Calcitonin gene-related peptide promotes angiogenesis via AMP-activated protein kinase

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Submitted 11 May 2010; accepted in final form 20 September 2010


CGRP is known to causes synthesis and release of calcitonin gene-related peptide (CGRP), it is not clear whether CGRP regulates angiogenesis under ischemia and how does it function. Thus we investigated the role of CGRP in angiogenesis and the involved mechanisms. We found that CGRP level was increased in the rat hindlimb ischemic tissue. The expression of exogenous CGRP by adenovirus vectors enhanced blood flow recovery and increased capillary density in ischemic hindlimbs. In vitro, CGRP promoted human umbilical vein endothelial cell (HUVEC) tube formation and migration. Furthermore, CGRP activated AMP-activated protein kinase (AMPK) both in vivo and in vitro, and pharmacological inhibition of CGRP and cAMP attenuated the CGRP-activated AMPK in vitro. CGRP also induced endothelial nitric oxide synthase (eNOS) phosphorylation in HUVECs at Ser1177 and Ser633 in a time-dependent manner, and such effects were abolished by AMPK inhibitor Compound C. As well, Compound C blocked CGRP-enhanced HUVEC tube formation and migration. These findings indicate that CGRP promotes angiogenesis by activating the AMPK-eNOS pathway in endothelial cells.

vasoactive peptide; endothelial cell; endothelial nitric oxide synthase.

ISCHEMIA is a pathophysiological cue leading to functional changes and adaptations of the cardiovascular system. The implications of ischemia-induced endothelial responses include angiogenesis and/or neovascularization involved in myocardial infarction and hindlimb ischemia (4, 28). Calcitonin gene-related peptide (CGRP), a neuropeptide containing 37 amino acid residues, distributes widely in central nervous system and peripheral nerves in cardiovascular tissues and intestine (36). Previous works by us and others have found that hypoxia, ischemia, as well as the related increase in lactic acid and decreased pH induce the synthesis and releasing of CGRP (34, 35, 37). In turn, CGRP promotes endothelial cell (EC) proliferation (9) and activates endothelial nitric oxide (NO) synthase (eNOS) via the cAMP-PKA pathway (2). In vivo, CGRP increases angiogenesis during wound healing and tumorigenesis (24, 30, 31). These findings suggest the involvement of CGRP in angiogenesis, but the underlying mechanisms remain to be elucidated.

Several kinases including AMP-activated protein kinase (AMPK), Akt, and PKA can phosphorylate eNOS, thereby increase the eNOS-derived NO bioavailability (7). Originally identified as an energy gauge (32), AMPK is highly sensitive to the changes of oxygen tension (7). AMPK has been shown to promote angiogenesis (22), especially under hypoxic and/or ischemic stresses (27). The involved mechanisms include that AMPK maintains EC metabolic homeostasis, protects ECs from being apoptotic, and activates eNOS (6, 11, 20, 29).

Although AMPK imposes such a potent angiogenic effect, whether CGRP can regulate AMPK in ECs and the consequent angiogenesis is currently unknown. Since CGRP activates eNOS via its Gs-coupled receptor, and AMPK is one of the downstream targets of G protein-coupled receptors (GPCR) (10), we hypothesize that AMPK-eNOS pathway is involved in the CGRP-elicited angiogenesis in response to ischemia. Both in vivo data of blood flow and capillary density from rat hindlimb ischemia model and in vitro data of human umbilical vein endothelial cells (HUVEC) angiogenic functions suggest that CGRP promotes angiogenesis, and further in vitro results indicate that AMPK plays a crucial role in CGRP-elicited angiogenesis.

