion (Memezawa *et al*, 1992b). Isolated MCA from nonoccluded contralateral hemisphere showed no contractile response to S6c. However, the occluded ipsilateral MCA showed a strong contraction to S6c, with an average E_{max} (maximum contraction, calculated as percentage of the contractile capacity of K⁺) around 68% (Stenman *et al*, 2002). Furthermore, there is a significant upregulation of ET_B receptor mRNA in the occluded MCA

compared with the nonoccluded MCA, indicating transcriptional ET_B upregulation in the occluded MCA.

Since ET_B receptors located on endothelial cells normally mediate a dilatatory response in the artery, we hypothesized that the appearance of contractile ET_B receptors reflected expression of ET_B receptors in smooth muscle cells of the cerebral arteries, where they are not normally present. Indeed, ET_B receptors were visualized in the smooth muscle cell layer of occluded MCA segments using immunohistochemistry, confocal microscopy, and colocalization of the receptors with the smooth muscle cell marker β -actin (Figure 1) (Henriksson *et al*, 2007a; Maddahi and Edvinsson, 2008). Interestingly, permanent MCAO did not result in ET_B upregulation in the occluded MCA (unpublished data). This suggests that ET_B upregulation is a metabolically active process, involving *de novo* gene transcription and protein synthesis, which cannot take place under chronic ischemic conditions.

Subsequently, transcriptional upregulation of vascular ET_B receptors after MCAO has been confirmed in several studies (Henriksson *et al*, 2007a, b). In one study, the authors reported a slight but significant increase also in ET_A receptor-mediated contraction of

Figure 1 Immunofluorescence staining for 5-hydroxytryptamine type 1B (5-HT_{1B}), angiotensin type 1 (AT₁), and endothelin type B (ET_B) receptors in the ischemic middle cerebral artery (MCA), cerebral microvessels (Mic.V), and surrounding brain tissue (brain). There was a clear increase in 5-HT_{1B}, AT₁, and ET_B receptor protein levels in the smooth muscle cell layer of ischemic vessels (MCA and Mic.V) as compared with vehicle control. Treatment with U0126 starting at 6 hours after MCA occlusion (MCAO) prevented the increase in expression of 5-HT_{1B}, AT₁, and ET_B receptors in smooth muscle cells of the MCA and Mic.V. There was no significant difference in expression of receptor protein levels in control brain tissue, in ischemic brain tissue, and tissue from animals treated with U0126. Data were obtained with confocal microscopy. Scale bar, 50 μ m.

occluded MCAs when compared with contralateral MCAs and ipsilateral MCAs from sham-operated rats (Henriksson *et al*, 2007a). This difference was not seen in other studies (Henriksson *et al*, 2007a; Stenman *et al*, 2002, 2007) and may reflect a relatively small ET_A receptor upregulation much less pronounced than in the case of the ET_B receptor.

Most recently, we studied changes in ET_B receptor expression in the Tamura model of distal MCAO (Tamura *et al*, 1981a, b), where the main MCA branch is occluded permanently at a point distal from the Circle of Willis, after narrowing and branching of the MCA. In this model, the area supplied by the occluded MCA is much smaller with better possibilities for collateral perfusion of the occluded area. Laser Doppler studies confirmed that, at least in our hands, there is not complete ischemia of the distal MCA region (unpublished data). In this model, ET_B receptor upregulation in the occluded MCA was demonstrated when the MCA was both occluded permanently with a suture (Rasmussen *et al*, 2011) or transiently (Kristiansen *et al*, 2011) using a microsurgical hook (Bonfils *et al*, 2006). This work revealed two interesting aspects: (1) ET_B receptors were upregulated distal to the occlusion but not proximally (Rasmussen *et al*, 2011), suggesting that loss of tension in the vascular wall is a key phenomenon in initiating receptor upregulation and (2) upregulation of ET_B-mediated vasoconstrictive responses was evident after only 1 hour of reperfusion following a 2-hour MCAO, whereas no increase in smooth muscle ET_B receptor protein levels could be detected at this time point by immunohistochemistry (Kristiansen *et al*, 2011). This suggests that there may be a rapid early phase of functional ET_B receptor upregulation and a later phase with stronger upregulation, involving *de novo* synthesis of ET_B receptors (Kristiansen *et al*, 2011).

Several other studies point to a major role for the endothelin system in the pathophysiology of ischemic stroke. For instance, it has been suggested that increased endothelin-induced contractility after cerebral ischemia leads to decreased perfusion of the ischemic area and subsequently an enlargement of the ischemic core (Asano *et al*, 1989; Robinson *et al*, 1990). In addition, exogenous ET-1 is able to decrease CBF to levels that induce ischemia (Macrae *et al*, 1993). After both ischemic stroke and SAH, the levels of ET-1 are increased in plasma, cerebrospinal fluid, and cerebral tissue (Lampf *et al*, 1997; Neuschmelting *et al*, 2009; Thampatty *et al*, 2011; Viossat *et al*, 1993), and although the detected levels are lower than the ones necessary to cause vasoconstriction under normal conditions, increased ET-1 sensitivity of cerebral arteries due to receptor upregulation may allow secreted ET-1 to cause detrimental cerebral vasoconstriction. However, attempts to treat ischemic stroke with specific ET receptor antagonists have produced conflicting results. Selective ET_A receptor antagonists increase cerebral perfusion and decrease the ischemic area in

some studies (Dawson *et al*, 1999; Patel *et al*, 1996) but not others (Bhardwaj *et al*, 2000; Umemura *et al*, 1995). Similarly, the ET_A/ET_B receptor antagonist Bosentan gives varying results when used in animal models of ischemia (Li *et al*, 1995; McAuley *et al*, 1996; Patel and McCulloch, 1996). Furthermore, an ET_B receptor antagonist, BQ788, actually exacerbated ischemic damage (Chuquet *et al*, 2002), possibly due to blockade of endothelial-dependent ET_B receptor-mediated vasodilatation.

