2.4. Calculations and statistics

The values of plasma hormones and metabolites were expressed as least squares means of 4 wethers with SE or SEM. The decrement area was calculated for ghrelin, and the incremental areas were calculated for glucose and insulin, with the values expressed as least squares means of 4 wethers with SE as well. Data were analyzed as a 4 × 4 Latin square with a mixed linear model that used restricted maximum likelihood of the JMP program package (Version 5.01 for Windows computer system; SAS Institute Inc, Cary, NC, USA). For the statistical analysis of the differences in the values before injection (mean from −10 and 0 min), the decrement for ghrelin or increment for glucose and insulin areas, the models included the treatment as the fixed effect, and wethers and period as the random effects. The comparisons among treatments were evaluated by Student *t* test. For statistical analysis of the values of the concentrations after injections (5 to 60 min), time and the interaction between treatment × time were added to the fixed effects. Factorial contrasts tested the effect of SCFA injections (CON vs ACE, CON vs PRO, and CON vs BUN). A P value < 0.05 was considered significant.

3. Results

At pre-injection time (−10 and 0 min), no differences were observed among treatments in plasma ghrelin, glucose, insulin, ACE, and BHBA concentrations (Fig. 1 and Fig. 2).

In all SCFA treatments, plasma ghrelin concentrations after injection were lower compared with CON ($P < 0.05$; Fig. 1A). The decrement areas of plasma ghrelin were greater with all SCFA treatments than with CON ($P < 0.05$; Table 1) but not different among ACE, PRO, and BUT.

After injection, plasma glucose concentrations did not differ between CON and ACE, but in PRO and BUT plasma glucose concentrations were greater than with CON ($P < 0.05$; Fig. 1B). The increment area of plasma glucose was largest in BUT, followed by PRO, whereas the increment areas in CON and ACE were smaller than with PRO and BUT ($P < 0.05$; Table 1).

After injection, plasma insulin concentration did not differ in CON and ACE, but in PRO and BUT plasma insulin concentrations were higher than with CON ($P < 0.05$; Fig. 1C). The increment area of plasma insulin in ACE was smaller than with PRO and BUT, although it was greater than with CON ($P < 0.05$; Table 1). The increment areas of plasma insulin were similar in PRO and BUT.

Plasma acetate concentration in ACE was higher than with CON after injection ($P < 0.05$; Fig. 2A). Plasma BHBA concentration in BUT was higher than with CON after injection ($P < 0.05$; Fig. 2B).

4. Discussion

The aim of our study was to determine whether SCFAs affect plasma ghrelin concentrations. We determined plasma ACE concentration by sodium acetate injection in ACE to ascertain the increase and confirmed that plasma ACE concentrations increased clearly after ACE injection. Plasma BHBA concentration increased with BUT; however, this indicated that injected BUT was rapidly converted to BHBA. Similarly, the increase of plasma glucose concentration in PRO indicated that injected PRO was converted to glucose.

Our results showed that intravenous injections of ACE, PRO, and BUT reduced plasma ghrelin concentrations. Some reports have suggested that glucose or insulin or both could decrease plasma ghrelin concentration in nonruminants [8–10,15,16]. We observed increases of plasma glucose concentrations in PRO and BUT. Injected PRO was converted to glucose by itself, and BUT enhanced glucagon that eventually induced a plasma glucose increase [17]. We also observed increases of plasma insulin concentrations in PRO and BUT. These findings suggest that increased plasma concentrations of glucose and insulin might mediate depressions of plasma ghrelin concentrations by PRO and BUT. However, Sugino et al [18] demonstrated neither glucose nor insulin changed plasma ghrelin concentration in sheep with the use of the hyperglycemic clamp test. In our study, the increment areas of plasma glucose and insulin were different among treatments. No changes were observed in the decrement areas of plasma ghrelin concentration. Therefore, we assumed that plasma ghrelin concentrations might have decreased as direct responses to SCFAs. This was because the decrement areas of plasma ghrelin concentrations after SCFA injection were not different between ACE, PRO, and BUT, although their energy contents and metabolism were different. Presumably, the ghrelin response to SCFAs might be a molar-dependent action generated directly via their chemoreceptors. Ghrelin secretion is regulated by autonomic nerves in rats [19]. Sugino et al [3] demonstrated that the intravenous infusion of cholinergic blockers inhibited a meal-induced decrease in plasma ghrelin concentration, suggesting that cholinergic activity sup-

