Role of calcitonin gene-related peptide in cerebral vasospasm, and as a therapeutic approach to subarachnoid hemorrhage

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Calcitonin gene-related peptide (CGRP) is one of the most potent microvascular vasodilators identified to date. Vascular relaxation and vasodilation is mediated via activation of the CGRP receptor. This atypical receptor is made up of a G protein-coupled receptor called calcitonin receptor-like receptor (CtLR), a single transmembrane protein called receptor activity-modifying protein (RAMP), and an additional protein that is required for Ga coupling, known as receptor component protein (RCP). Several mechanisms involved in CGRP-mediated relaxation have been identified. These include nitric oxide (NO)-dependent endothelium-dependent mechanisms or cAMP-mediated endothelium-independent pathways; the latter being more common. Subarachnoid hemorrhage (SAH) is associated with cerebral vasospasm that occurs several days after the hemorrhage and is often fatal. The vasospasm occurs in 30−40% of patients and is the major cause of death from this condition. The vasospasm is associated with a decrease in CGRP levels in nerves and an increase in CGRP levels in draining blood, suggesting that CGRP is released from nerves to oppose the vasospasm. This evidence has led to the concept that exogenous CGRP may be beneficial in a condition that has proven hard to treat. The present article reviews: (a) the pathophysiology of delayed ischemic neurologic deficit after SAH (b) the basics of the CGRP receptor structure, signal transduction, and vasodilatation mechanisms and (c) the studies that have been conducted so far using CGRP in both animals and humans with SAH.

Keywords: GPCR, CGRP, subarachnoid hemorrhage, cerebral vasospasm, G proteins

INTRODUCTION

In the US, over 30,000 persons each year experience a subarachnoid hemorrhage (SAH). Whereas intracranial aneurysms are found in 2−5% of all autopsies, the incidence of rupture is only 2−28/100,000 individuals/year (Ingall et al., 2000). SAH is more frequent in women than men (3:2 ratio) over the age of 40, but the reverse is the case in those younger than 40 (The ACROSS Group, 2000; Ohkuma et al., 2002). Peak rupture rates occur between the ages of 50 and 60 years (The ACROSS Group, 2000; Ohkuma et al., 2002). Intracranial aneurysms account for approximately 85% of cases of non-traumatic SAH, whereas 10% have the pattern of non-aneurysmal perimesencephalic hemorrhage (van Gijn et al., 2007). Whereas intracranial aneurysms are the major cause of death from this condition, the vasospasm is associated with a decrease in CGRP levels in nerves and an increase in CGRP levels in draining blood, suggesting that CGRP is released from nerves to oppose the vasospasm. This evidence has led to the concept that exogenous CGRP may be beneficial in a condition that has proven hard to treat. The present article reviews: (a) the pathophysiology of delayed ischemic neurologic deficit after SAH (b) the basics of the CGRP receptor structure, signal transduction, and vasodilatation mechanisms and (c) the studies that have been conducted so far using CGRP in both animals and humans with SAH.
CEREBRAL VASOSPASM AFTER SAH

DEFINITIONS

Throughout the literature, authors have used various means of defining vasospasm including terms like angiographic vasospasm, symptomatic vasospasm, and delayed cerebral ischemia (DCI). Angiographic vasospasm is a narrowing of the lumen of the major cerebral arteries, which is usually focal but may be diffuse. Vasospasm has its onset usually on day 3 after SAH, is maximal at days 6–8, and usually lasts for 2–3 weeks (Wilkins, 1990). Symptomatic vasospasm is characterized by the insidious onset of confusion and decreased level of consciousness, followed by focal motor and/or speech impairments. It is mainly a diagnosis of exclusion, when clinical deterioration occurs and hydrocephalus, rebleeding, hypoxia, and metabolic abnormalities have been ruled out. DCI is defined as symptomatic vasospasm, infarction attributable to vasospasm, or both (Frontera et al., 2009). Although about 70% of patients may develop arterial narrowing, only 30% will manifest neurological deficits. The outcome of DCI itself is death in about one-third and permanent deficit in another third (Dresch, 1995). In the present review the term vasospasm is defined as arterial vessel narrowing.

VASOSPASM PATHOPHYSIOLOGY

Nitric oxide

Loss of the biological effect of nitric oxide (NO) is considered to play a pivotal permissive role in the development of cerebral vasospasm. The principal effect of NO on cerebral vessels is the relaxation of vascular smooth muscle cells, with decreased bioavailability of NO being implicated in the formation of SAH-induced vasospasm. The depletion of NO has been assumed to occur via several mechanisms in the setting of SAH. First, due to its high affinity for hemoglobin (Hb), NO is scavenged by Hb released during the breakdown of subarachnoid blood (Goeretski and Holocher, 1988; Ignarro, 1991). Second, it is possible that the production of NO is decreased in SAH, as a result of the down-regulation of endothelial NO synthase (eNOS) and neuronal NOS (nNOS; Pluta, 2005). This is supported by studies that revealed the down-regulation/dysfunction of eNOS, and loss of nNOS in spastic arteries after SAH (Hino et al., 1996; Pluta et al., 1996), as well as the finding that levels of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of eNOS, are elevated in the CSF of patients after SAH and the extent of hemorrhage are correlated with the distribution, severity, and time course of CSF (Macdonald et al., 1991). Indeed, the presence of OxyHb in the intracisternal injections of this agent result in cerebral vasospasm (Macdonald et al., 1991). Lastly, it has been hypothesized that ROS can activate the protein kinase C (PKC) pathway directly and indirectly, through enhancement of the metabolism of membrane phospholipids resulting from peroxidative damage. This, in turn, can lead to vasospasm (Asano and Matsui, 1999). Other possible vasoactive compounds are bilirubin oxidation products (B0Xes). Once bilirubin is formed, it is subsequently oxidized into B0Xes, reaching maximum concentrations during the peak vasospasm period of 4–11 days. They are thought to be potentiators of cerebral vasospasm once it has been initiated, rather than primary initiators (Clark and Sharp, 2006).