MATERIALS AND METHODS

Reagents and antibodies. CGRP peptide and CGRP8–37 were from Phoenix Pharmaceuticals. AICAR (5-aminimidazole-4-carboxamide 1-ribofuranoside), Compound C [6-[4-(2-piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-pyrazol-5-yl]-a pyrimidine, also known as Dorsomorphin], and Rp-cAMPS were from Sigma (St. Louis, MO). Matrigel was from Vigo Biotechnology (Beijing, China). Primary antibodies to phospho-AMPKα (Thr172), AMPKα, phospho-ACC (Ser79), ACC, and horseradish peroxidase (HRP)-conjugated secondary antibodies were from Cell Signaling Technology, antibodies to phospho-eNOS (Ser1177 and Ser633) and eNOS were from BD Biosciences Pharmingen, antibodies to β-actin and von Willebrand factor (vWF), as well as biotin-labeled anti-rabbit secondary antibody and alkaline phosphatase-labeled streptavidin were from Santa Cruz (Santa Cruz, CA). Green fluorescence labeled donkey-anti-rabbit secondary antibody (H+L) was from Invitrogen. IRDye-conjugated secondary antibodies of Odyssey system were from LI-COR.

Animal model of hindlimb ischemia. Hindlimb ischemia was created in male Sprague-Dawley (SD) rats (200–220 g) based on published protocols (12, 21, 23) with modifications. Briefly, animals were anesthetized with chloral hydrate (10% w/vol in physiological saline, 4 ml/kg ip), and the left hindlimbs were made ischemic by surgery, the right hindlimb was used as nonischemic self control. To ensure ischemia efficiency, the proximal portion of femoral artery and vein and the distal portion of their major branches were ligated, followed by resection of the sections between ligations; such treatment led to more than 80% reduction in blood flow. As a parallel control, sham-operation only included the skin incision, vessels exposure, and skin suture.

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For exogenous CGRP gene transfer, adenovirus vector encoding human full-length CGRP with a cytomegalovirus (CMV) promoter was generously provided by Dr. Donald Heistad (University of Iowa, Iowa City, IA) (33) and amplified according to the protocol of the manufacturer of BD Biosciences Clontech. An adenovirus vector encoding green fluorescence protein (Adv-GFP) was used as a negative control. After vessel section was resected, \( 1 \times 10^9 \) plaque forming units (pfu) adenovirus in 100 \( \mu l \) saline were injected into adductor muscle of left hindlimb at five sites.

Rat hindlimb blood flow was measured on days 1, 3, 7, 14, 21, and 28 postsurgery by laser Doppler perfusion image (LDPI) analyzer (PeriScan PIM3, Perimed, Sweden). Total blood flow below the knee was calculated by the software of LDPIwin for PIM3. The relative blood perfusion index was calculated as the ratio of ischemic to nonischemic self-control hindlimb.

The animal protocol was approved by the Animal Care Committee of the Peking University Health Science Center.

**Evaluation of in vivo CGRP level and capillary density.** CGRP peptide level in the adductor muscles homogenate was measured by a CGRP radioimmunology kit, with the service provided by Beijing Sino-uk Institute of Biological Technology. CGRP content was normalized by total protein (pg/mg). For capillary density analysis, adductor muscles surrounding adenovirus injection area of ischemic hindlimbs were harvested from each animal and then sliced and fixed in 4% formaldehyde. The tissues were embedded in paraffin, and multiple tissue slices of 7-\( \mu m \) thickness were prepared. Capillary ECs were identified by immunohistochemical staining with anti-vWF antibody (Santa). Fifteen-serial sections were taken from each sample, five random microscopic fields of each section were examined for the presence of capillary ECs, and capillary density was expressed as the