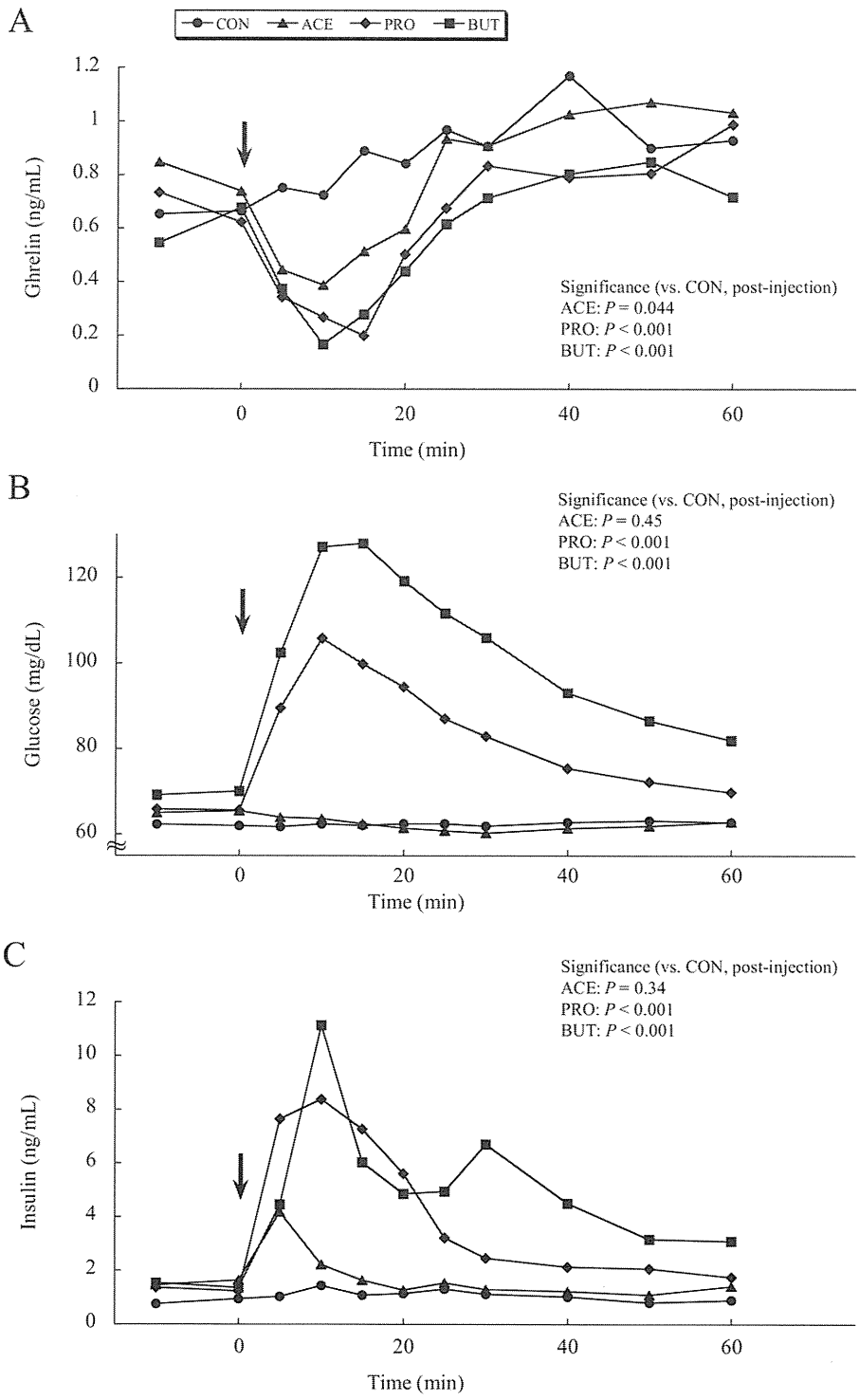

Fig. 1. Plasma ghrelin (A), glucose (B), and insulin (C) concentrations in CON ([circf]), ACE ([trif]), PRO ([diaf]) and BUT ([squlf]). Values are expressed as least squares means (n = 4). Their pooled SEM was 0.037 ng/mL for ghrelin, 3.00 mg/dL for glucose, and 0.36 ng/mL for insulin. The arrow shows the injection time. The pre-injection values of (mean from −10 and 0 min) plasma ghrelin, glucose, and insulin were not different among treatments. Statistical contrasts (figure inset) refer to decrement (A) or increment (B,C) areas.

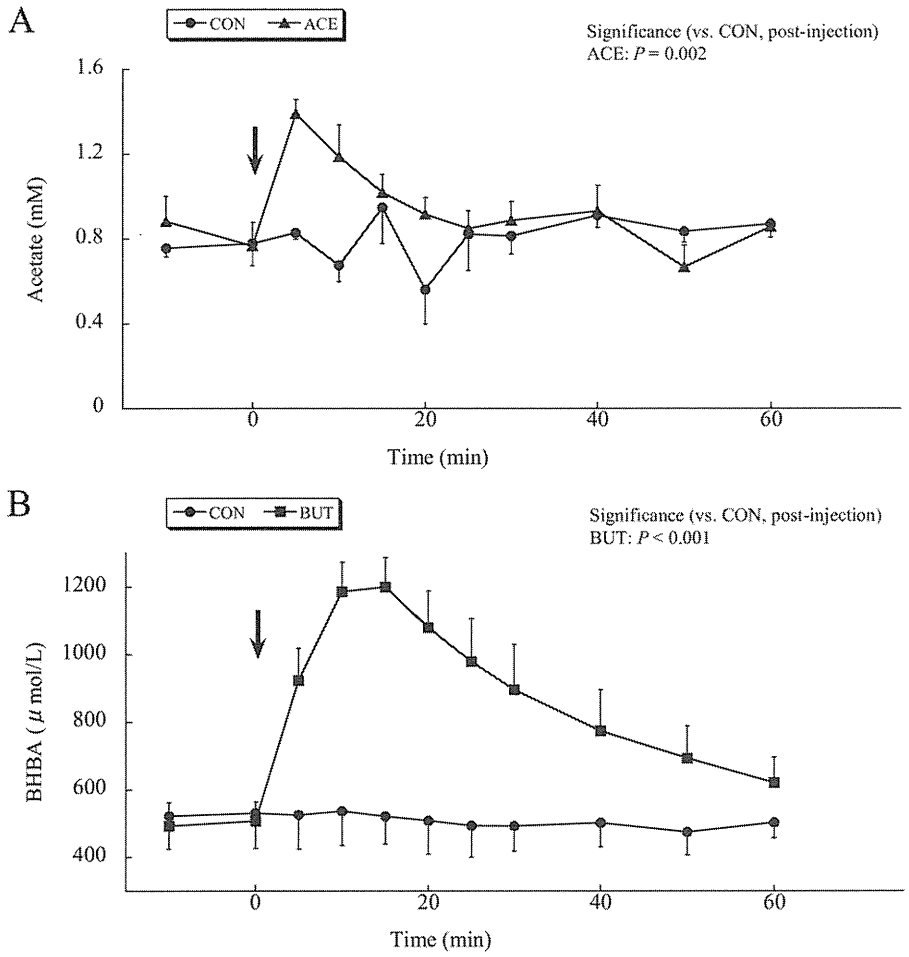

Fig. 2. Plasma acetate (A) and BHBA (B) concentrations in CON (]circf]), ACE ([trif]), and BUT ([squlf]). Values are expressed as least squares means ± SE (vertical bar; n = 4). The arrow shows the injection time. At pre-injection values (mean from −10 and 0 min) of plasma acetate and BHBA were not different among treatment. Statistical contrasts (figure inset) refer to increment areas.

pressed ghrelin secretion. Thus, we suggest here that activation of vagal afferents sensitive to SCFAs might be involved in the depression of plasma ghrelin concentration, although we could not identify any SCFA-stimulated sites because of intravenous injections of SCFAs. Although we could not elucidate the mechanisms by which SCFAs decreased plasma ghrelin concentration, we supposed that other factors aside from glucose and insulin might be at work in this study.