Upregulation of cerebrovascular receptors during ischemic stroke also occurs in the angiotensin system. A somewhat delayed increase in angiotensin AT₁ receptors was observed in the rat transient MCAO model (Stenman and Edvinsson, 2004; Stenman *et al*, 2007). This was evident by significantly stronger contractile responses to angiotensin II in the MCA 48 hours after the occlusion. The effects were blocked by the AT₁ receptor inhibitors candesartan or losartan but not by the AT₂ receptor inhibitor PD123319. Furthermore, AT₁ receptor protein levels are increased in the occluded MCA (Figure 1) (Maddahi and Edvinsson, 2008). Interestingly, the authors also demonstrated increased levels of angiotensin converting enzyme mRNA in the occluded MCA, indicating that the level of angiotensin II production may be increased in the vessel wall, which would further exacerbate the vasoconstrictive angiotensin II signaling axis. Also in the Tamura model of permanent distal MCAO, at 48 hours after the occlusion, AT₁-mediated contractile responses were upregulated in the occluded MCA in segments distal to the point of occlusion, not proximally (Rasmussen *et al*, 2011). Several clinical and experimental studies point toward important roles for the angiotensin system in the pathophysiology of ischemic stroke. Infusion of angiotensin II impairs a favorable outcome after cerebral ischemia (Hosomi *et al*, 1999), and blockade of angiotensin AT₁ receptors in the acute experimental ischemic stroke is beneficial (Engelhorn *et al*, 2004; Hosomi *et al*, 2005). Clinically, favorable effects of AT₁ receptor blockade in acute ischemic stroke were observed in the Acute Candesartan Cilexetil in Stroke Survivors (ACCESS) study, which showed that treatment with an AT₁ receptor blocker during 7 days after the onset of ischemic stroke improved cardiovascular morbidity and mortality during the following 12 months (Schrader *et al*, 2003).

A third type of vasoconstrictor receptors, 5-hydroxytryptamine (5-HT) receptor subtype 5-HT_{1B}, is also upregulated in the MCA after focal ischemic stroke (Figure 1) (Maddahi and Edvinsson, 2008). Increased levels of 5-HT_{1B} receptors were demonstrated in the smooth muscle layer of occluded (2 hours) and reperfused MCAs. Later, 5-HT_{1B} receptor upregulation was demonstrated in the Tamura MCAO model in MCA segments distal to the occlusion (Rasmussen *et al*, 2011). Together, these findings indicate that phenotypic receptor changes after focal ischemic stroke are not limited to a specific receptor type, but

perhaps indicate that ischemic arteries undergo a more generalized change in their vasoconstrictor receptor expression profile.

A key mechanistic question is if the described receptor upregulation events are limited to the large cerebral arteries or if microvessels in the affected brain area are similarly affected. This was addressed in a recent study using the proximal 2 hours MCAO model (Maddahi and Edvinsson, 2008). By means of immunohistochemistry, confocal microscopy, and Western blots, it was demonstrated that microvessels within the ischemic area also overexpress ET_B, 5-HT_{1B}, and AT₁ receptors compared with microvessels in sham-operated rats (Maddahi and Edvinsson, 2008) (Figure 1).

In the above-described studies, occlusion of a cerebral artery gives rise to a local vasoconstrictor receptor upregulation in the occluded artery only distal to the point of occlusion. This raises the question whether similar receptor changes may be found in stroke types, where the blood supply to a larger part of the brain is transiently disrupted by an extracranial cause, such as carotid artery thrombosis. In the human brain, carotid artery stenosis or occlusion leads to a focal forebrain ischemia which, depending on the collateral supply, can range from no infarct to partial middle/anterior cerebral artery infarct to malignant hemispheric infarction. This can be modeled in that rat by transient occlusion of both common carotid arteries and concomitant lowering of the systemic blood pressure by hypovolemia (reversible withdrawal of blood from the jugular vein). This causes cerebral ischemia mainly in the forebrain, whereas the posterior brain parts supplied by the vertebral arteries appears almost unaffected by the insult (Smith *et al*, 1984). In this model, significant upregulation of contractile ET_B and 5-HT_{1B} receptors in cerebral artery smooth muscles was recently demonstrated after 15 minutes carotid occlusion and hypovolemia followed by 48 hours of reperfusion (Johansson *et al*, 2011). Other receptor subtypes, such as the ET_A and 5-HT_{2A} receptors, were unchanged by the ischemia. Interestingly, the receptor changes were most pronounced in the anterior cerebral arteries, less pronounced in the MCA, and did not occur in the basilar artery. The anterior cerebral arteries are also the arteries in which the blood flow drops the most during the occlusion and hypovolemia, and they supply the brain area where ischemic brain damage is found. The striking similarity in the receptor changes observed after focal cerebral ischemia induced by intracranial MCAO and this forebrain ischemia model indicates that these changes represents a general vascular response to transient disturbances in blood flow in cerebral arteries.

Subarachnoid Hemorrhage

Similar to ischemic stroke, vascular contractile receptors increased after SAH. Using an experimen-

tal model of SAH in rats originally developed by Prunell *et al* (2002), it was demonstrated that after the SAH, ET_B receptors appear on cerebrovascular smooth muscle cells and mediate vasoconstriction (Hansen-Schwartz *et al*, 2003b). The ET_B receptor upregulation was demonstrated by functional contractility studies, immunohistochemistry, Western blotting, and real-time polymerase chain reaction, the latter indicating that the receptor upregulation occurs at the transcriptional level. The ET_B receptor upregulation has been demonstrated in both basilar arteries and MCA, reflecting the global nature of the cerebral ischemia associated with SAH. Although cerebrovascular ET_B receptor upregulation observed after SAH corresponds well with our findings in the ischemic stroke models, the functional manifestations of the increased number of smooth muscle ET_B receptors appear more complex. The upregulated ET_B receptors show no response to S6c but a potentiated response to ET-1, which could be antagonized by both an ET_B antagonist and an ET_A antagonist (Hansen-Schwartz *et al*, 2002a, 2003b). This may indicate that after SAH, the new smooth muscle ET_B receptors exert constriction only in conjunction with smooth muscle ET_A receptors (Hansen-Schwartz *et al*, 2003b) either via direct interactions in homodimers or heterodimers, which have been reported in other systems, or via indirect functional interactions between the two receptor subtypes (Evans and Walker, 2008; Harada *et al*, 2002).