Endothelin-1

Endothelin-1 (ET-1) is an extremely potent vasoconstrictor. In the brain, it is primarily produced by endothelial cells in response to ischemia, though it can also be produced by neurons, astrocytes, and activated leukocytes (Fassbender et al., 2000; Chow et al., 2002; Dumont et al., 2003). Levels of ET-1 are high in the plasma and cerebrospinal fluid (CSF) of SAH patients, correlate with the persistence of cerebral vasospasm (Seifert et al., 1995; Jouvet, 2000), and decline in the absence of vasospasm (Seifert et al., 1995).

Conversely, the administration of ET-1 antagonists or endothelin converting enzyme inhibitors prevents vasospasm (Kousa et al., 2002; Macdonald et al., 2008). Lastly, ET-1 induces NADPH oxidase expression and oxidative stress in human endothelial cells (Duerrschmidt et al., 2000).

Inflammation

Expression of adhesion molecules facilitates leukocyte adherence to the endothelium. Adhesion molecules, such as ICAM-1, VCAM-1, and E-selectin, have been found to be elevated in the CSF of patients with SAH and in blood vessel walls exposed to clot (Polim et al., 1998; Dumont et al., 2003). Leukocytes can contribute to vasospasm by promoting free radical formation that may evoke endothelial dysfunction (Gribam et al., 1998; Sullivan et al., 2000), and by producing a variety of vasoactive substances, including ET-1 and cytokines (Fassbender et al., 2000). Several cytokines have been found to be up-regulated in cerebral vasospasm, including TNF-alpha, IL-1, IL-6, and IL-8 (Hirashima et al., 1997; Fassbender et al., 2001; Takizawa et al., 2001).

Oxidative stress

Oxymyoglobin (OxyHb) may catalyze generation of reactive oxygen species (ROS). Free radicals are considered to play a pivotal role in cerebral vasospasm through various mechanisms. First, they can initiate lipid peroxidation, whose products, lipid peroxides, are capable of producing vasospasm and damaging the structure of arteries (Lin et al., 2006). Second, it has been hypothesized that ROS can activate the protein kinase C (PKC) pathway directly and indirectly, through enhancement of the metabolism of membrane phospholipids resulting from peroxidative damage. This, in turn, can lead to vasospasm (Asano and Matsui, 1999). Other possible vasoactive compounds are bilirubin oxidation products (B0Xes). Once bilirubin is formed, it is subsequently oxidized into B0Xes, reaching maximum concentrations during the peak vasospasm period of 4–11 days. They are thought to be potentiators of cerebral vasospasm once it has been initiated, rather than primary initiators (Clark and Sharp, 2006).

Hemoglobin

A large body of evidence suggests that OxyHb, the ferrous form of hemoglobin, released from lysed erythrocytes, is a mediator of vasospasm. More specifically, OxyHb causes prolonged constriction of isolated cerebral arteries (Toda et al., 1991), and intracisternal injections of this agent result in cerebral vasospasm (Macdonald et al., 1991). Indeed, the presence of OxyHb in the CSF of patients after SAH and the extent of hemostage are correlated with the distribution, severity, and time course of vasospasm (Mayberg et al., 1990). Ferrous hemoglobin released from subarachnoid clot could lead to delayed arterial narrowing by a number of mechanisms, such as scavenging or decreased production of NO (Pluta, 2005), free radical production, modification of K+ and Ca2+ channels (Ishiguro et al., 2008), differential up-regulation of genes (Vikman et al., 2006), and activation of the Rho/Rho kinase and PKC pathways (Wickman et al., 2003).

Intracellular Ca2+

Vasospasm can be regarded as an abnormal and prolonged contraction of vascular smooth muscle. The intracellular free Ca2+...
Expression of an additional type of voltage-dependent Ca$^{2+}$ channels (R-type) occurs after SAH, leading to increased Ca$^{2+}$ channel density, increased Ca$^{2+}$ influx, and vasoconstriction (Ishaguro et al., 2005).

**Neurogenic factors**

The cerebral arteries have sympathetic, parasympathetic, and sensory innervation. It has been postulated that SAH causes a derangement of neuronal regulatory mechanisms, which in turn leads to vascular smooth muscle contraction. The vasoconstriction is associated with a decrease in cGMP levels in cerebral perivascular nerves (Edfvissen et al., 1991) and an increase in cGMP levels in blood draining from the external jugular vein (Burl et al., 1990), suggesting that cGMP is released antidromically from trigeminal sensory perivascular nerves to oppose the vasoconstriction. This evidence has led to the concept that administration of cGMP may be beneficial in SAH-associated vasospasm. The molecular characteristics of cGMP and its use as a treatment option in SAH are reviewed in Sections “Calcitonin Gene-related Peptide Biology” and “Calcitonin Gene-related Peptide and SAH,” respectively, of the present article.

**TREATMENT OF VASOSPASM**

The management of vasospasm involves routine “prophylactic” measures as well as more aggressive interventions, reserved for situations where there are signs or symptoms of DCI.

**Hemodynamic therapy**

The use of triple-H therapy (hypervolemia, hypertension, and hemodilution) stems from numerous clinical observations noting improvement in patients’ clinical symptoms following induced hypertension and volume expansion (Kosnik and Hunt, 1976; Kassell et al., 1982). The relative contribution of each component is debated. However, there are many uncertainties for the use of prophylactic hemodynamic therapy following SAH. Two studies randomly assigned normovolemic or hypervolemic therapy to patients and reported no difference in the incidence of DCI between groups (Lennihan et al., 2000; Egge et al., 2001).