In conclusion, this study documents the effects of SCFAs on ghrelin secretion in ruminants for the first

Table 1
Decrement area for plasma ghrelin and increment areas for plasma glucose and insulin in wethers.

Item	CON, mean	ACE, mean	PRO, mean	BUT, mean	SEM	*P* value for treatment
Ghrelin: decrement area (ng.min/mL)	−0.024[b]	−0.282[a]	−0.279[a]	−0.230[a]	0.053	0.022
Glucose: increment area (mg.min/dL)	0.908[c]	0.106[c]	21.1[b]	32.4[a]	2.45	0.028
Insulin: increment area (ng.min/mL)	0.310[c]	0.864[b]	2.69[a]	3.53[a]	0.658	0.020

Abbreviations: CON, saline; ACE, saline with sodium acetate; PRO, saline with sodium propionate; BUT, saline with sodium butyrate.
Values with different superscripts (a,b,c) are different between treatments ($P < 0.05$).

time. We showed that ACE, PRO, and BUT equally decreased plasma ghrelin concentration.

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Review

Therapeutic applications of ghrelin to cachexia utilizing its appetite-stimulating effect

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ABSTRACT

Ghrelin, which is a natural ligand for the growth hormone (GH)-secretagogue receptor (GHS-R), stimulates food intake in both animals and humans. Ghrelin is the only circulating hormone known to stimulate appetite in humans. Ghrelin also stimulates GH secretion and inhibits the production of anorectic proinflammatory cytokines. As GH is an anabolic hormone, protein stores are spared at the expense of fat during conditions of caloric restriction. Thus, ghrelin exhibits anti-cachectic actions via both GH-dependent and -independent mechanisms. Several studies are evaluating the efficacy of ghrelin in the treatment of cachexia caused by a variety of diseases, including congestive heart failure, chronic obstructive pulmonary disease, cancer, and end-stage renal disease. These studies will hopefully lead to the development of novel therapeutic applications for ghrelin in the future. This review summarizes the recent advances in this area of research.

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1. Introduction

Ghrelin, which is a natural ligand for the growth hormone (GH)-secretagogue receptor (GHS-R) [49], plays a critical role in a variety of physiological processes, including the stimulation of growth hormone secretion, and regulation of energy homeostasis by stimulating food intake and promoting adiposity via a GH-independent

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mechanism [50,52,85]. GH, which regulates insulin-like growth factor (IGF)-I levels, is an anabolic hormone that spares protein stores at the expense of fat utilization during conditions of caloric restriction. GH and IGF-1 are the major mediators of metabolism involved in the regulation of energy balance. Ghrelin inhibits the production of anorectic proinflammatory cytokines, including interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α [23,25]. The combination of these actions suggests this peptide has benefits for the treatment of cachexia.

Cachexia is defined as a complex metabolic syndrome associated with underlying illness and characterized by loss of muscle with or without loss of fat mass [29]. The prominent clinical feature of cachexia is weight loss in adults or growth failure in children. Anorexia, inflammation, insulin resistance and increased muscle protein breakdown are frequently associated with cachexia. Cachexia is distinct from starvation, age-related loss of muscle mass, primary depression, malabsorption and hyperthyroidism and is associated with increased morbidity. Although there is real need for pharmacological treatment for cachexia, the track record of pharmacological interventions is limited. They include anabolic/androgenic steroids, appetite stimulants, protein anabolic agents and cytokine modulators [28,36]. Recently, several trials attempting to treat cachexia of different etiologies with ghrelin have been expanded [4]. This review summarizes the recent advances in this area of research.

2. Actions of ghrelin

2.1. Orexigenic action

Ghrelin has a well-established role in stimulating appetite and increasing food intake [17,90]; peripheral administration of ghrelin stimulates GH secretion and food intake in both animals and humans [91,93]. Ghrelin is the only hormone known to stimulate appetite after peripheral administration. Ghrelin, which increases c-fos expression in the arcuate nucleus, also activates hypothalamic neuropeptide Y (NPY)/Y1 receptors and agouti-related peptide (AgRP) pathways [16,46,74]. In addition, ghrelin induces food intake via the orexin pathway [82]. These functions are mediated at least in-part by vagal nerve pathways [19]. Repeated administration of ghrelin resulted in significant weight gain in rats [92] and patients with chronic obstructive pulmonary disease (COPD) [60]. Increases in adiposity were associated with body weight gain in animal experiments [27,84,92], while adiposity decreased in patients after total gastrectomy [1,3,98], esophagectomy [1,3,98] with gastric tube reconstruction and total hip replacement for osteoarthritis [1,3,98]. (Gastrectomized and esophagectomized patients were cancer patients and, in the study of gastrectomized patients, the placebo group also showed a decline in fat mass.) This discrepancy may result from the differences in the doses and frequencies of ghrelin administration. Long-term, twice-weekly injection with low-dose ghrelin (40 μg/kg) significantly decreased fat mass in aged mice [7]. While weekly food intake did not increase under these conditions, ghrelin-induced GH secretion may have contributed to low adiposity. In contrast, lean body mass increased in rodents [7,20] and humans [1,3,60,98] following ghrelin administration. Also, lean body mass increases following ghrelin mimetic administration [66,78]. These effects, which reflect increases in muscle mass, are promising for cachexia treatment, as losses in body weight and sarcopenia are characteristic features of cachexia.

2.2. Stimulation of GH secretion

Ghrelin strongly stimulates GH secretion in humans [5,8,40,80], several-fold more potently than GHRH under similar circumstances. Furthermore, ghrelin and GHRH synergistically increases GH release [40]. Ghrelin might also play a role on GH release in a non-acute setting [65]. GH regulates IGF-I levels, promotes anabolism, and increases muscle strength [35,86]. While GH enhances lipolysis, IGF-1 stimulates protein synthesis, myoblast differentiation, and muscle growth. Recombinant GH is currently approved by the U.S. Food and Drug Administration for use in HIV/AIDS wasting, parenteral nutrition-dependent short bowel syndrome, pediatric chronic kidney disease, and adult and pediatric GH-deficiency states [38]. Pharmacological doses of this agent, however, cause problematic side effects, such as dose-related arthralgias, carpal-tunnel syndrome, paresthesias, insulin resistance, sodium retention, and peripheral edema. In contrast, stimulation of GH production to supraphysiological levels following ghrelin administration has a paucity of severe side effects [3].