In studies using a rat double hemorrhage model of SAH, a similar leftwards shift of the ET-1 dose-contraction curve was observed in cerebral arteries from SAH rats compared with sham rats (Konczalla *et al*, 2006; Vatter *et al*, 2007a). However, no change was detected in the immunoreactivity for ET_B receptors in cerebral arteries after SAH (Vatter *et al*, 2007b), in contrast with the above findings in the Prunell SAH model. The most likely explanation for these contrasting findings is the different methodologies used for inducing experimental SAH. In the double hemorrhage model, blood is injected into the cisterna magna with low pressure. This injection is repeated after 48 hours; and 3 to 5 days after the second blood injection, CVS develops. In the Prunell model, a single injection of blood into the prechiasmatic subarachnoid space is made at a pressure equal to the mean arterial blood pressure, thus mimicking bleeding from an artery. This gives rise to CVS and delayed cerebral ischemia ~48 hours after the blood injection (see Figure 2). A study performed to clarify the critical events in initiation of the vasoconstrictor receptor changes in the Prunell model (Ansar and Edvinsson, 2009) showed that injection of saline into the prechiasmatic cistern with a pressure equal to mean arterial blood pressure caused significant cerebrovascular ET_B upregulation, whereas saline injections at low pressure caused no changes in ET_B expression. This suggests that the intracranial pressure increase itself and the resulting acute drop in

Figure 2 Time course of changes in cerebral blood flow following subarachnoid hemorrhage (SAH) in humans and in the rat prechiasmatic injection SAH model. **(A)** Schematic prediction of the typical biphasic nature of the cerebral ischemia associated with SAH in humans and delineates the characteristics of the two phases. **(B)** Time course of the biphasic reduction in cerebral arterial diameter (assessed by X-ray-based angiography) after experimental SAH induced by prechiasmatic injection of blood in the rat. Arterial diameter data in panel **(B)** are from Delgado *et al* (1985).

CBF in this model is sufficient to cause cerebrovascular ET_B upregulation. However, high-pressure injections of blood yielded more pronounced ET_B upregulation, indicating that the presence of subarachnoid blood also contributes to the receptor upregulation (Ansar and Edvinsson, 2009). On this basis, the lack of acute rise in intracranial pressure may be the reason for the lack of cerebrovascular ET_B upregulation in the rat double hemorrhage model.

As is the case in ischemic stroke models, cerebrovascular receptor changes after SAH are not limited to endothelin receptors but also includes $5-HT_{1B}$ (Hansen-Schwartz *et al*, 2003a) and AT_1 (Ansar *et al*, 2008) receptors. Furthermore, a recent study demonstrated SAH-induced upregulation of thromboxane A_2 receptors (Ansar *et al*, 2010). However, not all contractile receptors change in experimental SAH as other amine and neuropeptide receptors, contractile as well as relaxant, are unaffected (Ansar, unpublished data). Most interestingly, the time course of upregulation of the ET_B , $5-HT_{1B}$, and AT_1 receptors was demonstrated to correlate with the time course of delayed cerebral ischemia, suggesting an important pathophysiological role for upregulated vasoconstrictor receptors (Ansar *et al*, 2008).

In summary, a number of vasoconstrictor GPCRs expressed in cerebral arteries show expressional plasticity in both hemorrhagic and ischemic stroke models, indicating that this may be a common response of the cerebral vasculature leading to increased vasoconstriction after ischemic insults.

Organ Culture as a Method to Investigate Cerebrovascular Receptor Changes

The first evidence that cerebral arteries can undergo dynamic changes in endothelin receptor expression came from rat basilar arteries and MCAs placed in organ culture for 1 to 2 days (Hansen-Schwartz and Edvinsson, 2000; Leseth *et al*, 1999). This treatment caused a leftwards shift of the dose-response curves for ET-1 (indicating increased sensitivity to ET-1) and the appearance of a strong ET_B -specific contractile response to S6c (Hansen-Schwartz *et al*, 2002a; Nilsson *et al*, 1997). The time course of endothelin receptor changes in cultured MCAs is relatively fast, with a small contractile response to S6c after just 6 hours of organ culture (Henriksson *et al*, 2003; Kristiansen *et al*, 2011). After 24 or 48 hours of culture, there is a strong S6c response accompanied by a leftwards shift of the ET-1 concentration-contraction curve (Hansen-Schwartz *et al*, 2002b; Henriksson *et al*, 2003). After 24 hours of organ culture, ET_B mRNA and protein levels in the smooth muscle layer are increased compared with fresh vessels, while ET_A mRNA and protein levels remain unchanged (Henriksson *et al*, 2003). Moreover, both the transcriptional inhibitor actinomycin D and the translational inhibitor cycloheximide abrogate the ET_B receptor upregulation during organ culture, underscoring the dependence of the protein on

de novo gene transcription and protein synthesis (Henriksson *et al*, 2003). Organ culture also causes an increased contractile response to 5-carboxamidotryptamine (5-CT) due to transcriptional upregulation of the 5-HT_{1B} receptor subtype (Hoel *et al*, 2001).

Since changes in vasoconstrictor responses after *in vitro* organ culture show a striking similarity to the changes observed in animal models of ischemic and hemorrhagic stroke, organ culture can be used as a convenient *in vitro* method to study the pharmacological characteristics, time course, and underlying cellular and molecular mechanisms of cerebrovascular receptor alterations.