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**Fig. 1. Calcitonin gene-related peptide (CGRP) promotes angiogenesis in vivo.** A: adductor muscles were excised and made into homogenate from hindlimbs with indicated treatments 3 days after operation. CGRP content was measured by radioimmunology kit. Data were normalized by total proteins (pg/mg). \( n = 3 \) in sham group, \( n = 8 \) in ischemia group, \( n = 7 \) in ischemia plus adenovirus vector encoding green fluorescence protein (Adv-GFP) group, \( n = 6 \) in ischemia plus Adv-CGRP group. B: Adv-GFP or Adv-CGRP was injected into ischemic muscle, and blood flow was assessed at the indicated time points after surgery. The representative LDPI scanning images are shown. C: post-ischemia blood flow recovery was assessed with use of the ratio of blood perfusion from ischemic to nonischemic self-control hindlimb. \( n = 10 \) in each group. D: skeletal muscle samples among adenoviral vectors injected area were excised on day 3 and 7 postsurgery and embedded in paraffin. Representatives of immunohistochemical assay (left) and hematoxylin-eosin (H-E) staining (right) are shown (magnification \( \times 400 \), and von Willebrand factor (vWF) expression was detected to indicate capillaries. Capillaries were stained into Lyons blue points, which were between muscle fibers. E: 15 serial sections were taken from each sample and underwent vWF immunostaining, and 5 randomly selected fields were calculated in each section. Capillary density was expressed as the ratio of capillary to muscle fiber, \( n = 7 \) in GFP 3-day group, \( n = 9 \) in CGRP 3-day group, \( n = 4 \) in both GFP 7-day and CGRP 7-day groups. Data are means \( \pm SE \). *\( P < 0.05 \).
ratio of capillary to muscle fiber. In parallel, hematoxylin-eosin staining was also performed in one muscle section of each sample.

**Cell culture.** HUVECs were digested by type I collagenase (100 IU/ml) from human umbilical vein and isolated by differential attachment rate from other cells. Then the cells were cultured in medium 199 (Hyclone) containing 15% fetal bovine serum (FBS, Hyclone), 1.4 IU/ml heparin sodium (Sigma), 2.7 mg/l thymidine (Sigma), 0.214 g/l HEPES, 1.98 g/l NaHCO3, and 22.5 g/l amphotericin and passed by 0.05% trypsin digestion. After isolation, HUVECs were identified by using rabbit-anti-human vWF antibody. The purity of the HUVECs was more than 97% by calculating the ratio of vWF-positive cell to total nucleus. HUVECs of passage 5–8 were used for experiments.

**Tube formation assay.** Matrigel assay was performed according to the manufacturer’s instruction. Briefly, HUVECs (8 × 104 cells in 600 μl M199) were seeded onto 24-well plates coated with Matrigel. Tube formation was observed using an inverted phase contrast microscope 2.5 h after various treatments. According to published methods (1), the degree of tube formation was quantified by measuring the number of tubes tube-like structures in five randomly chosen fields (×100) from each dish using the NIH Image Program. Tube-like structure was decided according to Li et al. (18). Each experiment was repeated for three times.

**Cell migration assay.** For the scratching assay, HUVECs (8 × 104 cells in 3 ml M199) were seeded in 60-mm diameter culture dishes. The medium was changed to serum-free OPTI.MEM the next morning for synchronization. Four hours later, scratching was made, and fresh medium containing 1% FBS was added with or without CGRP treatment. Five fields were randomly selected in each dish for recording gap distances immediately following scratching and 6 h afterwards, so that cell migration within 6 h could be calculated.

For Boyden Chamber assay, according to published method (14), 50 μl of HUVECs suspension (2 × 107/ml in M199 containing 1% FBS) were placed in the upper chamber, and 27 μl of M199–1% FBS with 10−7 M CGRP or vehicle were placed in the lower chamber. After incubation for 4 h, the cells on the upper surface were removed, and the cells on the underside were fixed and stained. Each treatment was performed in triplicate, and the mean number of cells in each well was counted from four randomly chosen fields under light microscopy (×200).

**Evaluation of in vivo phosphorylate AMPK.** Paraffin sections of ischemic adductor muscle were incubated with anti-phospho-AMPK...
primary antibody and green fluorescence-labeled secondary antibody subsequently. The mean fluorescence intensity of high light capillary-like structures around muscle fibers were measured by using the NIH Image Program and calculated to compare the phosphorylation levels of AMPK in different groups.