Ghrelin's ability to increase circulating IGF-1 levels has been demonstrated in human studies of congestive heart failure (CHF) and COPD, in which 3-week ghrelin injections tended to increase IGF-1 levels [60,63]. This effect is less evident as that seen for GHS, such as MK-677 and anamorelin (RC-1291). Long-term treatment (6 months) with MK-677 in patients with hip fractures increased IGF-1 levels by 84% in comparison to 17% after placebo [12]. Anamorelin treatment induced impressive increases in food intake in a 12-week trial of cancer cachexia; post-treatment IGF-1 levels were 36.5 ng/mL after anamorelin treatment in comparison to 5.95 ng/mL after placebo. In a 6-day trial of healthy volunteers, post-treatment IGF-1 levels increased to greater than 60 ng/mL after anamorelin treatment in comparison to <0 ng/mL after placebo [32,33]. In our study of patients undergoing total hip replacement for osteoarthritis, serum IGF levels changed significantly following eight daily ghrelin injections; the changes in post-treatment IGF-1 levels were 30.0 ng/mL for ghrelin in comparison to 5.6 ng/mL for placebo. These changes were not observed after 21 daily treatments. In males, however, serum IGF-1 levels after ghrelin treatment remained elevated in comparison to the placebo group. Thus, the timing and duration of ghrelin injection and the subjects receiving treatment (e.g., disease features and sex) may influence the effect of ghrelin on IGF-1 levels. In addition, serum levels of IGF-1, which is primarily produced by the liver, reflect the systemic effects of IGF-1. GH also induces the synthesis of IGF-I in non-hepatic tissues. The local (autocrine/paracrine) effects of IGF-1 may play distinct roles in various tissues, including muscle mass regulation [54,86]. For instance, local muscle-restricted IGF-1 transgene expression accelerates the regeneration of injured skeletal muscle in mice, modulating inflammatory responses and limiting fibrosis [69].

2.3. Anti-inflammatory action

Evidence that ghrelin exerts anti-inflammatory actions has been accumulating. Ghrelin suppresses the production of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α both *in vitro* [25,55] and *in vivo* [37,81,94]. In clinical trials, daily administration of ghrelin for 3 weeks decreased inflammatory cytokine levels and neutrophil density in sputum from patients with chronic respiratory infections [48]. In contrast, ghrelin induces the anti-inflammatory cytokine IL-10 [37,88].

Ghrelin inhibits the activation of NF-κB, a transcription factor known to control the production of multiple proinflammatory cytokines during inflammatory insults [55,88,94]. Although the molecular mechanisms and cellular targets mediating ghrelin inhibition of NF-κB activation remain to be determined, the vagus nerve may play an important role in the ghrelin-mediated inhibition of proinflammatory cytokine release [83,94]. Cachexia and muscular wasting occur via protein degradation by the ubiquitin–proteasome pathway [44]. Two muscle-specific

ubiquitin ligases, muscle RING-finger protein-1 (MuRF1) and atrogin-1/muscle atrophy F-box (MAFbx), are up-regulated under catabolic conditions. NF-κB activation may regulate skeletal muscle proteasome expression and protein degradation. The elevations in MuRF1 and MAFbx expression seen in skeletal muscle after thermal injury, arthritis, and dexamethasone administration were normalized, attenuated, and prevented, respectively, by ghrelin or GHS administration [13,72,97]. IGF-1 prevents the expression of MuRF1 and MAFbx by inhibiting Forkhead box O (FOXO) transcription factors via stimulation of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway. The IGF-1 receptor triggers activation of several intracellular kinases, including phosphatidylinositol-3-kinase (PI3K) [76]. Thus, the effects of ghrelin on NF-κB activation and IGF-1 synthesis are favorable for minimizing inflammatory responses and sarcopenia in patients with cachexia.

In addition, ghrelin promotes thymopoiesis during aging, suggesting an important biological role of ghrelin in generation of naive T cells and age-associated thymic involution [26]. This also suggests a possible therapeutic benefit of harnessing ghrelin signaling pathway in the reconstitution of thymic function in immunocompromised subjects such as cachectic patients. Moreover, ghrelin may be a key player in coupling metabolism to immunity [24], considering that reduction in ghrelin levels is associated with increased inflammation during obesity [89] and that increased ghrelin by calorie restriction is associated with decreased inflammation [99].

2.4. Other actions

The role of ghrelin in stimulating gastric emptying and acid secretion is well-established [68]. This effect may ameliorate gastrointestinal symptoms in patients with anorexia–cachexia syndrome. Ghrelin also increases endogenous nitric oxide (NO) release [75,96], which may influence the orexigenic and anti-inflammatory actions of ghrelin [51,59]. These qualities may be important in the treatment of cachexia.

Ghrelin influences insulin secretion and glucose metabolism [22,85]. Ghrelin may have obesogenic/diabetogenic properties. These properties may be direct effects of ghrelin on pancreatic islet function and/or indirect effects through GH secretion modulation. Also, ghrelin is part of a mechanism that integrates the physiological response to fasting [100]. This is very likely of physiological relevance to protection against food scarcity by building energy reserves. Moreover, this action may be beneficial for catabolic states such as cachexia. At least, no harmful effects of ghrelin on glucose metabolism or diabetic patients have been observed in our clinical studies of repeated ghrelin administration to patients with cachexia [60,63] or undergoing surgery [3].