Cerebrovascular Receptor Changes in Human Arteries

Because of the clinical relevance, it is important to know if human cerebral arteries show the same GPCR expression profiles as found in rats, and if they display a similar degree of receptor plasticity with cerebral ischemia and *in vitro* organ culture. To address the first part of this question, small samples of cortex arterioles were obtained in conjunction with neurosurgical tumor resections or operations to remove epileptic seizure areas. These vessels indeed express ET_A and ET_B receptors (Nilsson *et al*, 1997), AT₁ (Ahnstedt *et al*, 2011; Ansar *et al*, 2009), 5-HT_{1B} (Nilsson *et al*, 1999), and thromboxane A₂ (Uski *et al*, 1983) receptors. Moreover, freshly obtained human cerebral arteries exhibit ET-1-induced responses that were found to be similar to the responses observed in fresh rat cerebral arteries; ET-1 elicited an ET_A receptor-mediated contraction with $E_{\max}$ 112% and pEC₅₀ 9.2, whereas S6c elicited an ET_B receptor-mediated dilatation on precontraction (Nilsson *et al*, 1997).

To investigate receptor plasticity in human cerebral arteries, the *in vitro* organ culture method has been used. However, in cultured human cortical cerebral arteries, the picture was somewhat different compared with cultured rat cerebral arteries; organ culture of human arteries enhanced the response to ET-1 in terms of both sensitivity and potency, $E_{\max}$ 152% and pEC₅₀ 10.3 (Hansen-Schwartz *et al*, 2002a). The ET-1-induced contraction was solely ET_A receptor dependent. In contrast, S6c did not elicit any contraction (Hansen-Schwartz *et al*, 2002a). Furthermore, contractile responses mediated by AT₁ and 5-HT_{1B} receptors were also found to be increased in human cerebral arteries on organ culture, and the upregulation of ET_B, 5-HT_{1B}, and AT₁ receptors in the smooth muscle layer was confirmed with immunohistochemistry (Ahnstedt *et al*, 2011; Ansar *et al*, 2009). Thus, human cerebral arteries clearly have the ability to modulate their vasoconstrictor receptor expression profile during culture. The changes were similar to those seen in rat cerebral arteries, although the importance of the

ET_A receptor appears to be greater in human than in rat cerebral arteries, where only the ET_B receptor is upregulated on organ culture.

To discuss whether GPCR changes are induced by cerebral ischemia *in vivo* in humans, a series of studies examined postmortem cerebral artery samples from stroke patients. In the first series of cerebrovascular samples, it was observed that both large vessels and microvessels had more ET_A and ET_B receptor mRNA as compared with control specimens (Hansen-Schwartz *et al*, 2002c). A later study compared MCA samples from 10 subjects who had died from a stroke and 10 subjects who died due to a myocardial infarct. Analysis of mRNA expression and the expression and localization of the encoded proteins validated the earlier results on changes in receptor expression for endothelin receptors but also showed enhanced expression of receptors in the smooth muscle cells for AT₁ and 5-HT_{1B} (Vikman and Edvinsson, 2006).

Intracellular Signaling Mechanisms Involved in Cerebrovascular Receptor Upregulation

Following discovery and characterization of cerebrovascular receptor changes, an extensive series of studies have been aimed at identifying upstream signal transduction mechanisms and transcription factors involved in the alteration of vascular GPCR gene transcription and protein synthesis in cerebral ischemia (summarized in Table 2).

Mitogen Activating Protein Kinases

One of the key findings is that mitogen activating protein kinase (MAPK) kinases have an important role in cerebrovascular receptor plasticity. There are three major MAPKs, p38, ERK1/2, and JNK (c-Jun NH₂-terminal kinase), each of them located downstream of a dynamic chain of kinases. Each MAPK signaling pathway involves an MAPKKK that can activate MAPKKs, which in turn activate MAPKs. p38 and SAPK/JNK are considered inflammatory MAPKs and tend to be activated by inflammatory cytokines and cellular stress. ERK1/2 is considered mainly mitogenic and has a predominant role in growth factor receptor signaling (Boulton *et al*, 1991; Grethe *et al*, 2004; Hsieh and Nguyen, 2005; Kumar *et al*, 2003). The MAPKs elicit some of their effects through phosphorylation of transcription factors, thus initiating DNA binding and transcriptional regulation. Transcription factor activation is somewhat redundant between the various MAPKs, with the same transcription factor sometimes being activated by two or more MAPKs. There are also transcription-independent effects of MAPK activity such as cytokine mRNA transportation from the nucleus (Karin, 2005), increased translation, and

Table 2 Overview of signal transduction components involved in cerebrovascular receptor changes in various stroke models