**Nimodipine**

Nimodipine is safe, cost-effective, and reduces the risk of poor outcome and secondary ischemia (Neil-Dwyer et al., 1987; Welty, 1987; Kostron et al., 1988; Mee et al., 1988), but has very modest effects. It is used prophylactically in all patients with SAH. Its precise mechanism of action remains unclear. Despite being shown to reduce the incidence of DCI and cerebral infarction in clinical trials, it has negligible effects on angiographic vasospasm; nimodipine may be neuroprotective by blocking Ca$^{2+}$ influx at a neuronal level (Al-Tamimi et al., 2010).

**Endovascular techniques**

Endovascular techniques frequently play a role in the aggressive treatment of vasospasm. They include transluminal angioplasty and intra-arterial infusion of vasodilators (papaverin, nicardipine, verapamil, etc.; Brismar et al., 2006). Transluminal balloon angioplasty is very effective at reversing angiographic spasm of large proximal vessels and produces a sustained reversal of arterial narrowing (Brismar et al., 2006; Jestaedt et al., 2008). The optimal timing of angioplasty in relation to medical therapy is uncertain. Major complications occur in ~5% of procedures and include vessel rupture, occlusion, dissection, hemorrhagic infarction, and hemorrhage from unsecured aneurysms (Zwienenberg-Lee et al., 2006).

**Statins**

Statins have been shown to possess cholesterol-lowering-independent pleiotropic effects in different clinical settings, including a decrease in the incidence and duration of severe vasospasm as well as a reduction in the mortality rate after SAH (Lynch et al., 2005; Tseng et al., 2005, 2007). Statins are thought to be beneficial in the prevention of cerebral vasospasm by down-regulating inflammation and up-regulating the expression of eNOS and therefore NO (Sugawara et al., 2011).
study (a phase 2 trial) did not show a reduction in patient mortality, though the study was underpowered for this endpoint (the primary end point of this study was moderate or severe vasospasm within 14 days; Macdonald et al., 2008). CONSCIOUS 2 study (a phase 3 trial) included 1157 patients and its primary composite end point comprised all-cause mortality and vasospasm-related morbidity. This study showed that clopidogrel at 3 mg/h had no significant effect on mortality and vasospasm-related morbidity or functional outcome (Macdonald et al., 2011).

Erythropoietin (EPO) has also been examined in the setting of cerebral vasospasm. Apart from being potentially neuroprotective, EPO may play a role in preventing vasospasm by increasing the phosphorylation of eNOS (Banthaman et al., 2005), a potentially important mechanism for increasing NO production.

A recent randomized controlled trial (MASH 2) including 1204 patients did not show any benefit from intravenous (i.v.) magnesium sulfate administration in clinical outcome after aneurysmal SAH (Dohout Mees et al., 2012).

Other drugs under investigation are tirilazad, a free radical scavenger (Elalwy et al., 1997), fusidil, a Rho-kinase inhibitor that inhibits vascular smooth muscle contraction (Shibuya et al., 1992), sodium nitrite, an NO donor (Pluta et al., 2005) and cisternal placement of prolonged-release nicardipine-loaded polymers (Kasuya et al., 2005).

**Calcitonin Gene-Related Peptide Biology**

Calcitonin gene-related peptide (CGRP) is derived from the calcitonin gene (Amara et al., 1982). CGRP, in humans, which was found to contain an alternative peptide tide that was identified in 1982 by molecular biological techniques (Benarroch, 2011).

In the human circulation, CGRP has a half-life of approximately 7–10 min (Kraenzlin et al., 1985; Struthers et al., 1986). CGRP is known to be transcribed from its own distinct gene (Steenbergh et al., 1985, 1986). The majority of CGRP within the body is α-CGRP and primarily expressed in the peripheral and central nervous system. α-CGRP is mainly expressed in the gut (Mulderry et al., 1988). However, it has also been identified in the central nervous system, pituitary, thyroid, and in medullary thyroid carcinoma as a major CGRP form together with α2CGRP (Petermann et al., 1987).

Data from NMR studies suggest that CGRP consists of a characteristic N-terminal disulfide bridge-linked loop between cysteines Cys2 and Cys7, followed by an alpha-helix in amino acids Val8-Arg18 (Breeze et al., 1991). The next domain at residues 19–27 forms a hinge region (Conner et al., 2002). The C-terminus lies at residues 28–37, and contains two turn regions which form a putative binding epitope (Carpenter et al., 2001). It appears that the N-terminal cyclic portion of the CGRP molecule, containing a ring structure with a disulfide bond, is essential for agonistic activity (Maggi et al., 1980). It is interesting to note that the C-terminal fragment, CGRP1–37, is devoid of any agonist activity at CGRP receptors, although it behaves as a competitive antagonist against the intact peptide (Chiba et al., 1989).

Calcitonin gene-related peptide is widely distributed in the central and peripheral nervous systems, primarily in sensory fibers that are closely associated with blood vessels (Uddman et al., 1986). CGRP is often co-localized with other peptides in these fibers, especially the tachykinin substance P (Uddman et al., 1986). In the cerebral circulation, CGRP is released from sensory fibers originating in the trigeminal ganglia and acts to dilate cerebral vessels (McCluskey et al., 1986). In the gut, CGRP is also released from spinal afferents, where it dilates mucosal blood vessels and may protect against the acidic environment (Holzer, 2000). CGRP-containing fibers also innervate coronary arteries of the heart (Gulbenkian et al., 1993).

The regulation of CGRP production is poorly understood. At a cellular level, nerve growth factor (NGF) up-regulates CGRP via the Ras/Rat/mitogen-activated protein kinase-1 (MEK) 1/p42/p44 pathway (Freeland et al., 2000).