3. Plasma ghrelin levels in cachexia

Plasma ghrelin levels are elevated in cachectic conditions caused by a variety of underlying disorders [31,43,47,64,73]. Although this phenomenon has been called "ghrelin resistance", these elevations may be a compensatory response reflecting the negative energy balance state. While there is usually an inverse relationship between plasma ghrelin levels and BMI, no significant difference in ghrelin levels between normal subjects and cachectic patients after matching for BMI. Thus, difference in ghrelin levels between them is less apparent after controlling for BMI. In patients with ESRD, conflicting results (i.e., increases [11,70,71], decreases [42], or no change [42,79]) for circulating ghrelin concentrations have been reported [57]. Aygen et al. recently reported elevations in both ghrelin and des-acyl ghrelin in ESRD patients undergoing hemodialysis (HD) in comparison to age-matched healthy controls [11]. Iglesias et al. indicated that patients undergoing HD possessed similar ghrelin concentrations to the control group; only peritoneal dialysis (PD) patients exhibited significantly lower ghrelin concentrations at baseline than those found in patients on conservative management [42]. These conflicting results are due, at least in-part, to cross-sectional studies using different ghrelin assays that compared patients with different residual renal functions, ages, genders, and nutritional status. Residual renal function may affect the metabolism and clearance of ghrelin. Longitudinal studies following patients with renal disease using ghrelin assays measuring both ghrelin and des-ghrelin are required to determine the pathophysiologic role of ghrelin in cachexia associated with ESRD. Post-hemodialysis serum ghrelin levels are significantly lower than pre-hemodialysis ghrelin levels, supporting the view that ghrelin is cleared by hemodialysis [11,45,57].

4. Clinical studies

Trials seeking to apply the effects of ghrelin to the treatment of cachexia have been expanding. These studies have sought to evaluate ghrelin as a treatment for patients with the cachexia associated with CHF, COPD, cancer, ERSD, etc. Cachexia, which manifests as excessive weight loss in the setting of an underlying chronic disease [58], is typically associated with anorexia as a major cause of weight loss. Weight loss and decreased appetite are the major causes of morbidity and mortality in patients with anorexia-cachexia syndrome. There is an immediate need for effective, well-tolerated treatments to stimulate appetite [18], prompting several trials to explore the application of ghrelin as a treatment for patients with cachexia.

4.1. CHF cachexia

Ghrelin induces a positive energy balance state through both GH-dependent and -independent mechanisms and has protective cardiovascular effects [61]. GH treatment may be especially useful in a subgroup of patients with cardiac cachexia [6]. Ghrelin stimulates food intake, induces adiposity, regulates the central nervous system to decrease sympathetic nerve outflow, and inhibits apoptosis of cardiomyocytes and endothelial cells in a GH-independent manner. Nagaya et al. investigated the effects of ghrelin on cardiac cachexia in ten patients with CHF [63] (Table 1). Daily administration of ghrelin for 3 weeks increased both food intake and body weight. This study also demonstrated improvements in patient exercise capacity, muscle wasting, and left ventricular function. Ghrelin treatment also resulted in significantly decreased plasma

Table 1
Clinical studies of ghrelin for cachexia treatment.

Diseases	Reference	Year	Study design	Number of patients	Ghrelin administration
CHF	[58]	2004	Open-label pilot study	10	Ghrelin, 2 μg/kg b.i.d. for 3 weeks, i.v.
COPD	[55]	2005	Open-label pilot study	7	Ghrelin, 2 μg/kg b.i.d. for 3 weeks, i.v.
Cancer cachexia	[62]	2004	Acute, randomized, placebo-controlled, cross-over study	7	Ghrelin, 5 pmol/kg/min i.v. for >180 min
Cancer cachexia	[72]	2008	Randomized, placebo-controlled, cross-over study	21	Ghrelin, 2 or 8 μg/kg, i.v. for 4 days, once a day
ESRD	[89]	2005	Acute, randomized, placebo-controlled, cross-over study	9	Ghrelin, 3.6 nmol/kg, s.c.
ESRD	[9]	2009	Randomized, placebo-controlled, cross-over study	12	Ghrelin, 12 μg/kg, s.c. for 1 week, once a day

norepinephrine levels. Although this study was neither randomized nor placebo-controlled, the eight CHF patients who did not receive ghrelin (control group) were followed to rule out any time-course effects during hospitalization. None of the aforementioned parameters changed in patients with CHF who did not receive ghrelin therapy. Further studies will be necessary to identify the pathways involved in this ghrelin effect and to determine the best therapeutic strategies for ghrelin use to combat the wasting process found in cardiac cachexia [6]. Clinical trials are currently attempting to reproduce these data in a double-blind, placebo-controlled fashion.

4.2. COPD cachexia

Patients with COPD often exhibit some degree of cachexia [62], which is an independent risk factor for mortality in COPD; GH treatment increases muscle mass in such patients. COPD and CHF are both associated with multiple pathophysiological disturbances, including anemia and neurohormonal activation [53]. In COPD patients, ghrelin exhibits anti-inflammatory effects. Chronic respiratory infections, characterized by neutrophil-dominant airway inflammation, lead to end-stage cachexia [10]. The cytotoxicity of accumulated neutrophils against bronchial and alveolar epithelial cells induces a deterioration of pulmonary function in COPD, resulting in excess energy expenditure and weight loss in patients. Intravenous ghrelin treatment for 3 weeks reduced both neutrophil counts in sputum samples and the volume of sputum, suggesting suppression by ghrelin of excess neutrophilic influx [48].

An open-label pilot study examined the ability of ghrelin to improve cachexia and functional capacity in patients with COPD; ghrelin was administered intravenously for 3 weeks to seven cachectic patients with COPD [60]. Repeated ghrelin administration significantly increased food intake, body weight, lean body mass, and peripheral and respiratory muscle strength. Ghrelin treatment ameliorated the exaggerated sympathetic nerve activity, as indicated by marked decreases in plasma norepinephrine levels. In cachectic patients with COPD, treatment with ghrelin improved appetite, body composition, muscle wasting, functional capacity, and sympathetic augmentation. Subsequently, another placebo-controlled trial demonstrated that ghrelin increased both appetite and body weight with an apparent dose-dependent trend toward improved physical performance (chair stand score) [34]. A larger clinical trial is currently being conducted to confirm these data in a double-blind, placebo-controlled fashion. Comparisons of this treatment to current standard medications will be required [53].