Western blot analysis and densitometry analysis. Western blot analysis was performed as previously reported (19, 29). Briefly, total protein lysates from HUVECs were resolved on 8% SDS-PAGE, then transferred to nitrocellulose membrane or polyvinylidene fluoride membrane, and underwent immunoblotting with different antibodies. Densitometry analysis was carried out by using Odyssey or ImageJ software.

Statistical analysis. Values are expressed as means ± SE. Student’s unpaired t-test was used to compare difference between two groups. Comparisons of parameters more than two groups were made by One-way ANOVA, followed by Student-Newman-Keuls tests. In all experiments, unless otherwise indicated, P < 0.05 was considered statistically significant.

RESULTS

CGRP promotes blood flow recovery and angiogenesis in ischemic hindlimb. CGRP has been reported to be upregulated in the forebrain after ischemic stress (8), thus we first tested whether ischemia altered endogenous CGRP level in hindlimb skeletal muscle. Radioimmunoassay showed that, 3 days post-surgery, the level of CGRP in adductor muscle of ischemic hindlimbs was significantly higher than that in sham-operated hindlimbs (Fig. 1A). We then delivered CGRP into the ischemic hindlimb by using adenovirus vector and used Adv-GFP as control. CGRP level was significantly increased in ischemic muscle receiving Adv-CGRP when compared with single ischemic muscle, whereas there was little difference between single ischemic muscle and Adv-GFP injected ischemic muscle (Fig. 1A). These results proved that ischemia could induce CGRP

![CGRP activates AMP-activated protein kinase (AMPK) in HUVECs](http://ajpcell.physiology.org/)
level, and Adv-CGRP was efficiently expressed in hindlimb muscle, whereas adenovirus vector had little effect on CGRP level.

Based on CGRP overexpression treatment, serial blood flow perfusion of ischemic hindlimb was measured by LDPI analyzer. On day 14 postoperation, blood flow was restored in both Adv-CGRP- and Adv-GFP-injected rats. However, more blood flow recovery was observed in Adv-CGRP-injected rats (Fig. 1, B and C).

To further confirm the angiogenic effect, the capillary density in the ischemic hindlimbs was analyzed. Anti-vWF immunostaining showed more positive staining between muscle fibers (Fig. 1D) in the Adv-CGRP injected ischemic muscles. The ratio of capillary to muscle fiber was significantly increased in Adv-CGRP-injected rats when compared with Adv-GFP control on both day 3 and day 7 postsurgery (Fig. 1E). These results suggest that ischemia induces CGRP expression in the hindlimb, which in turn promotes angiogenesis.

**CGRP enhances angiogenesis in vitro.** To explore the mechanisms underlying CGRP-induced angiogenesis, we performed Matrigel assay to examine the effect of CGRP peptide on HUVEC tube formation. CGRP with concentrations ranging from 1 to 100 nM increased the number of tube-like structure in Matrigel-mixed HUVECs in a CGRP concentration-dependent manner (Fig. 2A). We also tested the potency of CGRP on HUVEC migration. As shown in Fig. 2B, CGRP concentration dependedently promoted endothelial cell migration during wound closure after scratching, and statistical significance came out at 100 nM concentration. Accordingly, 100 nM CGRP also enhanced HUVECs migration in the Boyden Chamber assay, raising the migrated cells to about twofold of control (Fig. 2C). These results suggest that CGRP could promote endothelial cell angiogenic functions.