In the human circulation, CGRP has a half-life of approximately 7–10 min (Kraenzlin et al., 1985; Struthers et al., 1986). Regarding its metabolism, it seems that there is not an obvious mechanism, and it is probably broken down via a number of routes. First, mast cell tryptase has a potent effect in cleaving CGRP into inactive fragments, both in vivo and in vitro. More specifically, if both CGRP and substance P are released simultaneously, then CGRP could be inactivated by enzymes (tryptases), released by mast cells in response to substance P. This mechanism has been demonstrated in skin (Brain and Williams, 1988, 1989). Second, a matrix metalloproteinase II has the ability to metabolize CGRP and remove its vasodilator activity (Fernandez-Patron et al., 2000). Third, Sams-Nielsen et al. (2004) have provided evidence that CGRP is taken back up into sensory nerve terminals after repolymerization in vitro. Finally, in the CNS, α2CGRP is degraded by an endopeptidase that cleaves the peptide at the Leu28-Arg29 bond (Le Greves et al., 1989).
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FIGURE 1 | Tissue-specific expression of the calcitonin gene by alternative splicing. Splicing of alternative exons leads to two different mRNAs. The mRNA encoding calcitonin is found predominantly in the thyroid C cell; the mRNA encoding a CGRP is found predominantly in the hypothalamus and other nervous tissue. CGRP, calcitonin gene-related peptide; mRNA, messenger RNA; CCP, calcitonin carboxyl-terminal peptide. From Amara et al. (1982), with permission.

STRUCTURE OF CGRP RECEPTORS

Many peptides, including the CGRP family, mediate their actions via G protein-coupled receptors (GPCR). The GPCRs form the largest family of cell-surface proteins that are capable of interacting with an extracellular stimulus and transducing that stimulus to produce a reaction inside a cell (Pierce et al., 2002). All GPCRs have seven transmembrane spanning domains, an extracellular N-terminus and an intracellular C-terminus and can be divided into three families based on signature amino acid sequences. Family A is the largest and generally binds small molecules and short peptides. Receptors in this class have been studied extensively, including photoreceptor rhodopsin, as well as adrenergic

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and olfactory receptors. Family B receptors bind larger peptides in the range of 27 to approximately 50 amino acids (secretin, glucagons, VIP, etc.). These receptors mediate the actions of CGRP and related peptides (Poyner et al., 2002; Hoare, 2005).

Family C receptors include glutamate and GABA\_B receptors (Pierce et al., 2002).

Calcitonin receptor-like receptor (CLR), which belongs to family B of the GPCRs, comprises the main functional unit of the CGRP receptor (Figure 2). It was not until McLatchie’s work (McLatchie et al., 1998) was published that it was recognized that a novel family of single transmembrane domain proteins, called receptor activity-modifying proteins (RAMPs), were required to allow CLR to bind peptide and transduce signal. Each RAMP has a single transmembrane-spanning domain, a short intracellular C-terminal tail (∼9 amino acids) and a long extracellular-terminus (∼100 amino acids; McLatchie et al., 1998).

As a result of CLR and calcitonin receptor (CTR) interactions with RAMP, the International Union of Pharmacology (IUPHAR) nomenclature recognizes that CGRP interacts with CLR/RAMP\_1 (CGRP\_1) receptors, whereas AM interacts with CLR/RAMP\_2 (AM\_1) or CLR/RAMP\_3 (AM\_2) receptors. The CTR without RAMP is sufficient for calcitonin binding, but CTR with RAMP \_1, \_2, or \_3 are AM\_1, AM\_2, and AM\_3 receptors, respectively (Poyner et al., 2002). The discovery of RAMPs has led to evolution of our understanding of how receptor diversity is implemented, providing a novel mechanism for generating receptor subtypes within a subset of family B GPCRs (Sexton et al., 2006).

The primary function of CLR is thought to be related to ligand binding, whereas the RAMP molecule plays a crucial role in receptor trafficking to the membrane and determination of receptor pharmacology. The RAMP family regulate the glycosylation and transport of the CLR. However, they are not CGRP receptors by themselves (McLatchie et al., 1998; Sexton et al., 2009). Terminal glycosylation of the receptor and transit from the endoplasmic reticulum/Golgi apparatus to the cell surface require interaction of CLR with RAMP (Sexton et al., 2009).

Calcitonin gene-related peptide receptor activation is known to involve several crucial elements, in common with other GPCRs, such as the presence of a proline “kink” in transmembrane helix (TM\_6 (Conner et al., 2005), and a putative “DRY” motif equivalent (Conner et al., 2007), similar to family A GPCRs. There is also evidence suggesting stabilization of the CLR interaction with G “alpha”s (G\_α) by another 17Da intracellular membrane protein, called RCP (Evans et al., 2000).

The existence of two receptors, CGRP\_1 and CGRP\_2, was originally proposed in the late 1980s, with the CGRP\_1 receptor being the predominant mediator of cardiovascular effects. This receptor classification was developed as a consequence of pharmacological studies carried out with different agonists and antagonists in a range of tissue preparations, especially the positive inotropic effect in the guinea pig or rat atrium for determination of CGRP\_1 receptor activity, and the inhibition of electrically evoked twitch responses in the rat vas deferens for determination of CGRP\_2 receptor activity (Dennis et al., 1989, 1990; Dumont et al., 1997).

In general, receptors that can be antagonized by the 30-amino acid fragment of CGRP, CGRP\_8–37, with an approximate pA\_2 value of 7.0 are designated as CGRP\_1 receptors, while those that CGRP\_8–37 blocks with a pA\_2 of 6.0 or less are classified as CGRP\_2 receptors (Quirion et al., 1992; Poyner, 1995). However, it is questionable whether the CGRP\_2 receptor is a single receptor type or whether it is, in fact, explained by multiple molecular entities (Hay, 2007).

**FIGURE 2 | Structure of CGRP receptor.** CGRP receptor components and important residues for receptor signaling and internalization. The CGRP receptor is formed by CLR (blue), RAMP\_1 (yellow), and RCP (orange). Functionally important residues are shown as single letter abbreviations.
In contrast, CGRP1 is a well-defined receptor type consisting of CLR and RAMP1.