Signal transduction component	Evidence for role in cerebrovascular receptor changes	References
<i>Raf/MEK/ERK1/2</i>		
Organ culture of cerebral arteries	<ul style="list-style-type: none"> ● Raf inhibitor SB386023 and ERK inhibitor AG126 prevents upregulation of ET_B contractile function and mRNA levels ● MEK1/2 inhibitors U0126 prevent upregulation of ET_B and 5-HT_{1B} contractile function and mRNA levels 	Henriksson <i>et al</i> (2003, 2004, 2007a); Sandhu <i>et al</i> (2010a, b)
Focal ischemic stroke	<ul style="list-style-type: none"> ● Phosphorylation of ERK1/2 in cerebral arteries and microvessels at 24 and 48 hours after MCAO ● MEK1/2 inhibitor U0126 prevents ET_B, 5-HT_{1B}, and AT₁ upregulation in cerebral arteries and microvessels, reduces infarct size, and improves neurology score 	Henriksson <i>et al</i> (2007a); Maddahi and Edvinsson (2008)
Subarachnoid hemorrhage (SAH)	<ul style="list-style-type: none"> ● Phosphorylation of ERK1/2 in cerebral arteries at 1–6 hours after SAH ● Raf inhibitor SB386023 prevents ET_B, 5-HT_{1B}, and AT₁ upregulation and delayed reduction of cerebral blood flow ● MEK1/2 inhibitor U0126 prevents upregulation of ET_B and 5-HT_{1B} and improves neurology score 	Beg <i>et al</i> (2006); Ansar and Edvinsson (2008); Larsen <i>et al</i> (2010)
<i>p38 and JNK</i>		
Organ culture of cerebral arteries	<ul style="list-style-type: none"> ● p38 inhibitor SB239063 does not prevent ET_B upregulation but increases ET_A contractile function and mRNA levels 	Henriksson <i>et al</i> (2004)
SAH	<ul style="list-style-type: none"> ● Phosphorylation of p38 and JNK in cerebral arteries at 48 hours after SAH 	Ansar and Edvinsson (2008)
<i>Protein kinase C</i>		
Organ culture of cerebral arteries	<ul style="list-style-type: none"> ● PKC inhibitors Ro-31-7549, Ro-31-8220, Ro-32-0432, and bisindolylmaleimide I prevent upregulation of ET_B contractile function and mRNA levels ● Ro-31-7549 does not prevent 5-HT_{1B} upregulation ● PKC inhibitor 20–28 prevents upregulation of ET_B contractile function and protein levels, but not mRNA levels 	Hansen-Schwartz <i>et al</i> (2002b); Henriksson <i>et al</i> (2003, 2006)
Focal ischemic stroke	<ul style="list-style-type: none"> ● PKC inhibitor Ro-32-0432 prevents ET_B upregulation, reduces infarct size, and improves neurology score 	Henriksson <i>et al</i> (2007b)
SAH	<ul style="list-style-type: none"> ● Phosphorylation of PKCδ in cerebral microvessels at 1 hour after SAH ● PKC inhibitor Ro-32-0432 prevents ET_B and 5-HT_{1B} upregulation and delayed reduction in cerebral blood flow 	Beg <i>et al</i> (2007); Ansar and Edvinsson (2008)
<i>Calcium channels and CaMKII</i>		
Organ culture of cerebral arteries	<ul style="list-style-type: none"> ● CaMKII inhibitor KN93, voltage-operated calcium channels inhibitor nifedipin, and IP3 channel inhibitor xestospongine C prevent upregulation of ET_B contractile function after organ culture 	Waldsee <i>et al</i> (2010)
<i>Transcription factors</i>		
Focal ischemic stroke	<ul style="list-style-type: none"> ● Phosphorylation of Elk-1 in cerebral arteries and microvessels at 24 and 48 hours after MCAO 	Henriksson <i>et al</i> (2007a); Maddahi and Edvinsson (2008)

ET_A, endothelin type A; ET_B, endothelin type B; JNK, c-Jun NH₂-terminal kinase; MCAO, middle cerebral artery occlusion; PKC, protein kinase C; CaMKII, calcium calmodulin-dependent kinase II; ERK1/2, extracellular-regulated kinase 1/2; MEK, mitogen-activated protein kinase kinase.

mRNA stability (Clark *et al*, 2003; Kumar *et al*, 2003; Saklatvala *et al*, 2003).

One of our earliest findings was that cytokines, which activate p38 MAPK, are able to increase the

ET_B receptor-mediated contractions in arteries subjected to organ culture (Uddman *et al*, 1999). Other studies have shown an involvement of the MEK/ERK1/2 pathway in cerebral ischemia, and inhibitors

toward this pathway are able to diminish the ischemic area (Alessandrini *et al*, 1999; Namura *et al*, 2001). In addition, Lennmyr *et al* (2002) demonstrated activation of ERK1/2 in cerebral arteries after MCAO.

On this basis, the involvement of ERK1/2 and p38 MAPK pathways was assessed in the upregulation of contractile ET_B receptors in artery culture (Henriksson *et al*, 2004, 2007a). Inhibitors tested were U0126, which inhibits MEK1/2 (the MAPKK of ERK1/2), SB386023, which inhibits Raf (the MAPKKK of ERK1/2), and SB239063, which inhibits p38. In MCAs cultured in the presence of ERK1/2 pathway inhibitors, the upregulation of ET_B receptor-mediated contractile responses was diminished. This was not seen with the p38 inhibitor (Henriksson *et al*, 2004). Furthermore, U0126 and SB386023 diminished ET_B mRNA levels after organ culture, whereas SB386023 and SB239063 showed a tendency to elevate ET_A receptor mRNA levels. The difference between the functional ET_A responses and the ET_A mRNA level could simply be due to the fact that the mRNA level is increased at an earlier stage of the organ culture and is returning to their original values at the 24-hour time point. The raf inhibitor SB386023 also modified expression of ET_B receptor protein in smooth muscle cells (Henriksson *et al*, 2004). In a recent study, a number of MAPK inhibitors were compared for their ability to prevent the upregulation of various cerebrovascular vasoconstrictor receptors during organ culture (Sandhu *et al*,

2010a). It was found that the MEK/ERK inhibitor U0126 was effective in preventing ET_B and 5-HT_{1B} upregulation, while other MAPK inhibitors (PD98059, SL327, AG126) were not. Most interestingly, U0126 and SB386023 were effective in inhibiting vasoconstrictor receptor upregulation even when applied at 6 and 12 hours, respectively, after the start of the incubation (Jamali and Edvinsson, 2006; Sandhu *et al*, 2010a). These findings suggest the possibility that this treatment could be effective even if given hours after the stroke occurrence.

Several *in vivo* situation studies using the rat MCAO model have confirmed the role of MEK-ERK1/2 signaling in focal cerebral ischemia. Treatment with U0126, given systemically in mg doses, prevented phosphorylation of ERK1/2 and the downstream transcription factor Elk-1 as well as suppressed upregulation of ET_B, 5-HT_{1B}, and AT₁ receptors in cerebral arteries and microvessels in the brain area affected by ischemia (Figures 1 and 3). Middle cerebral artery occlusion resulted in an infarct of about 25% of the brain volume, as visualized by 2,3,5-triphenyltetrazolium chloride staining. In animals treated with U0126, the ischemic region was reduced to 10% to 15% of the brain volume at 2 days after MCAO, representing a 50% reduction in infarct size (Henriksson *et al*, 2007a; Maddahi and Edvinsson, 2008). There was also a significantly improved neurologic function of the animals (Henriksson *et al*, 2007a). Interestingly, the inhibitor exerted equally positive effects whether