4.3. Cancer cachexia

Anorexia, frequently encountered in cancer patients, is one of the major causes of malnutrition and cachexia in this patient population. Ghrelin administration resulted in significant increases in weight and food intake in rodent models of cancer cachexia [21,39,87]. In all studies, ghrelin improved both food intake and weight gain in rodent models. DeBoer et al. determined that weight gain resulted from a reversal in the loss of lean body mass, a critical component of cachexia [21].

Several randomized, double-blind placebo-controlled trials have demonstrated the efficacy and safety of ghrelin or GHS in patients with cancer-associated cachexia [32,67,77]. Nearry et al. performed a randomized, placebo-controlled, cross-over clinical trial to determine if ghrelin could stimulate appetite in seven cancer patients with severe anorexia [67]. Ghrelin infusion resulted in a marked increase in energy intake in comparison to saline-treated controls; all patients in the study demonstrated increased food consumption. The meal appreciation score was also higher in ghrelin-treated individuals. Strasser et al. detailed a randomized, double-cross-over, phase 1/2 study in 21 patients with advanced cancer [77]. They infused a low or high dose of ghrelin or placebo before lunch daily for 4 days in each course. Nutritional intake or eating-related symptoms did not differ between the ghrelin- and placebo-treated groups. More patients, however, preferred ghrelin to placebo at the middle and end of study, although this finding was not dose-dependent. In contrast to the results of Neary et al., this study did not demonstrate any increases in food intake. As the patient characteristics and study designs were very different in the two studies, further investigation will be required.

An important concern regarding the use of ghrelin in cancer cachexia is that ghrelin may increase growth factors, such as GH and IGF-1, to stimulate tumor growth. Additionally, ghrelin itself may have mitogenic potential. As far as we know, no *in vivo* data have examined the differences in tumor growth after ghrelin or GHS treatment. Long-term, large-scale clinical trials are required to determine if ghrelin treatment promotes tumor growth.

4.4. End-stage renal disease (ESRD)

ESRD is a chronic condition frequently associated with nutritional dysfunction [15]. This type of malnutrition is highly resistant to intervention and a major predictor of morbidity and mortality for patients on either peritoneal dialysis (PD) or hemodialysis. Wynne et al. sought to determine if a single injection of ghrelin could enhance food intake in patients with evidence of malnutrition receiving maintenance peritoneal dialysis [95]. Nine PD patients exhibiting mild to moderate malnutrition administered either ghrelin or a saline placebo subcutaneously were examined in a randomized, double-blind, cross-over protocol. Ghrelin administration significantly increased mean absolute energy intake during the study meals and maintained nonsignificant increases observed in energy intake over the first 24 h without a subsequent rebound. This research group has subsequently sought to analyze the efficacy of repeated ghrelin administrations in malnourished dialysis patients [9]. They performed a double-blind randomized cross-over study of a week of daily subcutaneous ghrelin injections in a group of 12 malnourished dialysis patients. Ghrelin administration significantly increased appetite, with increases in energy intake noted at the first study meal. Persistence of this effect throughout the week was confirmed by food diaries and final study meals, indicating that daily ghrelin treatment achieved a sustained positive change in energy balance in malnourished dialysis patients. In support of these data, an animal study using a nephrectomized rat model of renal cachexia demonstrated that daily treatment for 2 weeks with ghrelin or two GHS agents (BIM-28125 and BIM-28131) resulted in increased food intake, improved lean body mass accrual, and decreased circulating inflammatory cytokines [20]. Long-term studies are needed to demonstrate the efficacy in improving appetite, weight gain, lean body mass, and quality of life.

4.5. Others

Ghrelin treatment has also been applied to other causes of anorexia, sarcopenia, and emaciation, including anorexia nervosa (AN) [41], functional dyspepsia [2], aging [3], postgastrectomy anorexia [1], esophagectomy [98], chemotherapy [30,56], and thermal injury [14]. In addition, the effects of ghrelin mimetics were examined in the age-dependent sarcopenia [3,66]. These applications certainly provide additional insight into the successful treatment of cachexia, a wasting syndrome developing in the setting of a variety of chronic illnesses.

5. Conclusion

Ghrelin exhibits anti-cachectic effects in a number of animal and human studies. Ghrelin treatment is safe and well-tolerated.

Several larger-scale clinical trials are currently attempting to reproduce these effects for the treatment of cachexia, including that associated with CHF, cancer, COPD, and ESRD. Long-term, large-scale trials are eagerly awaited to determine if ghrelin is an effective therapy for cachexia.

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Angiogenic and Vasoprotective Effects of Adrenomedullin on Prevention of Cognitive Decline After Chronic Cerebral Hypoperfusion in Mice

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Angiogenic and Vasoprotective Effects of Adrenomedullin on Prevention of Cognitive Decline After Chronic Cerebral Hypoperfusion in Mice

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Background and Purpose—Although subcortical vascular dementia, the major subtype of vascular dementia, is caused by a disruption in white matter integrity after cerebrovascular insufficiency, no therapy has been discovered that will restore cerebral perfusion or functional cerebral vessels. Because adrenomedullin (AM) has been shown to be angiogenic and vasoprotective, the purpose of the study was to investigate whether AM may be used as a putative treatment for subcortical vascular dementia.

Methods—A model of subcortical vascular dementia was reproduced in mice by placing microcoils bilaterally on the common carotid arteries. Using mice overexpressing circulating AM, we assessed the effect of AM on cerebral perfusion, cerebral angioarchitecture, oxidative stress, white matter change, cognitive function, and brain levels of cAMP, vascular endothelial growth factor, and basic fibroblast growth factor.

Results—After bilateral common carotid artery stenosis, mice overexpressing circulating AM showed significantly faster cerebral perfusion recovery due to substantial growth of the capillaries, the circle of Willis, and the leptomeningeal anastomoses and reduced oxidative damage in vascular endothelial cells compared with wild-type mice. Vascular changes were preceded by upregulation of cAMP, vascular endothelial growth factor, and basic fibroblast growth factor. White matter damage and working memory deficits induced by bilateral common carotid artery stenosis were subsequently restored in mice overexpressing circulating AM.