**SIGNAL TRANSDUCTION OF CGRP RECEPTOR**

Several mechanisms involved in CGRP-mediated vasorelaxation have been identified. These mechanisms include either NO-dependent endothelium-dependent mechanisms or cAMP-mediated endothelium-independent pathways. The most common pathway is NO- and endothelium-independent. Activation of the CGRP receptor is generally accepted to result in Ga2i-mediated activation of adenylate cyclase, with a subsequent increase in cAMP and activation of protein kinase A (PKA). In the absence of endothelium, CGRP is able to cause relaxation, suggesting it must directly act on the smooth muscle cells to stimulate adenylate cyclase (Edvinsson et al., 1985, 1998; Crossman et al., 1990). The resulting rise in cAMP then activates PKA, which phosphorylates and opens up ATP-sensitive K+ channels, thus leading to relaxation (Figure 3A; Nelson et al., 1990).

Endothelium-independent relaxation to CGRP occurs in the majority of tissues examined to date. Exceptions include the rat aorta, where the relaxation to CGRP occurs only in the presence of an intact endothelium and is attenuated by inhibitors of NO synthase, implying an NO-dependent mechanism (Brain et al., 1985; Gray and Marshall, 1992b). A significant increase in both cAMP and cGMP occurs and is also dependent on the presence of endothelium (Gray and Marshall, 1992a). This implicates the release of NO from the endothelium, which then relaxes the smooth muscle cells through activation of guanylate cyclase and accumulation of cGMP. Moreover, it has been shown that cAMP is able to stimulate eNOS activity, leading to increased synthesis and release of NO (Ferro et al., 1999; Queen et al., 2000). The activation of eNOS via cAMP is probably mediated via PKA, as a study demonstrated that various protein kinases can phosphorylate and activate eNOS (Burt et al., 2000). It is a possibility that CGRP causes an increase in cAMP in endothelial cells, which leads to PKA activation. PKA, in turn, activates eNOS, which results in NO release, and thus relaxation of the smooth muscle (Figure 3A).

There is some evidence for Ga2i signaling by the CGRP receptor, which is traditionally identified by sensitivity to pertussis toxin (PTX; Figure 3B). The CGRP-mediated stimulation of Ca2+ transients in rat nodose neurons and the activation of c-Jun N-terminal kinase (JNK) in SK-N-MC cells (which express endogenous CGRP receptors) both displayed PTX sensitivity (Willey et al., 1992; Dua et al., 2000).

**FIGURE 3 | CGRP receptor-mediated intracellular signaling.** (A) Ga2i signaling increases AC (green) activity, elevating intracellular cAMP, activating PKA and subsequently many potential downstream effectors. (B) The CGRP receptor might also couple to Ga2i, reducing AC (red) activity, decreasing intracellular cAMP and reducing PKA activity. (C) CGRP signaling via Ga2i activates PLCβ, which cleaves PIP2 into IP3 and DAG, resulting in elevated intracellular Ca2+ and PKC activation. (D) The CGRP receptor might also utilize Ga-independent signaling, and Gaβγ or b-arrestin-mediated signaling pathways. Arrows represent reported pathways; broken arrows represent potential or inferred pathways. CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; Ga2i, α subunit of the G protein; NO, nitric oxide; NOS, nitric oxide synthase; AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; PKC, protein kinase C; RCP, receptor component protein; AC, adenylate cyclase; ER, endoplasmic reticulum; PIP2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol. From Walker et al. (2010), with permission.
An animal study of experimental SAH in rats revealed that the sen-
HEK293 cells, leading to an increase in intracellular Ca^{2+} through inos-
titol triphosphate (IP3) activity. This increase in Ca^{2+} occurred concomitantly with the stimulation of adenylyl cyclase and accumu-
lation of cAMP. Activation of PLC is considered to occur through G_{q11}{\alpha}, rather than through G_{q}, suggesting that the activated CGRP receptor is able to interact with both types of G protein. If this mechanism is present in endothelial cells, it provides an alternative explanation for CGRP activation of eNOS (which is traditionally considered to be dependent on Ca^{2+}/calmodulin for activation), independently of cAMP accumulation. The possibil-
ity that CGRP receptors may be coupled to phosphatidylinositol 
activation, independently of cAMP accumulation. The possibil-
ity that CGRP receptors may be coupled to phosphatidylinositol

Recently, Meens et al. (2012) reported that activated CGRP receptors induce cyclic nucleotide-independent relaxation of vas-
ular smooth muscle cells in mesenteric resistance arteries and terminate arterial effects of ET-1 via G_{q11}{\alpha}. More specifically, CGRP receptor activation causes cAMP production but the relaxation of rat mesenteric resistance arteries induced by activation of this receptor involves G_{q11}{\alpha} and is not dependent on cAMP (Figure 3C).

Another study by Meens et al. (2010) discovered that CGRP released from peri-arterial sensory motor nerves terminates long-
lasting vasoconstrictor effects of ET-1 by promoting dissociation
of ET-1/ET_{A}-receptor complexes.

The CGRP receptor can also potentially activate other down-
stream signaling molecules, such as PKC and mitogen-activated
protein kinase (MAPK) cascades, such as p38, JNK, and extracel-
lular receptor activated kinase 1/2 (ERK ½; Walker et al., 2010).
CGRP receptor signaling is regulated by desensitization, internal-
ization, and trafficking, which, as with other GPCRs, involves
GPCR kinases (GRK), ß-arrestin, and clathrin- and dynamin-
dependent endocytosis (Walker et al., 2010). Padilla et al. (2007)
proposed a mechanism by which endosomal endothelin con-
verting enzyme-1 (ECE-1) degrades CGRP in endosomes to
disrupt the peptide/receptor/ß-arrestin complex, freeing inter-
nalized receptors from ß-arrestins and promoting recycling and
resensitization, resulting in long-lasting vascular relaxing response
to CGRP.

