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Intranasal delivery of calcitonin gene-related peptide reduces cerebral vasospasm in rats

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1. ABSTRACT

Cerebral vasospasm is the primary cause of sequelae and poor clinical conditions of subarachnoid hemorrhage (SAH); therefore, it is imperative to relieve vasospasm and improve cerebral blood supply. Calcitonin gene-related peptide (CGRP) is a potent vasodilator that is normally released by trigeminal sensory fibers but depleted following SAH. We propose that intranasal application may be an effective way to deliver CGRP to the brain and ameliorate vasospasm after SAH. In this study, we intranasally applied CGRP to rats and induced SAH by double-injection of autologous blood into the cisterna magna. Compared to intravenous injection, intranasal delivery led to a 10-fold higher level of CGRP in the brain. Intranasal CGRP significantly ameliorated vasospasm, improved cerebral blood flow, and reduced cortical and endothelial cell death. Moreover, CGRP increased the levels of vascular endothelial growth factor and stimulated angiogenesis. Altogether, our data demonstrate that intranasal CGRP delivery is a promising method for moderating vasospasm and reducing the associated ischemic brain injury after SAH in rats, and suggest that it may be a potential approach in clinic.

2. INTRODUCTION

Subarachnoid hemorrhage (SAH) is a type of hemorrhagic stroke in which blood enters subarachnoid space, and aneurysm rupture is the cause in more than 80% of cases (1). SAH is devastating because of its super-acute nature, striking at a relatively young age and with a high mortality rate (1, 2). SAH accounts for only 6-7% of all stroke cases but 22-25% of stroke deaths (3, 4). The survivors of the initial bleeding constantly face two life-threatening challenges: rebleeding and cerebral vasospasm (1, 3, 5). With endovascular and neurosurgical repair of aneurysms, the rate of secondary bleeding decreases dramatically, but the management of cerebral vasospasm is far from satisfactory. The incidence of cerebral vasospasm remains high (5, 6), and its consequent early brain injury and delayed cerebral ischemia are the major reasons for neuronal death, cerebral infarct and overall poor clinical conditions (1, 3, 5, 6). Although calcium antagonists, magnesium and antiplatelet agents have been used to relieve vasospasm, the results remain disappointing (1, 6).

Vasospasm is caused by the irregular and prolonged contraction of vascular smooth muscle. It

typically starts at day three following the bleeding events, peaks around days 6-8, and subsides after 2-3 weeks (1, 6, 7). Delayed ischemic neurological deficit (NIND) occurs in parallel with the time course of the vasospasm. The mechanism responsible for vasospasm has been poorly understood up to now, though an imbalance is suggested between vasoconstrictive and vasodilatory factors (5-7). These factors can be classified into two origins, paracrinal and neurogenic. The paracrinal factors are mainly from endothelial cells, such as nitric oxide (NO) and prostaglandins as vasodilators, and endothelins as vasoconstrictors. Oxy-hemoglobin after SAH could be an additional vasoconstrictor (6, 8). The neurogenic factors of brain vasculature are all released by the terminals of the trigeminal nerve. Vasoconstrictors include noradrenaline (NA) from sympathetic fibers. Vasodilators include acetylcholine from parasympathetic fibers, as well as neurokinin A, substance P and calcitonin gene-related peptide (CGRP) from sensory fibers (5, 6, 9).

Among these vascular activators, CGRP is especially attractive. This 37-amino-acid neuropeptide is a product of the alternative processing of calcitonin RNA transcript (10). In the head, CGRP expresses abundantly in ganglion trigeminale and its sensory fibers that innervate intracranial small cerebral vessels (11-13). It acts through binding its G-protein coupled receptors that are located in vascular smooth muscles, Schwann cells and mononuclear cells, leading to vascular dilation in an endothelium- and NO-independent manner (11, 14). Functionally, CGRP is a potent endogenous vasodilator, stronger than acetylcholine, substance P and neurokinin (15, 16). Following SAH, it is CGRP but not co-localized substance P that is depleted, which may especially contribute to the pathogenesis of vasospasm (15, 17). Supplements of CGRP were able to prevent or attenuate vasoconstriction induced by SAH in both experimental and clinical settings (18-22).

The application of CGRP is limited, however, by its low BBB permeability and cardiovascular side effects. As a large peptide, CGRP can not cross the BBB; it relaxes arteries following abluminal administration, but has no effect when used luminally (23). Systemic use of CGRP was effective, but caused profound hypotension and tachycardia (16, 22). Alternative routes of CGRP administration include intrathecal infusion (19), intracisternal injection or adenoviral gene transfer (20, 21); apparently, none of these is clinically practical.

Intranasal drug delivery has recently emerged as a promising approach to target brain, spinal cord and perivascular spaces of the cerebrovasculature (24). This method has been successfully used to deliver various neuroprotective agents to reduce brain damage induced by cerebral ischemia (25-27), seizure (28, 29), and Alzheimer's disease (30, 31). Therefore, we propose that intranasal delivery of CGRP may be one of the most appropriate modes for treating vasospasm after SAH. In this way, CGRP, the most potent vasodilator, can reach the cerebral perivascular space. This space is the natural place for CGRP from the sensory fibers of the trigeminal nerve to bind to CGRP receptors on vascular smooth muscles.