Conclusions—These data indicate that AM promotes arteriogenesis and angiogenesis, inhibits oxidative stress, preserves white matter integrity, and prevents cognitive decline after chronic cerebral hypoperfusion. Thus, AM may serve as a strategy to tackle subcortical vascular dementia. **(*Stroke*. 2011;42:1122-1128.)**

Key Words: angiogenesis ■ arteriogenesis ■ adrenomedullin ■ vascular dementia

Ischemic white matter (WM) lesions, which are most likely caused by cerebrovascular insufficiency after atherosclerosis and/or arteriosclerosis, are an established marker of risk for cognitive deterioration.[1] Thus, therapeutic vascular growth and vasoprotection, resulting in the preservation of WM integrity, may serve to maintain cognitive function in subjects at risk of developing dementia.

Adrenomedullin (AM) has a variety of effects on the vasculature that include vasodilation, regulation of permeability, inhibition of endothelial cell apoptosis and oxidative stress, regulation of smooth muscle cell proliferation, and promotion of angiogenesis.[2,3]

Thus, the purpose of this study was to investigate the mechanisms and therapeutic potential of AM-induced neovascularization and/or vasoprotection after chronic cerebral hypoperfusion in a mouse model of subcortical vascular dementia.[4,5]

Materials and Methods

An expanded Methods section is available in the Online Data Supplement (http://stroke.ahajournals.org).

Results

Adrenomedullin Facilitates Recovery of Cerebral Blood Flow After Placing Microcoils Bilaterally on the Common Carotid Arteries

Immediately after bilateral common carotid artery stenosis (BCAS), cerebral blood flow (CBF) decreased to the lowest

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The online-only Data Supplement is available at http://stroke.ahajournals.org/cgi/content/full/STROKEAHA.110.603399/DC1.

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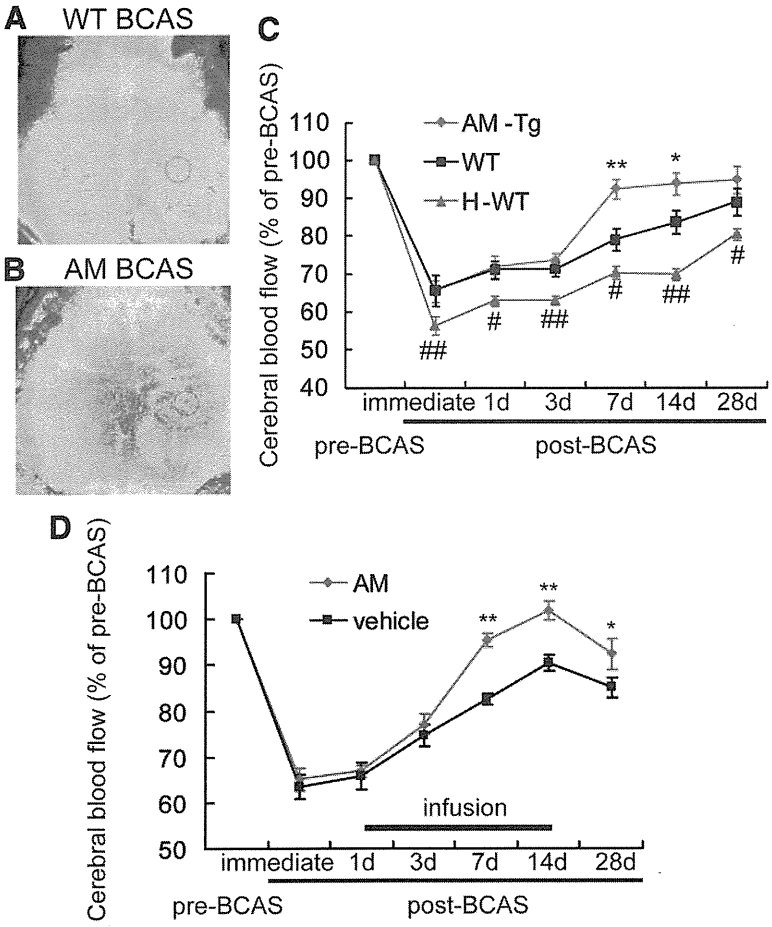

Figure 1. Adrenomedullin (AM) facilitates recovery of cerebral blood flow after the bilateral common carotid artery stenosis (BCAS). A–B, Representative images of CBF in wild-type or AM-Tg mouse subjected to BCAS operation (WT BCAS or AM BCAS) on Day 7. C, Temporal profile of CBF after BCAS in wild-type (WT; n=16 on Days 0 to 14, n=8 on Day 28), AM-Tg (n=16 on Days 0 to 14, n=8 on Day 28), and hydralazine-treated WT (H-WT; n=8 on Days 0 to 14, n=4 on Day 28) mice, in which the pre-BCAS value of CBF is adjusted to 100. Values are expressed as means±SEM. *$P<0.05$, **$P<0.01$ in AM-Tg vs WT; #$P<0.05$, ##$P<0.01$ in WT vs H-WT. D, Temporal profile of CBF after BCAS in AM-treated WT mice (n=11) and vehicle-treated WT mice (n=10). Values are expressed as means±SEM *$P<0.05$, **$P<0.01$. CBF indicates cerebral blood flow; AM-Tg, mice overexpressing circulating AM.

values but thereafter began to recover in all groups. On Days 1 and 3 post-BCAS, there was a slight, but not significant, increase in CBF (average±SEM) in mice overexpressing circulating AM (AM-Tg) compared with wild-type (WT) mice. On Day 7 post-BCAS, AM-Tg mice showed significantly faster CBF recovery: CBF was significantly higher in AM-Tg mice (93%±2%) compared with WT mice (79%±2%) and hydralazine-treated WT mice (71%±2%; Figure 1A–C). This trend continued on Days 14 and 28 post-BCAS (Figure 1C; Supplemental Figure IA). Slower CBF recovery in hydralazine-treated WT mice suggests that AM-induced CBF recovery may not be associated with the hypotensive effect of AM.

We further examined the effects of postoperative exogenous infusion of AM.[6,7] Continuous intraperitoneal injection of recombinant human AM at a rate of 50 ng/hr for 2 weeks, beginning on Day 1 post-BCAS, resulted in a significantly faster CBF recovery compared with the vehicle-treated mice (Figure 1D). These effects were comparable to those seen in BCAS-operated AM-Tg mice.