Figure 3 Immunofluorescence staining for phospho-extracellular regulated kinase 1/2 (pERK1/2) and transcription factor phospho-ELK-1 (pELK-1) in the ischemic middle cerebral artery (MCA), cerebral microvessels (Mic.V), and surrounding brain tissue (brain) in rats treated with vehicle, MCA occlusion (MCAO) + vehicle, or MCAO plus U0126 at different time points. There was an increase in activated (phosphorylation = p) ERK1/2 and ELK-1 in the smooth muscle cell layer of ischemic vessels (MCA and Mic.V) as compared with vehicle. Treatment with U0126 starting at 0, 6, and 12 hours after occlusion prevented the increase in expression of pERK1/2 in smooth muscle cells of the MCA and Mic.V as studied at 48 hours. Data were obtained with confocal microscopy. Scale bar, 50 μ m.

administered immediately after the MCAO or 6 hours after the insult, which is within the clinically relevant therapeutic time window (Maddahi and Edvinsson, 2008). This area of research has received little attention before; however, Slevin *et al* (2000) observed in microvessels within the penumbra that ERK1/2 is activated in human brain after acute ischemic stroke. Moreover, ET-1 levels are increased following a stroke and may regulate ERK1/2 in vascular smooth muscle cells (Brehm *et al*, 2002).

Functional consequences of MEK/ERK inhibition in stroke have been further substantiated by studies using the rat SAH model. In this model, ERK1/2 was found to be activated in the smooth muscle layer of cerebral arteries within minutes of initiation of the SAH, while other kinases (p38 and SAP/JNK) only appear phosphorylated at 2 days after the SAH (Ansar and Edvinsson, 2008). In an initial *in vivo* pharmacological study, the specific Raf inhibitor SB386023 was administered via a cisternal catheter before and immediately after the SAH as well as every 12 hours for 2 days after the SAH (Beg *et al*, 2006). This route of administration is clinically relevant since many hospitalized SAH patients with risk of developing CVSs have an intracranial catheter inserted to monitor and regulate the intracranial pressure. The animals tolerated the treatment well and the inhibitor showed capable of normalizing endothelin, AT₁, and 5-HT receptor expression in cerebral vessels in SAH animals. This effect correlated well with the regional CBF and neurology score, which were both compromised in SAH animals and normalized in SAH animals treated with SB386023 (Beg *et al*, 2006). Perhaps, the most exciting finding was that the Raf blocker could be given up to 6 hours after the SAH with an equally good result. In a subsequent study, the MEK inhibitor U0126 was evaluated for effects on functional outcome after SAH (Larsen *et al*, 2010). Treatment with U0126 commencing at 6 hours after the SAH was able to normalize both vasoconstrictor receptor expression and neurologic function at 2 days after SAH.

Protein Kinase C and Calcium–Calmodulin-Dependent Kinase II

Decades of research has revealed a central role for calcium overload in the cellular responses to neuronal ischemic injury (Siesjö *et al*, 1995). High levels of intracellular calcium result in the activation of various protein kinases, including protein kinase C (PKC) and calcium–calmodulin-dependent kinase II (CaMKII) (Bright and Mochly-Rosen, 2005; Waxham *et al*, 1996; Wieloch *et al*, 1991). In cerebral artery smooth muscle cells, PKC has been implicated in calcium sensitization, mechanotransduction, and myogenic constriction (Baek *et al*, 2010; Nakayama and Tanaka, 1993) as well as in the pathogenesis of CVSs after SAH (Laher and Zhang, 2001; Roman *et al*, 2006). Both PKC and CaMKII are also

well-known regulators of the smooth muscle contractile machinery (Kim *et al*, 2008).

Protein kinase C is a family of serine–threonine kinases, which consists of 11 cloned PKC subtypes that all regulate a broad spectrum of cellular functions. The first studies implicating PKC in the mechanisms behind cerebrovascular receptor plasticity demonstrated that PKC is critically involved upstream of the upregulation of ET_B receptors after organ culture (Hansen-Schwartz *et al*, 2002b; Henriksson *et al*, 2003). A subsequent study examining the involvement of specific PKC subtypes demonstrated expression of a limited number of PKC subtypes (α , γ , δ , and ϵ) (Henriksson *et al*, 2006). This study also revealed that different PKC inhibitors work differently on ET_B receptor contractile function, mRNA, and protein levels. The general PKC inhibitor, Ro-31-8220, attenuated both the contractile ET_B receptor-mediated response and the upregulated ET_B receptor mRNA levels after organ culture. However, Ro-31-8220 inhibits not only PKC, but also other factors such as c-jun, JNK, and mitogen-activated protein kinase phosphatase-1 (Beltman *et al*, 1996; Standaert *et al*, 1999), and thus the effects may not be solely dependent on PKC inhibition. A subsequent study tested inhibitors with different PKC subtype specificity and found that the PKC inhibitors bisindolylmaleimide I, Ro-32-0432, and 20 to 28 (a peptide mimicking an inactivating part of PKC) (Eichholtz *et al*, 1993) diminished ET_B receptor-mediated contractions and ET_B protein levels after organ culture while not affecting ET_A receptor-mediated contractions (Henriksson *et al*, 2006). Ro-32-0432 also effectively abolished the increase in ET_B mRNA during organ culture (Henriksson *et al*, 2006).

When Ro-32-0432 was administered *in vivo* to rats after MCAO and reperfusion, it reduced the infarct volume, improved neurology score, and normalized ET_B-mediated vasomotor responses in ipsilateral cerebral arteries (Henriksson *et al*, 2007b). Furthermore, RO-32-0432 administered intrathecally to rats with experimental SAH attenuated the delayed reduction in CBF and normalized vasomotor responses mediated by ET_B and 5-HT_{1B} receptors (Beg *et al*, 2007). Activation of various PKC isoforms as early as 1 hour after SAH was demonstrated in a quantitative Western blot study (Ansar and Edvinsson, 2008). This study also revealed an interesting interaction between PKC and MEK–ERK1/2 signaling in cerebral arteries after SAH, which is also supported by molecular data from others (Ginnan *et al*, 2006).