The purpose of the current study is to investigate the beneficial effects of intranasal CGRP on vasospasm, ischemic neuronal injury and angiogenesis following experimental SAH in rats. Our results demonstrate that intranasal application of CGRP led to increased concentrations in CSF and cerebral parenchyma compared to IV injection. This approach more efficiently ameliorated vasospasm and improved cerebral blood flow following SAH; it also reduced the severity of early brain injury. Furthermore, intranasal CGRP application stimulated angiogenesis after SAH *via* upregulating VEGF. Collectively, our data indicate that this novel method of vasodilator delivery possesses advantages over traditional systemic administration, and therefore could be a practical remedy in the clinical management of cerebral vasospasm in SAH patients.

3. MATERIALS AND METHODS

3.1. Animals and physiologic parameter monitoring

All procedures in this study were approved by the institutional review board of Taishan Medical College. Male Wistar rats weighing 300-350 g were purchased from the Experimental Animal Center of Lukang Pharmaceuticals (Shangdong, China). The rats were housed at room temperature with a 12:12-hour light-dark cycle with free access to food and water.

Rats were anesthetized by IP injection of 10% chloral hydrate solution at a dose of 300 mg/kg (32, 33). A left femoral arterial catheterization was established using PE-50 tubing for blood sampling and monitoring. Blood pressure was continuously monitored. At the indicated time points, arterial blood was collected from the femoral catheterization for blood gas analysis, or for cisternal injection to induce SAH.

3.2. Intranasal administration of CGRP

Intranasal drug administration (IN) was performed using a previously described method with minor modification (25, 34). Briefly, after anesthesia with chloral hydrate, rats were placed on their backs. Their heads were slightly elevated by gauze pads wedged underneath their heads and necks. A segment of PE-50 tubing, connected with a Hamilton syringe, was inserted into nasal cavities for drug delivery. A total of 1 microgram of CGRP (Sigma, St. Louis, MO, USA) in 50 microliters of water was administered per rat through 10 injections, with 5 microliters for each injection. The injections alternated between left and right nostrils with an interval of 2 minutes. The opposite nostril was closed during application to allow complete inhalation of the solution.

3.3. Measurement of CGRP distribution in CSF, blood and brain tissues

Iodine-125-labeled CRGP (^{125}I-CGRP, Northern BioTech Inc, Beijing, China) and liquid scintillation were used to measure the CGRP distribution (25). Twenty-four hours before the injection, the rats were fed potassium iodine solution to block the thyroid gland. Then 20 microCi of ^{125}I-CGRP in 50 microliters of solution were injected either intranasally or intravenously (IV) *via* the femoral

vein. Samples were collected 30 minutes after the injections. Venous blood samples were withdrawn, CSF was collected from cisternal puncture, and cortical and hippocampal tissues were collected after transcardial perfusion with normal saline. Cortical and hippocampal samples were further homogenized in buffer (50 mM Tris-HCl, pH 7.4). Samples were then prepared for isotope activity measurement (SN-695 gamma counter RIA, PerkinElmer, Waltham, MA, USA) by adding scintillation fluid. The mean cpm values were used to represent CGRP concentrations.

3.4. Rat model of SAH

Experimental SAH in rats was induced using Suzuki's double-blood injection model with modification (32, 33, 35). In brief, rats were anesthetized with chloral hydrate. Core body temperature was kept at approximately 37 degrees C with an electric heating pad and a light bulb. The left femoral artery was cannulated with a PE-50 tube for withdrawing blood and monitoring physiological parameters. The rat's head was then placed in a stereotactic frame (Stoelting Co. Ltd, Wood Dale, IL, USA) with the head angled down at about 30 degrees. A midline skin incision was made on the back of the neck. Under a surgical microscope, the atlanto-occipital membrane was exposed by separating muscular attachments. Then 0.4 ml of arterial blood was withdrawn *via* femoral cannulation with a 1-cc insulin syringe, which was then mounted onto the manipulating arm of the frame. The syringe was lowered, and the needle was punctured through the arachnoid membrane. The fresh autologous blood was then injected into the cisterna magna in three minutes. The second injection of blood was performed after a 48-hour recovery. Sham-operated rats went through the same procedures except for the cisternal injection of blood. The rats were allowed free access to food and water after recovery from anesthesia.

3.5. Measurement of basilar artery diameter

The diameter of the basilar artery was measured *via* a transclival approach as described previously (33). In brief, rats were anesthetized with chloral hydrate, and their core body temperature was maintained at 37 degrees C. Under a surgical microscope, the rat's trachea was exposed, transected, and intubated. The animal was then mechanically ventilated with a rodent ventilator. The clivus was then exposed, and a bone window about 15-20 mm^2 in size was created over the clivus using a dental drill. The window was then covered and flushed with warm artificial CSF. The basilar arteries were monitored *via* a camera connected to a computer with Ry video software.

For histological examination of BA, rats were sacrificed at day 3 after SAH. Perfusion was performed PBS *via* transcardial cannulation of the left ventricle. The brains were then removed and immersed in the same fixative overnight at 4 degrees C, followed by immersion in 30% sucrose for 3 to 4 days. The brainstem was carefully separated and cut with a cryostat. The brainstem sections were stained with hematoxylin & eosin (H&E). For each vessel, diameter, wall thickness and luminal areas were measured in 10 sequential sections using NIH Image software, and were presented as averages (n=5).

3.6. Measurement of regional cerebral blood flow

Regional cerebral blood flow (rCBF) of rats was measured using laser Doppler flowmetry (LDF, Periflux PF150, Perimed Co., Sweden) as described previously (32, 36). Under anesthesia, the rat's head was placed into a stereotactic frame, and the left parietal bone was exposed through a skull incision. A small burr hole was drilled on the parietal bone (bregma: -3.0 mm; lateral: 3.0 mm). The dura underneath was kept intact. A probe of laser Doppler was placed onto the dura through the burr hole and fixed with dental cement. Regional cerebral blood flow was continuously recorded for the indicated duration of time (n=5).