**CGRP activates AMPK in HUVECs via CGRP receptor 1.** As AMPK is involved in the hypoxia- and ischemia-induced angiogenesis (22, 27), we investigated whether CGRP activated AMPK in ECs. First, immunofluorescence assay showed that the fluorescence intensity of phospho-AMPK in capillary-like structures was significantly higher in Adv-CGRP-injected muscle tissue on 1 day postsurgery (Fig. 3A). Then we tested AMPK phosphorylation in HUVECs. Treating HUVECs with CGRP ranging from 1 nM to 1 μM for 30 min led to great increase in AMPK phosphorylation at Thr-172 (Fig. 3B). Also, the phosphorylation of ACC Ser-79, an AMPK downstream target, was increased. We then treated HUVECs with CGRP at 100 nM for various times. As shown in Fig. 3C, AMPK

![Fig. 4](http://ajpcell.physiology.org/)
phosphorylation was increased at 5 min, reached the plateau level between 15 and 30 min, and decreased at 60 min, while ACC phosphorylation lagged behind. Thus CGRP activates AMPK in ECs in a concentration- and time-dependent manner.

Mostly, CGRP binds to its type 1 receptor (i.e., CGRP R1) to initiate cAMP-PKA pathway (36). Thus we treated HUVECs with CGRP8–37 or Rp-cAMPS, the respective CGRP receptor 1 and cAMP antagonists, to investigate the involvement of CGRP receptor 1 and postreceptor cAMP pathway in CGRP-induced AMPK activation. As shown in Fig. 3D, CGRP8–37 and Rp-cAMPS treatment drastically depressed the CGRP-induced AMPK and ACC phosphorylation. These results suggest that AMPK activation in response to CGRP is mediated by CGRP receptor 1 and the consequent cAMP pathway.

**CGRP activates eNOS in HUVECs via AMPK.** Since AMPK phosphorylates eNOS at both Ser-633 and Ser-1177 to enhance the eNOS-derived NO bioavailability (5), we then studied whether CGRP can increase the eNOS phosphorylation, and if so, whether this activation is mediated through AMPK. We found that CGRP treatment indeed increased the phosphorylation of both sites (Fig. 4, A and B). However, phosphorylation of Ser-1177 was more rapid and transient when compared with that of Ser-633. Moreover, when we used Compound C to pharmacologically block AMPK, the CGRP-induced phosphorylation of ACC Ser-79, eNOS Ser-633, and Ser-1177 were all attenuated (Fig. 4C). These results suggest that the CGRP-activated eNOS is via AMPK.

**AMPK mediates CGRP-triggered HUVECs angiogenesis.** After finding that CGRP activation of eNOS is mediated through AMPK, we further investigated the possible role of AMPK in the CGRP-induced angiogenesis by using Matrigel and scratching assays. As shown in Fig. 5A, Compound C completely blocked EC tube formation induced by 100 nM CGRP. In contrast, the inclusion of AICAR, an AMPK agonist, increased the number of tubelike structure, similar to the effect of 10 nM CGRP. We also tested the possible role of AMPK in CGRP-induced HUVEC migration in scratching assay. Figure 5B indicates that Compound C not only reduced CGRP-promoted HUVECs migration but also inhibited the basal level migration. These results suggest that the angiogenic effect of CGRP involves the AMPK-eNOS pathway.

**DISCUSSION**

CGRP has many physiological and pathophysiological effects including promoting ECs proliferation (9), improving the
survival of surgical flaps of ischemic tissue (16), accelerating wound healing, increasing recovery from stomach ulcer, and supporting tumor growth (24, 30, 31). In this study, we showed that CGRP increased angiogenesis in vivo by using rat hindlimb ischemia model and adenoviral-delivered CGRP into ischemic tissue (Fig. 1). The angiogenic effect of CGRP was also supported by the tube formation and scratching assays in vitro (Fig. 2).