As mentioned above, calcium has a central role in neuronal cell death and in vascular smooth muscle contraction–excitation coupling, but its significance for cerebrovascular vasoconstrictor receptor plasticity is less well understood. The calcium-activated CaMKII is known to have effects on gene regulation, and in a recent study, Waldsee *et al* (2010) examined the involvement of CaMKII and various calcium channels in endothelin receptor regulation in

cerebral arteries on organ culture. The ET_B receptor upregulation was attenuated by coincubation with the inositol 1,4,5-triphosphate receptor (IP3R) inhibitor xestospongine C, L-type voltage-operated calcium channel inhibitor nifedipine, and the CaMKII inhibitor KN93. KN93 and xestospongine C attenuated, and nifedipine promoted K⁺, S6c, and ET-1-induced contractions after organ culture, indicating that both ET_B receptor upregulation and ET_B-independent (K⁺-induced) contraction involves CaMKII, voltage-operated calcium channels, and IP3 channels (Waldsee *et al*, 2010).

Upstream Initiators of Cerebrovascular Signal Transduction and Receptor Changes

An important question is 'What starts the phenotypic receptor changes?' We hypothesize that the change in vascular wall shear stress and luminal pressure that occur during a stroke or organ culture is one important factor (Olesen *et al*, 1988). An early study of isolated mesenteric arteries were perfused at different flow levels and perfusion pressures (Szok *et al*, 2001) showed that perfusion at increased pressure (150 mm Hg) results in enhanced ET_B receptor mRNA expression (Lindstedt *et al*, 2009). This study supports the hypothesis that pressure changes can induce receptor changes.

This hypothesis is also supported by *in vivo* studies (Rasmussen *et al*, 2011), in which activation of ERK1/2 and upregulation of AT₁, 5-HT_{1B}, and ET_B receptors was observed distal to a microsuture occlusion of a distal branch of the MCA (Tamura *et al*, 1981a) but not proximal. Since the same low degree of ischemia and inflammation was present around the MCA both proximally and distally to the knot, it suggests that the crucial initiating factor is the drop in wall tension distally to the knot. However, it is still not clear which mechanosensitive molecular factors that 'sense' this and how it is conveyed to the intracellular processes that lead to receptor upregulation. Furthermore, it is not clear whether it is the transient drop or the subsequent increase in shear stress during reperfusion, or a combination of the two that are the pivotal factor initiating the receptor changes. The *in vitro* experiments (Lindstedt *et al*, 2009) in which increased perfusion pressure induced ET_B receptor upregulation points to the reperfusion process as at least a contributing factor. Furthermore, it is possible that the initiation, propagation, and maintenance of the process of vasoconstrictor receptor upregulation involve other factor besides shear stress changes, such as cytokines, oxidative stress, growth factors, and vasoactive hormones.

It has been shown recently that lipid soluble smoke particles can act via MAP kinases and nuclear factor- κ B to enhance the ET_B receptor upregulation process during organ culture and coincubation with low-

density lipoprotein further enhances this process (Sandhu *et al*, 2010b; Xu *et al*, 2008, 2010). In addition, ET_B receptors are elevated in vascular smooth muscle following experimental chronic increases in intraluminal pressure (Lindstedt *et al*, 2009) and in human hypertension (Nilsson *et al*, 2008), which is a well-known risk factor for stroke. Thus, a range of stroke risk factors are able to enhance cerebrovascular vasoconstrictor receptor upregulation, and this mechanism may be a target for therapy to minimize stroke risk in certain patients.

The Role of Inflammation in Cerebrovascular Pathophysiology After Stroke

The reduction in CBF after a stroke results in a robust inflammatory response in the injured brain tissue that exacerbates tissue damage for several days after the insult (Mitsios *et al*, 2006). This response is characterized by expression of inflammatory genes with local activation and release of several cytokines, chemokines, adhesion molecules, and proteolytic enzymes (Endres *et al*, 2008). Some of the factors involved after ischemic stroke are tumor necrosis factor- α , interleukin-1 β (IL-1 β), IL-6, and inducible nitric oxide synthase, which are produced by a variety of activated cell types; endothelial cells, microglia, neurons, platelets, monocytes, macrophages, and fibroblasts (Huang *et al*, 2006). Furthermore, there is now clinical evidence of an upregulation of IL-6 after SAH (Sarrafzadeh *et al*, 2011).

Recently, it has become clear that the inflammatory process after stroke not only affects the brain tissue itself but also exerts detrimental effects in the walls of cerebral arteries. After cerebral ischemia, the blood-brain barrier is disrupted, which allows activated leukocytes to infiltrate the smooth muscle layer of cerebral arteries. Interestingly, recent work suggests that there is a strong link between inflammatory processes in cerebrovascular walls and upregulation of vasoconstrictor receptors after stroke. Thus, 2 hours of MCAO followed by reperfusion for 46 hours resulted in enhanced levels of tumor necrosis factor- α , IL-1 β , IL-6, and nitric oxide synthase in the smooth muscle layer of the ischemic MCA and in associated intracerebral microvessels (Maddahi and Edvinsson, 2010). In experimental SAH, the delayed global cerebral ischemia 2 days after the SAH was also shown to be associated with enhanced protein levels of IL-1 β , IL-6, and nitric oxide synthase in the smooth muscle layer of cerebral arteries (Maddahi *et al*, 2011). Blockage of the MEK/ERK pathway prevented this response in both models, and this effect could also be seen when treatment with the blocker was delayed for up to 6 hours after the insult (Maddahi and Edvinsson, 2010; Maddahi *et al*, 2011).

In both the MCAO and SAH models, costaining with smooth muscle marker β -actin showed that the upregulated cytokines were localized to the smooth muscle layer of the cerebral arteries and microvessels and detailed morphological analyses using confocal microscopy revealed only negligible amounts in the surrounding adventitia and brain parenchyma (Maddahi and Edvinsson, 2010; Maddahi *et al*, 2011). However, the authors did not find increased cytokine mRNA levels in the cerebral artery walls after SAH (Maddahi *et al*, 2011). This suggests that the cytokines in the smooth muscle layer are either synthesized outside the vessels or by a small subpopulation of infiltrating cells in the smooth muscle cell layer, such as leukocytes.