3.7. Cell death assay

Neuronal apoptosis was detected using terminal deoxynucleotidyl transferase mediated dUTP nick end labeling assay (TUNEL) according to the manufacturer's protocol (Roche, Basel, Switzerland), as described previously (37, 38). The rats were deeply anesthetized 72 hours after SAH (n=6 per experimental condition), and then perfused. Their brains were quickly removed, frozen in liquid nitrogen, embedded with OCT and stored at -70 degrees C until use. Brains were then cut with a cryostat into 8-micron sections that were then mounted onto slides for staining. Detection of cell death was carried out by incubating the sections with a solution of diaminobenzidine and hydrogen peroxide in phosphorous-buffered saline. To determine non-specific labeling, selected sections were incubated in the reaction buffer without the transferase.

3.8. Measurement of circulating endothelial cells

Circulating endothelial cells (CEC) were counted as a marker of endothelial damage after SAH (39, 40). For this purpose, we collected CEC using Hladovec's density centrifugation method (39). In brief, 4.5 ml of venous blood was mixed with 0.5 ml of 3.8% citrate, which was then centrifuged for 20 minutes. The supernatant was mixed with 0.2 ml of 0.1% ADP-Na_2 and centrifuged. The supernatant was collected and centrifuged again for 30 minutes. The pellet was then re-suspended with 0.1 ml normal saline. The harvested cells were smeared on slices for immunofluorescent staining with anti-VWF, the endothelial marker; or they were counted using a hemacytometer (n=6).

3.9. Immunohistochemistry

Immunohistochemical stainings were performed to identify endothelial cells with von Willebrand factor (VWF), angiogenesis with platelet endothelial cell adhesion molecule-1 (PECAM-1, also known as CD31) and VEGF antibodies. The animals were anesthetized 72 hours after SAH (n=6 per experimental condition), and were perfused transcardially with cold normal saline. The brain was quickly removed, frozen in liquid nitrogen, embedded with OCT and stored at -70 degrees C until use. Brains were then cut with a cryostat into 8-micrometer sections that were mounted onto slides. Immunohistochemical stainings for VWF (1:200, Beijing Biosynthesis Biotechnology Co.,

Ltd., Beijing, China), VEGF (1:100, Santa Cruz, CA, USA) and CD31 (1:100, Santa Cruz, CA, USA), were performed as previously described (32). Images were captured using a confocal microscope (Radiance 2100, Bio-Rad, Hercules, CA, USA).

3.10. Western blot

Westerns were performed using the previously described method (36, 41). Rats were deep anesthetized at 72 hours after SAH (n=4 per experimental condition), followed by transcardial perfusion with cold normal saline. The cortical tissues were then collected, frozen with dry ice, and stored at -70 degrees C until use. After thawing, the brain tissues were homogenized in lysis buffer. The suspension was then sonicated and centrifuged. The total protein extracts were separated using 12% SDS-PAGE gels, and subjected to Western blot analysis. The primary antibody was mouse anti-caspase-3 antibody purchased from Cell Signaling Technology (Danvers, MA, USA) and diluted at 1:500. The optical density of bands was measured with the assistance of Image-Pro Plus Software (Media Cybernetics, Inc., Bethesda, MD, USA). For gel analysis, the optical density of each band was first normalized with its corresponding beta-actin band, and then compared with that of a sham-operated animal on the same gel.

3.11. VEGF mRNA measurement

RT-PCR was performed to measure the mRNA level of VEGF (32). The rats were sacrificed at 72 hours after SAH, and the brains removed. Cortical and hippocampal tissues were collected and preserved in liquid nitrogen. Total RNA was extracted using the phenol/guanidine isothiocyanate method with TRIzol reagents (Gibco BRL, Gaithersburg, MD, USA) according to the manufacturer's protocol. The purity of total RNA was assessed by the 260/280-nm ratio (between 1.8 and 2.0) and by the absence of bands corresponding to contaminating DNA in the agarose electrophoresis. The first chain of cDNA was synthesized using Moloney murine leukemia virus (MMLV) reverse transcriptase. Two microliters of total RNA, 1 microliter of oligo dT, 1 microliter of dNTPs, 1 microliter of MMLV reverse transcriptase, and 1 microliter of RNasin in 10 microliters of diethylpyrocarbonate (DEPC)-treated water were maintained at 37 degrees C in a water bath for 60 minutes, followed by heating at 95 degrees C for 5 minutes. The products were kept at -20 degrees C.

Oligonucleotide primers (XXX) for VEGF are: upstream, 5'-TGGACCCTGGCTTTACTGCTG-3'; downstream, 5'-GGCAATAGCTGCGCTGGTAGA-3'; and the PCR product is 127 bp in length. Beta-actin primers are: upstream, 5'-GGGAAATCGTGCG TGACAT-3'; downstream, 5'-CAGGAGGAGCAATGATCTT-3'; and the PCR product is 386 bp. The PCR was performed using a PTC-150-microplate gradient thermal cycler (MJ Research, USA). The cycles were 36 for VEGF and 30 for beta-actin. PCR product was electrophoresed in 1.5% agarose gels containing 0.2 mg/ml of ethidium bromide. The gels were visualized under ultraviolet light and photographed.