CALCITONIN GENE-RELATED PEPTIDE AND SAH
PRELIMINARY OBSERVATIONAL STUDIES

An animal study of experimental SAH in rats revealed that the sen-
so innervation of the cerebral circulation by CGRP-containing
fibers appeared to be reduced after SAH (estimated by the number of
fibers present), and there was also a larger vasodilating response
to CGRP in basilar arteries after SAH as compared to vessels from
control animals. The reduction in CGRP could be due to release of
the transmitter from the perivascular nerve terminals caused by
blood in the subarachnoid space (Edvinsson et al., 1990).

In another study (Edvinsson et al., 1991), the proximal parts of
the middle cerebral artery (MCA) were collected within 24 h
after death from five humans suffering SAH (5–10 days before-
hand) and from six subjects dying from myocardial infarction. In
humans who had died from SAH the level of CGRP was nearly not
detectable, being in contrast to that seen in age and sex matched
subjects who had died of myocardial infarction. The trigemino-
cerebrovascular system was suggested by the authors to act as an
anti-vasoconstrictor system by releasing stored peptides, CGRP
being the most likely candidate.

Juul et al. (1995) measured CGRP levels with specific radioim-
unoassays (RIA) in patients with SAH, after operation with
aneurysm clipping and nimodipine treatment. They used sam-
ple taken either from the external jugular vein (n = 20) or from
the CSF (n = 14) during the postoperative course. They also used
samples from healthy volunteers. The degree of vasoconstriction
in the patients was monitored with Doppler ultrasound record-
ings. CGRP concentrations from the external jugular vein were
significantly higher than from controls. Also, the CGRP level was
measurable in SAH CSF but not in CSF of controls.

Others (Tran-Dinh et al., 1994) showed that the basal level of
endogenous CGRP in CSF was 0.77 nmol/L in rabbits. The CGRP
concentration peaked at 14 nmol/L within 30 min, and at 8 nmol/L
within 24 h, after SAH. They further showed that 3 days after SAH
the CGRP concentration in CSF declined to 3.5 nmol/L.

Nozaki et al. (1989a) produced a model of SAH by a single
injection of fresh autologous arterial blood into the cisterna magna
of dogs. Then, they examined changes of CGRP immunoreactivity
immunohistochemically in perivascular nerve fibers of the large
pial arteries. CGRP in cerebrovascular nerve fibers was suppressed
after SAH. The suppression was first detected on the third day after
SAH, and was most marked during the 7th to 14th day. CGRP,
however, recovered to a normal level by the 42nd day after SAH.

Arrieta et al. (1991) isolated the basilar artery from five rab-
bits subjected to SAH and five control animals. A mild or severe
vasospasm was observed in the basilar artery about 15 min after
injection of blood in the cisterna magna, while fluorescence
immunohistochemistry revealed a marked decrease of the perivas-
cular nerves containing CGRP in the animals of the experimental
group, as compared to the control group.

EFFECTS OF CGRP ADMINISTRATION ON CEREBRAL VASOSPASM
AFTER EXPERIMENTAL SAH IN ANIMALS (Table 1)

Nouzki et al. (1989b) produced experimental SAH in 30 dogs by
injecting autologous arterial blood into the cisterna magna. They
used two models of injection: in the first, single-injection model,
1 ml/kg of blood was injected on day 0, while 0.5 ml/kg of blood
was injected successively 48 h apart in the second, double-injection
model, on day 0 and day 2. The diameter of the basilar artery
was measured by angiography. The most marked constriction
of the basilar artery was seen on day 3 after SAH in the single-
injection model and on day 7 in the double-injection model. When
10^{-10} mol/kg of CGRP was administered intracisternally (i.c.) on
day 3 in the single-injection model, cerebral vasospasm reversed
completely. The effect began to appear 5 min after CGRP admin-
istration, continued for 4 h, and disappeared by 24 h after the
administration. When CGRP was administered at doses of 10^{-11}
to 2 × 10^{-10} mol/kg on day 7 after SAH in the double-injection
model, the cerebral vasospasm was reversed in a dose-dependent
manner: 2 × 10^{-10} mol/kg of CGRP reversed the vasospasm
completely. The effect began to appear 5 min after the CGRP
administration. When CGRP was administered intracisternally (i.c.) on
day 3 in the single-injection model, cerebral vasospasm reversed
completely. The effect began to appear 5 min after CGRP
administration.
administration, continued for 4 h, and disappeared by 24 h. Of note, when the amounts of CGRP mentioned above were administered i.c., both mean arterial blood pressure and heart rate were only slightly increased and returned to the previous levels within several minutes.

In a similar study by Imaizumi et al. (1996), experimental SAH was produced by i.c. injection of arterial blood in rabbits. The animals were treated with intrathecal administration of CGRP 3 days after SAH. The degree of vasospasm and the effect of CGRP were evaluated angiographically by measuring the basilar artery diameter. The basilar artery constricted to 73% of the pre-SAH values 3 days after SAH. Fifteen minutes after 10^{-10} mol/kg CGRP injection, the basilar artery dilated from 73 to 117% (n = 8), which was significantly larger than 67.1% in the vehicle group (n = 8, p < 0.01). At 6 h after 10^{-10} mol/kg CGRP injection, the basilar artery was still dilated to 90% (p < 0.05). In the 10^{-11} mol/kg CGRP group, the basilar artery was dilated to 87% (p < 0.05) 15 min after the injection. The injection of 10^{-12} mol/kg CGRP had no significant effect. The dilatory effect in the 10^{-10} mol/kg CGRP group was demonstrated up to 6 h after injection. Arterial blood pressure was stable after injection of CGRP.