AMPK has been reported to mediate angiogenesis under various conditions. Shear stress, ischemia, hypoxia, and many drugs could active AMPK and directly induce angiogenesis in ECs (22, 25, 27, 29). AMPK also enhances angiogenesis indirectly by regulating hypoxia-induced factor (17) and promoting EPC differentiation (18). Because CGRP increased phosphorylation of AMPK and its substrate ACC in a concentration- and time-dependent manner, AMPK would play a crucial role in CGRP-enhanced angiogenesis. Evidence supporting this finding includes that CGRP-induced AMPK activation was attenuated by Compound C—37 and Rp-cAMPS and that Compound C significantly inhibited the CGRP-induced tube formation and migration.

Although CGRP has been shown to increase the phosphorylation of eNOS Ser-1177 via its type 1 receptor and cAMP pathway (2), our data herein showed that Ser-633 phosphorylation could be enhanced by CGRP as well. Despite Ser-1177 phosphorylation was transient, Ser-633 phosphorylation was sustained. Such a distinct temporal response is consistent with a previous report that shears stress also caused a sustained Ser-633 phosphorylation (5). The molecular basis of this activity-related distinction is unknown. One possible explanation is that confirmation surrounding Ser-633 may restrict the catalysis by phosphotase. AMPK inhibition by Compound C resulted in the decrease in eNOS phosphorylation responding to CGRP, suggesting that AMPK regulates the eNOS phosphorylation in response to CGRP treatment. Indeed, AMPK blockage impaired the CGRP-induced angiogenesis and EC migration (Fig. 5).

CGRP receptor 1 belongs to the Gs-coupled receptor, and the cAMP-PKA pathway is a major downstream signaling event (10). Figure 3C shows that both CGRP receptor 1 and cAMP were involved in the CGRP-induced AMPK activation. Because LKB1 and CaMKK can phosphorylate AMPK (3), these two kinases may interplay with PKA in phosphorylating AMPK. Indeed, cAMP-PKA can activate LKB1 to promote axon initiation (26), and IL-6 activates AMPK via increased cAMP concentration (15).

According to the previous report (13), hindlimb ischemia could induce the phosphorylation levels of AMPK and eNOS (Ser1177) protein in hindlimb tissues. In our study, we also tested the phosphorylation levels of AMPK by immunofluorescence staining (Fig. 3). We indeed found higher fluorescence intensity of phospho-AMPK in capillary-like structures around muscle fiber in Adv-CGRP group than that in Adv-GFP controls. These data provided evidence that AMPK phosphorylation could be further enhanced by CGRP.

To further support our hypothesis, we shall have included parallel groups administered with CGRP inhibitors in our study, but because the usual administration of these drugs is intravenous or subcutaneously injection with high dosage (it is also very difficult to calculate the exact efficacious dosage in vivo) by osmotic pump, it would bring us at least two problems. First, such a whole body high-dosage administration would induce nonspecific reactions/responses in many organs and tissues other than hindlimb. Second, little of the inhibitors would reach the ischemic hindlimb tissue because of the drastic reduction of blood flow after the ligation of femoral artery. The alternative method is to directly inject inhibitors into ischemic hindlimb tissue. However, the inhibitory effects through direct injection may not last for an effective time, which means the requirement of repetitive. Thus we only tested the effects of CGRP antagonist in vitro. As well, more efficient methods shall be developed to further confirm the effects of CGRP signaling on angiogenesis in vivo.

In conclusion, our results show for the first time that the angiogenic effect of CGRP is mediated through a signaling cascade of CGRP R1-cAMP-AMPK-eNOS. This study provides some new insights that correlate the CGRP released from nervous system and angiogenesis in the vessel system.

ACKNOWLEDGMENTS

The authors appreciate Dr. Donald Heistad (University of Iowa) for the kind donation of Ad-CGRP constructs. We thank Na Zhao from Peking University Microcirculation Research Center for technical help in rat hindlimb blood flow assessment. We thank Li Chen and Bo Liu from Peking University Health Science Center for histological assessment.

GRANTS

This work was supported by the Major National Basic Research Program of China (no. 2006CB503802) to X. Wang and by the 111 Project (no. B07001) to X. Wang and J. Y.-J. Shyy.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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