3.12. Statistical analysis

The data were presented as mean plus or minus standard deviation (SD), and analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's test for *post-hoc* comparisons. A P-value <0.05 was considered statistically significant.

4. RESULTS

4.1. Intranasal administration of CGRP was more efficient than IV injection in its delivery to CSF and brain

We first determined whether intranasal CGRP administration has advantages over systemic injection in terms of delivery efficiency into CSF and brain parenchyma of rats. Equal amounts of ^{125}I-CGRP (20 microCi) were administered *via* either IN or IV. Regular CGRP was used in another set of rats as controls. Thirty minutes after injections, the rats were deeply anesthetized for blood and CSF samplings, and the rats were then sacrificed for collecting cortical and hippocampal tissues. Samples were subjected to liquid scintillation. No radioactivity was detected in the fluids and tissues of rats when un-labeled CGRP was administered (data not shown). Blood radioactivity was rather high after IV injection of ^{125}I-CGRP, but the count was very low after IN application, less than 3% of that of IV (Figure 1A). In contrast, the radioactivity in CSF was only mild after IV but very high after intranasal application, about 10 times higher than with IV (Figure 1B), indicating that CGRP has low permeability crossing the BBB when IV administered, but that it can easily transport across the BBB when IN applied. Likewise, the radioactivity in cortex and hippocampus following IN of ^{125}I-CGRP was also significantly higher than by the IV route (Figure 1B). Collectively, these data indicate that intranasal administration has a significantly higher efficiency than IV in delivering CGRP to CSF and brain tissues. We therefore chose IN as the delivery method for the rest of our studies.

We next determined whether intranasal CGRP delivery would be interfered with by experimental SAH in rats. Autologous arterial blood was injected into cisterna magna to model SAH, and ^{125}I-CGRP was then intranasally applied. Blood, CSF and cerebral tissues were sampled 30 minutes after administration for liquid scintillation. As shown in Figure 1C, no difference was detected between physical and pathological conditions, indicating that intranasal administration can effectively deliver CGRP into CSF and brain regardless of vasospasm and SAH.

4.2. CGRP attenuated vasospasm in SAH

Our next goal was to determine whether intranasal CGRP usage could attenuate the vasospasm induced by experimental SAH in rats. Three days after SAH induction, rats were anesthetized, and their basilar arteries were exposed through transclival windows. As shown in Figure 2A, SAH caused apparent vasospasm of the basilar artery, as indicated by decreased vascular diameter; intranasal CGRP attenuated vascular narrowing. H&E staining of basilar arteries demonstrated similar results (Figure 2B). It was also noticed that, in the sham-

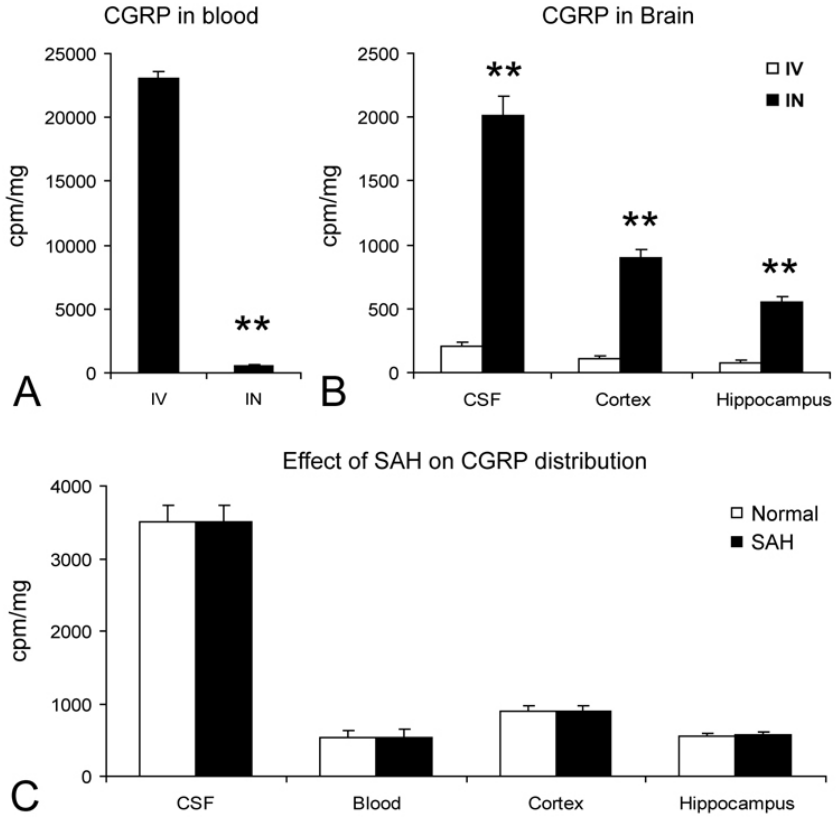

Figure 1. ^{125}I-CGRP distribution in rat blood and various brain structures after IV and intranasal administration. Twenty microCi of ^{125}I-CGRP was applied either IV or IN. Samples were collected 30 minutes later and subjected to liquid scintillation. (A) Radioactivities in blood. (B) Radioactivities in CSF, cortex and hippocampus. (C) Effects of SAH on the distribution of CGRP after IN application. No difference was detected. Data were presented as mean plus or minus SD and analyzed with ANOVA and Dunnett's tests, n=8, ** P<0.01 *vs.* IV administration.

operated group, BA was normal, with a smooth luminal surface and spread elastic lamina. SAH caused wall thickening, severe endothelial loss and elastic lamina shrinkage, and intranasal delivery of CGRP ameliorated these vasospastic effects of SAH.