Toshima et al. (1992) produced SAH in 41 rabbits by injecting i.c. autologous blood. The animals were randomly assigned to five groups and were sacrificed on day 2 post-SAH. Group 1 was the control group. Immediately prior to sacrifice, group 2 and 3 animals received a 2-h i.c. injection of vehicle or CGRP (100 ng/kg/min), respectively. Group 4 and 5 animals received a 2-h i.v. injection of vehicle or CGRP (100 ng/kg/min), respectively. The diameter of basilar artery in group 3 (i.c. CGRP) was significantly greater than that in group 2 (i.c. vehicle, p < 0.001). Similarly, the diameter of basilar artery in group 5 (i.v. CGRP) was significantly greater than that in group 4 (i.v. vehicle, p < 0.01). Although no significant difference was observed in mean arterial blood pressure between groups 2 and 3 (i.c. groups), there was a significant difference between i.v. groups 3 and 5 (lower in group 5, p < 0.01).

Ahmad et al. (1996) implanted a CGRP slow-release tablet i.c., containing either 24 or 153 μg of human αCGRP, 24 h after experimental SAH was induced in rabbits. Following implantation, the CGRP level in the CSF remained elevated for 5 days. The implantation of the tablet almost completely ameliorated angiographic vasospasm. Moreover, no significant systemic hypotension or neurological adverse event was associated with the treatment.

In a similar approach, Inoue et al. (1996) investigated the efficacy of a CGRP slow-release tablet for the prevention of cerebral vasospasm after SAH in monkeys. Experimental SAH was produced by the method of Espinosa et al. (1984). The animal underwent a right frontotemporal craniectomy under sterile conditions. The dura mater was opened, and the arachnoid membrane was microsurgically incised until the ipsilateral internal carotid

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Table 1 | Studies of CGRP administration after experimental SAH in animals.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Animal model</th>
<th>Total sample size (intervention/ control)</th>
<th>Way of CGRP administration</th>
<th>CGRP dose</th>
<th>Results</th>
<th>Adverse events</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nozaki et al. (1989a)</td>
<td>Dog</td>
<td>30 (22/8)</td>
<td>i.c.</td>
<td>2 × 10^{-10} mol/kg</td>
<td>Vasospasm was reversed completely</td>
<td>None</td>
</tr>
<tr>
<td>Toyoda et al. (1996)</td>
<td>Rabbit</td>
<td>16 (6/8)</td>
<td>i.c.</td>
<td>10^{-10} mol/kg</td>
<td>Basilar artery dilated from 73 to 117%, significantly larger than 67% in control (p &lt; 0.01)</td>
<td>None</td>
</tr>
<tr>
<td>Ahmad et al. (1996)</td>
<td>Rabbit</td>
<td>45 (22/3)</td>
<td>i.o. slow-release tablet</td>
<td>24 or 153 μg</td>
<td>Vasospasm was completely reversed</td>
<td>None</td>
</tr>
<tr>
<td>Inoue et al. (1996)</td>
<td>Monkey</td>
<td>10 (5/5)</td>
<td>i.o. slow-release tablet</td>
<td>1,200 μg</td>
<td>Cerebral vasospasm was significantly ameliorated</td>
<td>None</td>
</tr>
<tr>
<td>Toyoda et al. (2000)</td>
<td>Rabbit</td>
<td>16 (6/8)</td>
<td>i.o. adenovirus-mediated CGRP gene transfer</td>
<td>NA</td>
<td>Arterial diameter was similar before and after SAH in CGRP group</td>
<td>None</td>
</tr>
<tr>
<td>Satoh et al. (2002)</td>
<td>Dog</td>
<td>20 (9/12)</td>
<td>i.o. adenovirus-mediated CGRP gene transfer</td>
<td>NA</td>
<td>Vasospasm was significantly reduced compared with the control group</td>
<td>None</td>
</tr>
</tbody>
</table>

i.c., intracisternal; i.o., intraventricular; AP, arterial pressure; HR, heart rate; SAH, subarachnoid hemorrhage; NA, non-applicable.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Sample size/patient type</th>
<th>Study design</th>
<th>Way of CGRP administration</th>
<th>Dose</th>
<th>Primary outcome</th>
<th>Results</th>
<th>Adverse events</th>
</tr>
</thead>
<tbody>
<tr>
<td>Juul et al. (1994)</td>
<td>5/ postoperative course after SAH</td>
<td>Not randomized, not controlled study</td>
<td>i.v. infusion</td>
<td>0.6 μg/min</td>
<td>H.I.</td>
<td>Significant reduction in H.I. during CGRP infusion compared to that before infusion</td>
<td>HR increase during CGRP infusion</td>
</tr>
<tr>
<td>Johnston et al. (1990)</td>
<td>152/ neurological deficit after surgical clipping of the aneurysm</td>
<td>Multicenter randomized placebo-controlled study</td>
<td>i.v. infusion</td>
<td>0.036 μg/min, doubled every 10 min, max 1.15 μg/min</td>
<td>Modified GCS</td>
<td>Of the nine patients who showed a treatment preference, eight favored CGRP ($p &lt; 0.05$)</td>
<td>None</td>
</tr>
<tr>
<td>European CGRP in SAH study (1992)</td>
<td>117/ ischemic neurological deficit after operation for the aneurysm</td>
<td>Multicenter randomized placebo-controlled study</td>
<td>i.v. infusion</td>
<td>0.6 μg/min</td>
<td>Glasgow outcome scale</td>
<td>Relative risk of a bad outcome in CGRP-treated compared with controls 0.88 (95% CI: 0.6–1.26)</td>
<td>2/3 of the patients included in the CGRP group had reduced AP and did not complete treatment</td>
</tr>
</tbody>
</table>

i.v., intravenous; AP, arterial pressure; HR, heart rate; SAH, subarachnoid hemorrhage; H.I., hemodynamic index; GCS, Glasgow coma scale.
artery (ICA) and proximal portions of the MCA and anterior cerebral artery (ACA) were exposed. An autologous blood clot (1 ml/kg) was then placed around the exposed arteries to produce experimental SAH. For animals in the CGRP (n = 5) and placebo (n = 5) groups, a total of three tablets (total drug 1200 μg) were ipsilaterally placed under the frontal and temporal lobes at the time of SAH production. In the control group, cerebral vasospasm developed on day 7 (56% as an average of the ICA, MCA, and ACA). In the CGRP group, vasospasm was significantly ameliorated on average (75%; p < 0.02). The CGRP concentration in CSF was measurable only on day 7 for the CGRP group (6.5 nmol/L). No significant untoward reactions were recorded.