The formation of edema and opening of the BBB in cerebral ischemia is associated with enhanced expression of metalloproteinase-9 (MMP-9) and with tissue inhibitor of MMP-1 (TIMP-1) as a counter-regulatory mechanism. In experimental MCAO and SAH, enhanced protein and mRNA expression of MMP-9 and TIMP-1 has been demonstrated in cerebral vessel walls. Interestingly, MMP-9 and TIMP-1 was especially associated with smooth muscle actin but there was no overlap with the astrocyte/glial cell marker GFAP in the vessel walls, indicating that the transcriptional upregulation takes place in the vascular smooth muscle cells themselves (Maddahi *et al*, 2009, 2011). Treatment with inhibitors of the MEK-ERK1/2 pathway normalized the expression of MMP-9 and TIMP-1 (Maddahi *et al*, 2009, 2011), indicating that both upregulation of pro-inflammatory mediators and extracellular-matrix-related proteins is governed by at least some of the same signaling pathways as cerebrovascular receptor upregulation.

Signal Transduction Inhibitors Versus Vasoconstrictor Receptor Antagonists

In appreciation of the potential importance of local vasoconstriction for the development of cerebral ischemic damage after a stroke, several attempts have been made to treat ischemic stroke as well as SAH with receptor antagonists targeted to a specific vasoconstrictor receptor subtype. For example, in ischemic stroke, antagonists of angiotensin receptors (Chrysant, 2007; Hosomi *et al*, 2005; Lou *et al*, 2004) and endothelin receptors (Dawson *et al*, 1999; Edvinsson, 2009) have been tested in experimental models and/or clinical trials. In SAH, some of the first experimental treatments against the delayed vasoconstriction were designed to block specific vascular receptors that were thought to be involved (Wilkins, 1990). This approach is still ongoing today, for example the clazosentan trial program (Macdonald *et al*, 2008a; Schubert *et al*, 2008), which showed a reduction in the relative risk of angiographic vasospasm in SAH patients treated with the highest drug dose. However, this treatment failed to show significant reduction in the number of patients

exhibiting delayed neurologic deterioration; and, even more disappointingly, there was no beneficial effect on outcome after 3 months of observation after stroke (Macdonald *et al*, 2008b).

Since a large body of work now points to the combined, synergistic involvement of several vasoconstrictor receptors in vascular pathophysiology after a stroke, a study was performed to evaluate the effect of combined inhibition of the angiotensin and endothelin systems in experimental ischemic stroke (Stenman *et al*, 2007). AT₁ receptor antagonism has been shown to improve outcome after ischemic stroke, and it has been suggested that its beneficial effect is due to increased AT₂ receptor stimulation when the AT₁ receptor is blocked (Li *et al*, 2005) or to reduced production of the free radical superoxide, an important mediator of ischemic damage (Sugawara *et al*, 2005). In the latter combination study neither the AT₁ receptor blocker candesartan nor the ET_A receptor blocker ZD1611 by themselves significantly affected infarct volume or neurology score (Stenman *et al*, 2007). However, when candesartan and ZD1611 were administered in combination, the treatment reduced brain damage and improved neurology scores after MCAO, with no effect on mean arterial blood pressure (Stenman *et al*, 2007). This study clearly underscores the importance of a multitargeted approach in stroke therapy, and highlights the combined action of several vasoconstrictor systems to cause local vasoconstriction and augment brain damage after focal ischemic stroke.

Inhibition of intracellular signaling pathways that govern upregulation of multiple vasoconstrictor receptors in cerebral arteries represents another approach to prevent local vasoconstriction and vascular pathology after stroke. This strategy may prove more efficient and less prone to serious cardiovascular side effects than the combined blockage of various vasoconstrictor receptors. Inhibition of PKC isozyme-specific modulators have been examined and show clinical promise, in particular inhibitors of the δ PKC subtype (Bright *et al*, 2004). However, subsequent clinical trials could not support this hypothesis (O'Collins *et al*, 2006). We hypothesize that inhibition of the MEK-ERK1/2 pathway is a more promising approach, since this pathway appears to govern upregulation of a whole panel of cerebrovascular vasoconstrictor receptors after stroke as well as induction of vascular wall inflammation and blood-brain barrier breakdown.

Perspectives—Promises for Novel Stroke Treatments

There is an urgent need for new ways to treat cerebral ischemia since current neuroprotective agents have either proven ineffective or produced deleterious side effects. The work discussed in this review highlights the fact that the cerebral arteries participate actively in the response following an episode of cerebral

Figure 4 Schematic illustration of the main conclusions and hypotheses described in this review. BBB, blood–brain barrier; CaMKII, calcium–calmodulin-dependent kinase II; ERK1/2, extracellular-regulated kinase $\frac{1}{2}$; GPCR, G-protein-coupled receptor; JNK, c-Jun NH₂-terminal kinase; MMP, matrix metalloprotease; PKC, protein kinase C; SAH, subarachnoid hemorrhage.

ischemia in humans and rat (for a schematic summary, see Figure 4). The process involves ERK1/2 activation, which in turn activates the transcription of inflammatory and extracellular-matrix-regulating genes as well as genes for specific vasoconstrictor receptors in the smooth muscle of cerebral arteries (Figure 4). Because of the synergistic nature of these processes, which involve several gene groups, a single factor approach to the treatment of cerebral ischemia seems unlikely to be effective since not only vasoconstrictor receptors are involved but also induction of genes involved in inflammation, apoptosis, and blood–brain barrier modulation.

As discussed in this review, several central cell signaling pathways have been identified that are important for vasoconstrictor receptor upregulation and the subsequent local vasoconstriction as well as induction of proinflammatory and extracellular-matrix-related genes. By blocking these signaling components, cerebral infarct volume after ischemic stroke and delayed cerebral ischemia after SAH can be considerably reduced. These findings suggest that modulation of cerebrovascular intracellular signals in the subacute phase after a stroke may be a novel approach to protecting the brain from damage after a stroke.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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