Quantitative data show that CGRP increased the diameter of basilar arteries, decreased the thickness of vascular wall and increased luminal area after experimental SAH in rat (Figure 2C, D, and E), indicating a vasodilatory effect of CGRP.

To investigate whether CGRP-mediated vasodilation is associated with an increase of rCBF, we continuously monitored rCBF changes in rats after SAH. As shown in Figure 3, SAH caused a severe reduction of rCBF that started shortly after the injection and lasted for the duration of monitoring. Intranasal administration of CGRP significantly increased rCBF, which might be the result of its vasodilatory effect.

4.3. CGRP reduced cortical cell death after SAH

Ischemic neuronal death occurs after SAH mainly due to vasospasm-mediated brain ischemia. Although we demonstrated that intranasal CGRP effectively reduced vasospasm and improved rCBF, it was not clear whether it protects cortical neurons from ischemic injury (21). To shed light on this, we performed TUNEL stainings. As shown in Figures 4A and 4B, only sparse TUNEL-positive cells were seen in sham-operated rats. Three days after SAH, a large amount of dead cells was detected with TUNEL staining, and most of them were located in layers 3 and 4 of cortex. CGRP intranasal treatment significantly reduced cortical cell death (Figure 4A and 4B), indicating a neuroprotective role of CGRP. A parallel change in caspase-3 activation was also observed (Figure 4C), indicating an apoptosis-like cortical cell death.

4.4. CGRP decreased endothelial death in vasospasm and SAH

Endothelial damage frequently occurs in certain disorders, including vasospasm after SAH (42, 43), and offering neuroprotection can rescue endothelial cells (42). Using CEC counting as a novel method, we investigated whether intranasal CGRP could reduce endothelial death after SAH. As shown in Figure 5A, CECs are usually large, and are commonly shrunken, folded or twisted. They also differ greatly in shape, and they can be irregular, polygonal, or oval. However, there was no difference in CEC morphological aspect between the vehicle and CGRP

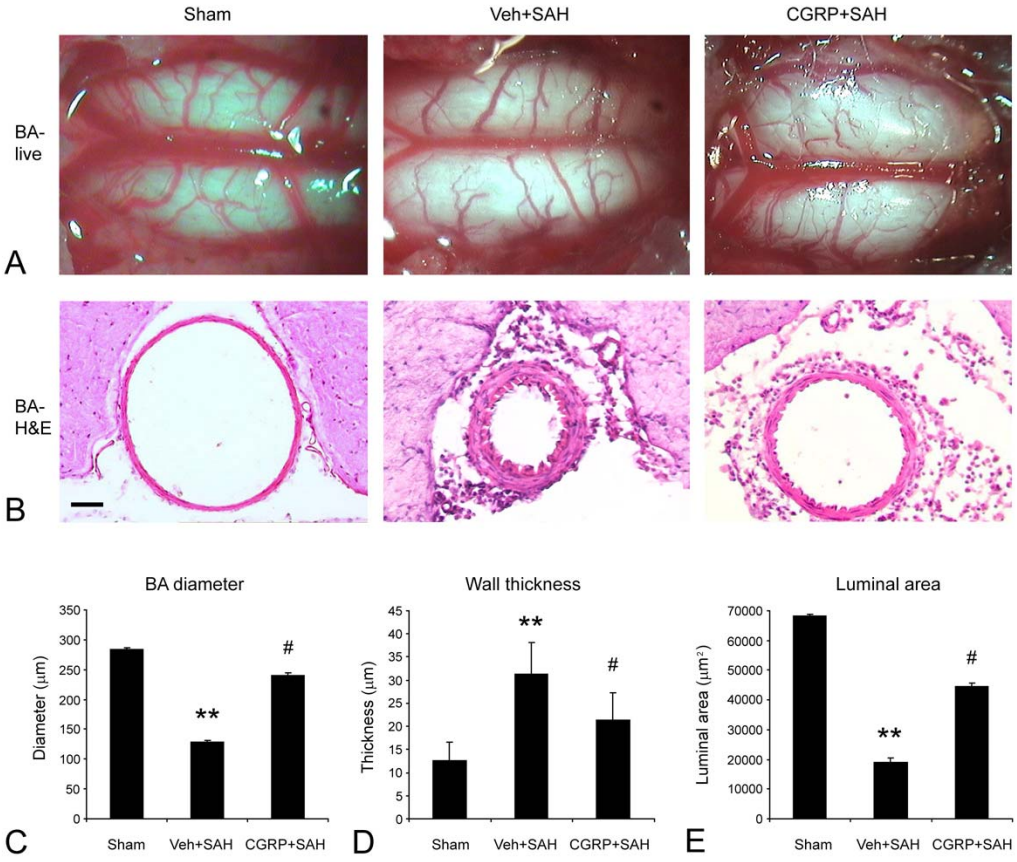

Figure 2. Intranasal CGRP ameliorated vasospasm of basilar artery (BA) after SAH in rats. (A) Representative images of BA as viewed through transclival windows, indicating that CGRP dilated constricted BA after SAH. (B) Representative H&E stainings of BA, showing the vasodilatory effect of CGRP on vasospasm caused by SAH. BA was regular in the sham-operated group with smooth lumina and elastic lamina. Following SAH, BA was narrowed, the wall thickened with endothelial loss, and the elastic lamina shrunken. Intranasal administration of CGRP reduced the damaging effects of SAH. Scale bar is 50 micrometers. Quantitative results are presented in (C) for diameter, (D) for wall thickness and (E) for luminal area. Data were presented as mean plus or minus SD and analyzed with ANOVA and Dunnett's tests, n=5, ** P<0.01 *vs.* sham; #P<0.05 *vs.* the vehicle-treated SAH group.

groups. The basal level of CEC was low, representing the normal turnover of endothelial cells (Figure 5B). Following SAH, however, the number of CEC significantly increased; in addition, intranasal CGRP application greatly reduced the CEC number, indicating that CEC is a useful method to measure endothelial death after SAH, and that CGRP has a protective function against endothelial damage.