Joyoda et al. (2000) sought to determine whether adenovirus-mediated gene transfer in vivo of CGRP ameliorates cerebral vasocostriction after experimental SAH. Arterial blood was injected into the cisterna magna of rabbits to mimic SAH 5 days after injection of adenovirus or vehicle. After injection of adenovirus (n = 8), there was a 400-fold increase in CGRP in CSF. In rabbits treated with vehicle (controls, n = 8), basilar artery diameter after SAH was 25% smaller than before SAH (p < 0.0005). In rabbits treated with adenovirus, arterial diameter was similar before and after SAH. Furthermore, treatment of rabbits with adenovirus after experimental SAH prevented spasm of the basilar artery 2 days after SAH.

Likewise, Satoh et al. (2002) investigated whether a delayed treatment with adenovirus encoding CGRP gene, 2 days after experimental SAH, reduces cerebral vasospasm in a double-hemorrhage model (on days 0 and 2) of severe vasospasm in dogs. Severe vasospasm was observed in control SAH dogs (n = 12) on day 7, and the mean basilar artery diameter was 53% of baseline. In the group treated with adenovirus (n = 8), vasospasm was significantly reduced (the basilar artery diameter was 78% of baseline, p < 0.05 compared with the control SAH group). High levels of CGRP were measured in CSF from dogs that received adenovirus (115-fold greater than baseline levels).

Intracisternal gene transfer of CGRP was initially thought to be more useful than i.v. infusion, because the local gene transfer might avoid systemic effects of CGRP and achieve its sustained release into the central nervous system. However, there are several concerns, such as the inflammatory process induced by adenovirus, the difficulty in approaching the target cells in the presence of a large subarachnoid blood clot, and its potential ability for cancerous transformation of the affected cells.

EFFECTS OF CGRP ADMINISTRATION ON CEREBRAL VASOSPASM AFTER SAH IN HUMANS (Table 2)

Juul et al. (1994) investigated the effect of i.v. CGRP infusion at a rate of 0.6 μg/min in five patients with vasospastic constrictor in the postoperative course after SAH, where the hemodynamic index (ratio between middle cerebral and ICA mean velocities) was used as an indicator of vasocostriction. A significant reduction was found in the hemodynamic index during the CGRP infusion as compared to that before infusion (4.3 vs. 6.2, p < 0.05). However, no significant change was observed in pulsatility index (another indicator of vasospasm, equal to the difference between the systolic and diastolic flow velocities divided by the mean flow velocity), blood pressure, or consciousness during CGRP infusion. A significant increase in heart rate was observed during the infusion, while blood pressure remained unaltered.

Johnston et al. (1990) undertook a multicenter, randomized, placebo-controlled trial to study the safety and efficacy of i.v. CGRP treatment to reverse neurological deficits after surgical clipping of a ruptured intracranial aneurysm. Patients were enrolled if they had postoperative neurological deficit. Patients received CGRP or placebo in random order, 24 h apart. Fifteen patients were eventually included in the study. Infusion started at a rate sufficient to deliver 0.035 μg/min CGRP, and was doubled every 10 min until either a clinical response was obtained or a maximum dose of 1.15 μg/min was reached at 1 h. If the neurological deficit had not deteriorated and the patient had no side-effects by that time, the maximum infusion rate was continued for another 20 min. Regarding neurological changes according to the modified Glasgow Coma Scale, five patients did not improve on either treatment, one improved on both, eight improved on CGRP but not on placebo, and one improved on placebo but not on CGRP. Of the nine patients who showed a treatment preference, eight (88.9%) favored CGRP (p < 0.05). The mean duration of neurological improvement was 25 min, after which patients returned to their previous neurological status. There was a significant decrease in both systolic and diastolic blood pressures during the infusion of CGRP.

A larger, multicenter, randomized controlled trial (European CGRP in SAH study, 1992) investigated the effect of a postoperative infusion of CGRP on outcome at 3 months. Patients with aneurysmal SAH who underwent surgery entered the trial if an ischemic neurological deficit developed after the operation. A total of 117 patients entered the study (62 patients received CGRP and 55 standard management). The CGRP-treated patients received the drug by i.v. infusion at a rate of 0.6 μg/min. If systemic hypotension developed, the infusion rate was reduced to 0.45 μg/min, then to 0.3 μg/min, if the hypotension was still apparent. CGRP treatment was given for at least 4 h. Patients who showed a satisfactory neurological response continued to receive treatment for up to 10 days (minimum of 4 days). The percentage of patients with a good outcome was slightly but not significantly higher in the CGRP than in the control group. The relative risk of a bad outcome in CGRP-treated compared with control patients was 0.88 (95% CI: 0.66–1.28). Interestingly, only a third of patients randomized to receive CGRP completed treatment, so two-thirds included in the treatment group for the analyses had limited exposure to CGRP, mainly due to arterial hypotension.

CONCLUSION

The pathogenesis of vasospasm after SAH is complex, multifactorial, and incompletely understood. CGRP has shown promising results both in vitro and in vivo, mainly in animal models of experimental SAH. However, there is a lack of studies in humans. Systemic hypotension induced by the i.v. administration of the drug seems to be a serious problem. The encouraging results from the i.c. application of CGRP in animals could warrant large studies in humans with CGRP instillation into the subarachnoid space, in order to avoid hypotension and achieve even more efficient dilatation of the cerebral arteries.
REFERENCES