4.5. CGRP upregulated VEGF transcription and expression

It is unclear whether CGRP has other beneficial effects in addition to its direct effect on vasodilation. Recent reports have shown that CGRP was able to stimulate VEGF expression and angiogenesis in non-brain tissues (44, 45). It has also been established that VEGF is neuroprotective against ischemic brain injury (46, 47). We therefore investigated whether CGRP upregulates VEGF expression. Three days after SAH, brain tissues were collected. RT-PCR revealed that SAH stimulated VEGF transcription, and CGRP further increased the mRNA level of VEGF (Figure 6A). Parallel with its mRNA level, VEGF protein was also upregulated as demonstrated by Western blot (see Figure 4B). The increase in VEGF seems to stimulate angiogenesis, as we simultaneously detected an increased expression of CD31 (Figure 6C), an endothelial marker.

5. DISCUSSION

As the chance of rebleeding ebbs due to aggressive surgical procedures, cerebral vasospasm remains as the primary reason for fatality and disability after SAH. Several clinical trials have been carried out using vasodilators to attenuate vasospasm after SAH, such as calcium antagonists, magnesium sulphate and antiplatelet agents; however, the results have not been satisfactory (1). Among the many factors responsible for the failure, an easily overlooked one is whether and how much the agents were delivered to the brain. Blood flow was supposed to be the carrier of these vasodilators when they were

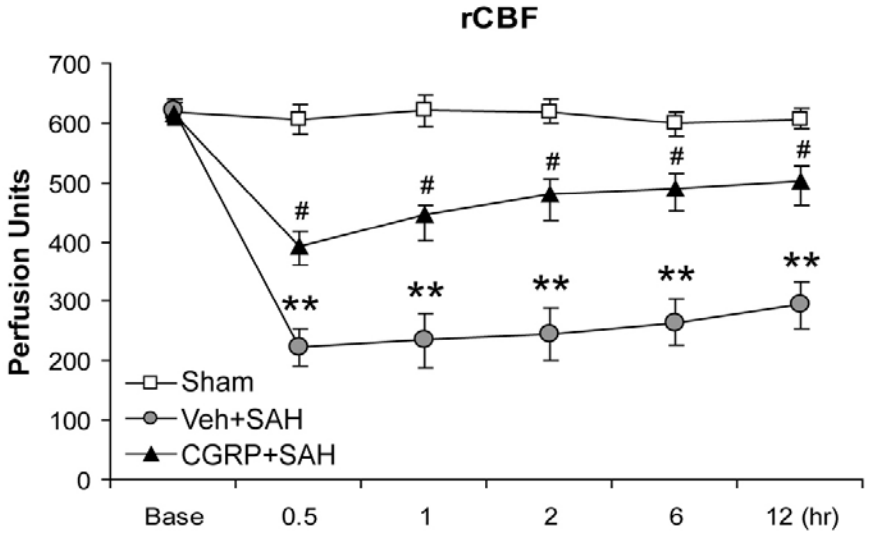

Figure 3. CGRP improved rCBF following SAH in rats. Regional cerebral blood flow changes were continuously monitored for 12 hours after SAH using laser Doppler flowmetry. Quantitative data are presented indicating that CGRP could improve rCBF after SAH as the possible result of its vasodilatory effect. Data were presented as mean plus or minus SD and analyzed with ANOVA and Dunnett's tests, n=5, ** P<0.01 *vs.* sham at the same time points; #P<0.05 *vs.* the vehicle-treated SAH group.

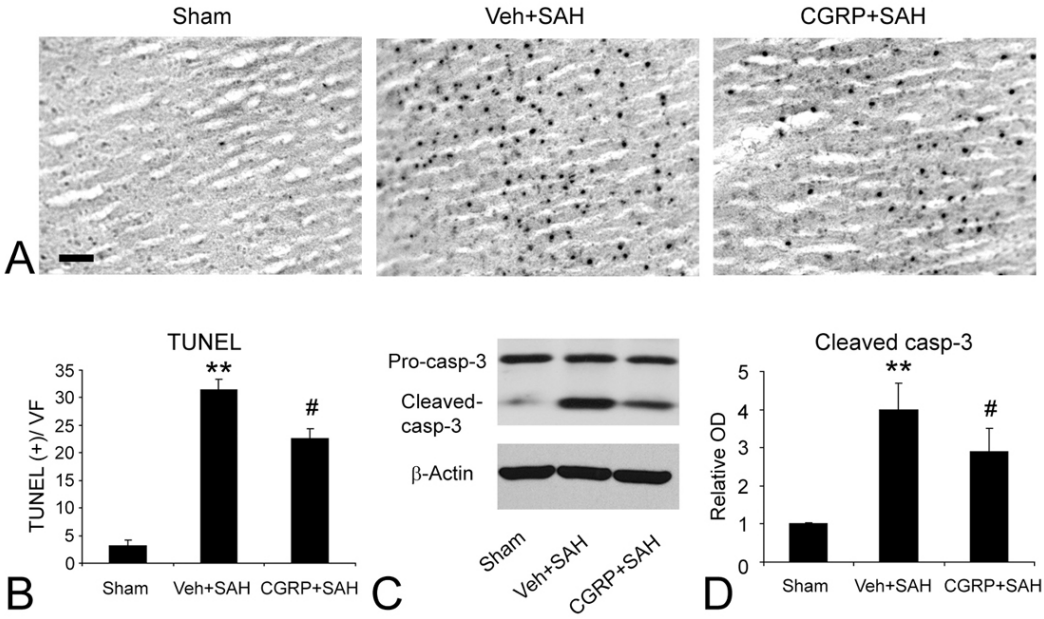

Figure 4. CGRP rescued cortical neurons from apoptotic death after SAH. (A) Representative microphotographs of parietal cortex stained with TUNEL assay. Scale bar is 100 micrometers. (B) Quantitative analysis of TUNEL-positive cells in each group, indicating a neuroprotective effect of CGRP. Data were presented as mean plus or minus SD and analyzed with ANOVA and Dunnett's tests, n=6, ** P<0.01 *vs.* sham; #P<0.05 *vs.* the vehicle-treated SAH group. (C) Representative Western of caspase-3. (D) Quantitative analysis of the optical density (OD) of cleaved caspase-3, standardized on the sham group. Data were presented as mean plus or minus SD and analyzed with ANOVA and Dunnett's tests, n=5, ** P<0.01 *vs.* sham; #P<0.05 *vs.* the vehicle-treated SAH group.

administered systemically. Due to vasospasm after SAH, however, rCBF was significantly reduced, and so was the drug delivery. How to increase delivery efficiency to the brain has been a hot topic in recent years. Promisingly, intranasal drug administration stands out as an efficient and realistic method (25, 26, 29-31, 48). Nasal mucosa is innervated by olfactory and trigeminal nerves, and both of them provide a direct connection between nose and brain. Therefore, olfactory and trigeminal pathways are major routes for intranasally administered drugs to bypass the

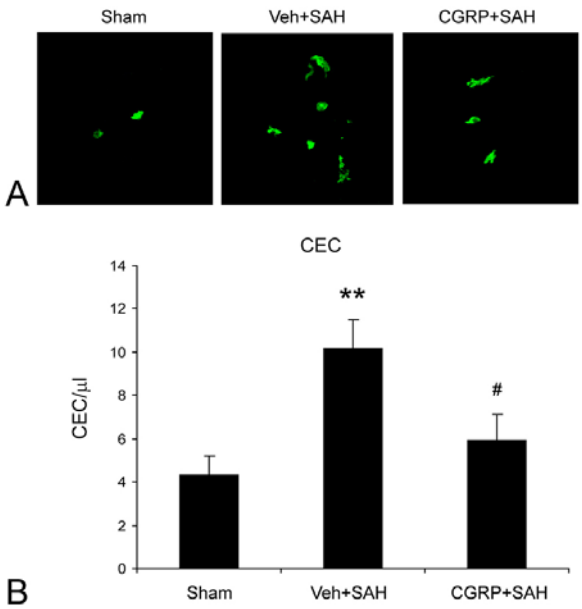

Figure 5. CGRP reduced endothelial cell death. CEC was harvested as described in Methods. (A) Representative microphotographs of CEC smears stained with anti-VWF. (B) Quantitative analysis of CEC numbers in each group using a hemacytometer. Data were presented as mean plus or minus SD and analyzed with ANOVA and Dunnett's tests, n=6, ** P<0.01 *vs.* sham; #P<0.05 *vs.* the vehicle-treated SAH group.

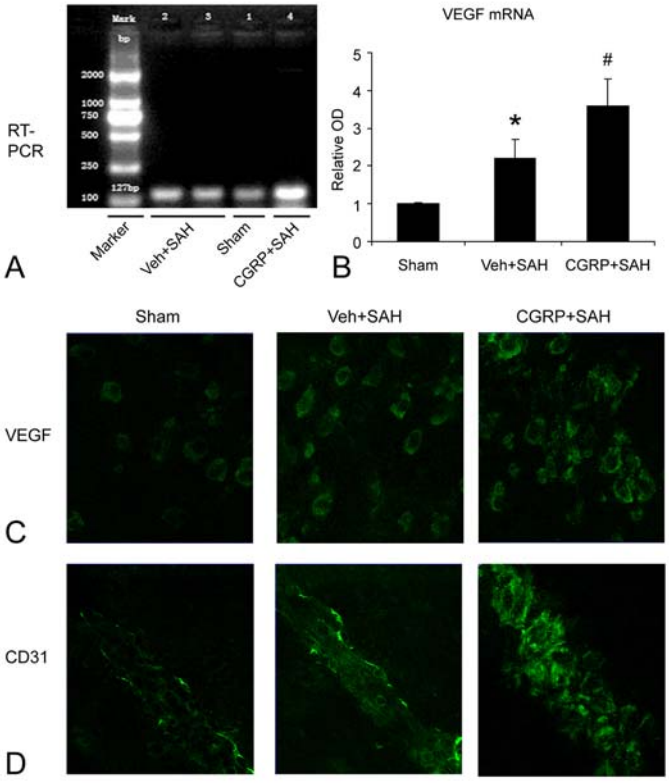

Figure 6. CRGP upregulated VEGF expression. (A) Representative photograph of VEGF RT-PCR. (B) Semi-quantitative analysis of VEGF mRNA, standardized on the sham group. Data were presented as mean plus or minus SD and analyzed with ANOVA and Dunnett's tests, n=5, * P<0.05 *vs.* sham; #P<0.05 *vs.* the vehicle-treated SAH group and P<0.01 *vs.* the sham group. (C) Representative microphotographs of immunofluorescent stainings of each group with anti-VEGF. 40x. (D) Representative microphotographs of immunofluorescent stainings of each group with anti-CD31, indicating increased expression of endothelial marker after intranasal CGRP administration. 40x.

BBB and reach the brain (24, 34). In addition to axonal transport, extracellular transport such as bulk flow and diffusion within perineuronal channels and perivascular space are considered to be major delivery mechanisms (24, 34). The perivascular space of trigeminal distribution may be an important place for the action of intranasal CGRP, because it is the place where the terminals of the trigeminal nerve, CGRP and CGRP receptors on perivascular smooth muscles co-localize (11, 14, 16). Our data for the first time demonstrate that intranasal administration of CGRP has advantages over IV injection in increasing its CNS delivery, 10-fold in CSF and 13-fold in brain.

When choosing vasodilators to ameliorate vasoconstriction, several factors need to be taken into account, such as the origin, the potency and action duration of the drugs. In this regard, CGRP is promising. First, CGRP is an endogenous neuropeptide released by certain nerve endings and enriched in the trigeminal sensory projection that innervates the intracranial arteries (13). Its local action is supported by the evidence that there is a deficiency in CGRP level in patients with Raynaud's disease (16, 49), and that CGRP is abnormally released during migraine headache (11, 50). Secondly, CGRP is a very powerful vasodilator; its potency is more than 10-fold greater than prostaglandins, and 100-1000 times greater than acetylcholine, adenosine and substance P (9, 16). Finally, unlike short-lasting vasodilators such as NO, acetylcholine and prostaglandins, the effect of CGRP is long-lasting. A small-dose (15 pM) skin injection of CGRP induced a long duration of vasodilation and erythema (5-6 hours) (9, 16). Thus, CGRP is considered one of the most appropriate drugs to relieve vasospasm induced by SAH.

In addition to its direct vasodilatory effect, intranasal CGRP administration upregulated VEGF expression as well, and this may account for part of the CGRP-mediated neuroprotection against ischemic injury caused by vasospasm and SAH. The protective effects of VEGF are at least twofold: pro-survival and angiogenic (46, 47, 51, 52). As an established neuroprotective agent, VEGF can reduce neuronal injury induced by *in vivo* and *in vitro* ischemia (46). More importantly, VEGF can promote angiogenesis, one of the major compensations for reduced oxygen levels under certain conditions such as hypoxia, vasospasm and rapid tumor growth. The angiogenic process itself is regulated by a group of factors; among them, VEGF is central (51, 52). Because VEGF specifically and potently stimulates endothelial proliferation and migration, it also modulates and relays the actions of other angiogenic factors (52). Recent studies have demonstrated that CGRP is a pro-angiogenic growth factor (44, 45). It stimulated VEGF expression and capillary-like tube formation, and knockout of CGRP significantly reduced VEGF level and subsequent angiogenesis (44, 45). In agreement with these findings, our data demonstrate that intranasal administration of CGRP increased the vascular density in cortex after SAH, which is associated with augmented levels of VEGF at both mRNA and protein phases. Taken together, our data suggest that CGRP benefits brains in multiple aspects.

The traditional way to measure endothelial death after SAH is to stain basilar arteries (42, 43). The drawbacks of this method include the fact that it reflects only the local condition of the examined vessels and that it requires the sacrifice of animals. When vascular endothelial cells are dead or damaged, they detach from the vessel walls and fall into the blood flow, becoming circulating endothelial cells. CEC counting has recently been used as a prospective marker of endothelial death, and it can be repeatedly performed *via* withdrawing venous blood to reflect dynamic changes. This method has been used in several disorders such as acute myocardial infarct, shock, ischemic brain stroke and some cancers (39, 40, 53). Similarly, SAH and vasospasm can also induce severe endothelial apoptosis (42, 43). However, it has not been reported previously whether CEC could be used as a tool to measure endothelial damage following SAH. In this study, we employed CEC as a novel marker to investigate endothelial damage after SAH and the neuroprotective effects of CGRP. We found that the CEC numbers were in proportion to the severity of vasospasm in SAH, suggesting that CEC is a useful tool to reflect endothelial damage in SAH.

In summary, this study has demonstrated that intranasal administration of CGRP is more efficient than IV in the delivery of CGRP to CNS. CGRP is a strong vasodilator that can profoundly attenuate vasospasm in SAH, and greatly reduce cell death in brain and endothelia as well. It also stimulates VEGF expression and angiogenesis. Together, these results suggest that intranasal application of CGRP could be a practical approach to diminish the vasospasm induced by SAH and to reduce the associated ischemic brain injury.

6. ACKNOWLEDGMENTS

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Abbreviations: BA, basilar artery; BBB, blood-brain barrier; CEC, circulating endothelial cell; CGRP, calcitonin gene-related peptide; cpm, counts per minute; CSF, cerebrospinal fluid; IN, intranasal administration; IV, intravenous injection; rCBF, regional cerebral blood flow; SAH, subarachnoid hemorrhage; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP nick end labeling assay; VEGF, vascular endothelial growth factor

Key Words: CEC, Cerebral Vasospasm, CGRP, SAH, VEGF, Vasodilator

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