Thus, both genetically overproduced AM and postoperative exogenous administration of AM facilitated recovery of CBF after BCAS.

Adrenomedullin Enhances Arteriogenesis After BCAS

At the dorsal surface of the brain, a significant increase in diameter of the leptomeningeal anastomoses was found in AM-Tg mice (28.7±1.6 μm) compared with WT mice (22.4±1.3 μm) on Day 7 post-BCAS (Figure 2A–C; Supplemental Figure IB, a–d). The number of leptomeningeal anastomoses was not different among the 4 groups. The diameter of the internal carotid artery, anterior cerebral artery, middle cerebral artery, and posterior communicating artery was significantly enlarged at the level of the circle of Willis in AM-Tg mice compared with WT mice (AM-Tg versus WT; anterior cerebral artery, 193±26 versus 161±24 μm; middle cerebral artery, 184±24 versus 153±13 μm; internal carotid artery, 206±30 versus 175±25 μm; posterior communicating artery, 191±16 versus 150±15 μm) on Day 7 post-BCAS (Figure 2D; Supplemental Figure IB, e–h).

To evaluate monocyte recruitments and proliferation of smooth muscle cells, both of which are essential in arteriogenesis, the immunofluorescent analysis of Ki-67 and F4/80, together with α-smooth muscle actin, was performed. BCAS-operated AM-Tg mice showed a significant increase in Ki-67-positive vascular smooth muscle cells compared with BCAS-operated WT mice (Supplemental Figure IC). In addition, a significant increase in vascular smooth muscle cells surrounded by F4/80-positive monocyte/macrophages was found in BCAS-operated AM-Tg mice compared with sham-operated WT mice (Supplemental Figure ID).

Adrenomedullin Enhances Angiogenesis After BCAS

A significant increase in platelet-endothelial cell adhesion molecule-1-positive capillary density of the cortex, corpus cal-

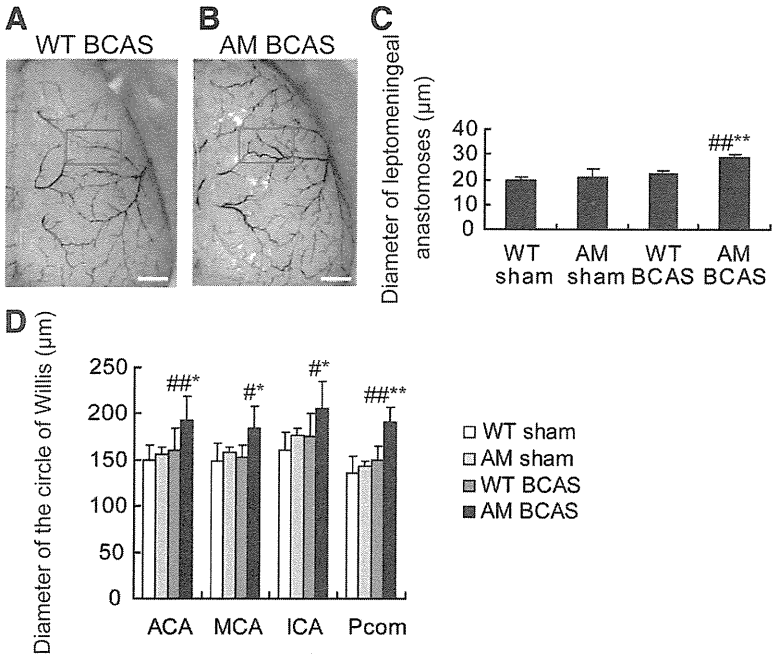

Figure 2. Adrenomedullin (AM) enhances arteriogenesis after BCAS. A–B, Representative images of the dorsal cerebral angioarchitecture by postmortem latex perfusion method of wild-type (WT) or AM-Tg mouse subjected to BCAS operation (WT BCAS or AM BCAS) on day 7. Scale bars, 1 mm. C–D, Histogram showing the diameter of the leptomeningeal anastomoses (C) and the anterior, middle, and posterior cerebral arteries (ACA, MCA, and PCA, respectively) and the posterior communicating artery (Pcom; D) of WT or AM-Tg mouse subjected to sham or BCAS operation on Day 7 (WT sham, n=4; AM sham, n=4; WT BCAS, n=6; AM BCAS, n=6). Error bars indicate SD. *P<0.05, **P<0.01 in AM BCAS vs WT BCAS; #P<0.05, ##P<0.01 vs WT sham. BCAS indicates bilateral common carotid artery stenosis; AM-Tg, mice overexpressing circulating AM.

losum, and caudoputamen was found in AM-Tg mice compared with WT mice (AM-Tg versus WT; cortex, 540±55/mm^2 versus 473±38/mm^2; corpus callosum, 273±7/mm^2 versus 213±18/mm^2; caudoputamen, 499±36/mm^2 versus 455±26/mm^2) on Day 7 post-BCAS. There was no significant difference in capillary density between AM-Tg and WT mice after sham operation (Figure 3A–C; Supplemental Figure IE).

Taken together, these results suggest that both chronic ischemic stress and AM overexpression are required to induce arteriogenesis and angiogenesis in the brain.

Adrenomedullin Attenuates Oxidative Damage in Cerebral Microvessels After BCAS

To evaluate oxidative damage in cerebral microvessels, double immunofluorescence staining for platelet-endothelial cell adhesion molecule-1 and 8-hydroxy-deoxyguanosine was performed on Day 3 post-BCAS. A significant decrease in oxidative damage in the cerebral microvessels was found in BCAS-operated AM-Tg mice compared with BCAS-operated WT mice (Figure 3D; Supplemental Figure IF).

Adrenomedullin Preserves WM Integrity After BCAS

BCAS-operated WT mice showed an increased density of glial fibrillary acidic protein-positive astrocytes and ionized calcium binding adapter molecule 1-positive microglia and a decreased density of glutathione-S-transferase-pi-immunoreactive mature oligodendrocytes in the corpus callosum and the anterior commissure compared with sham-operated WT or AM-Tg

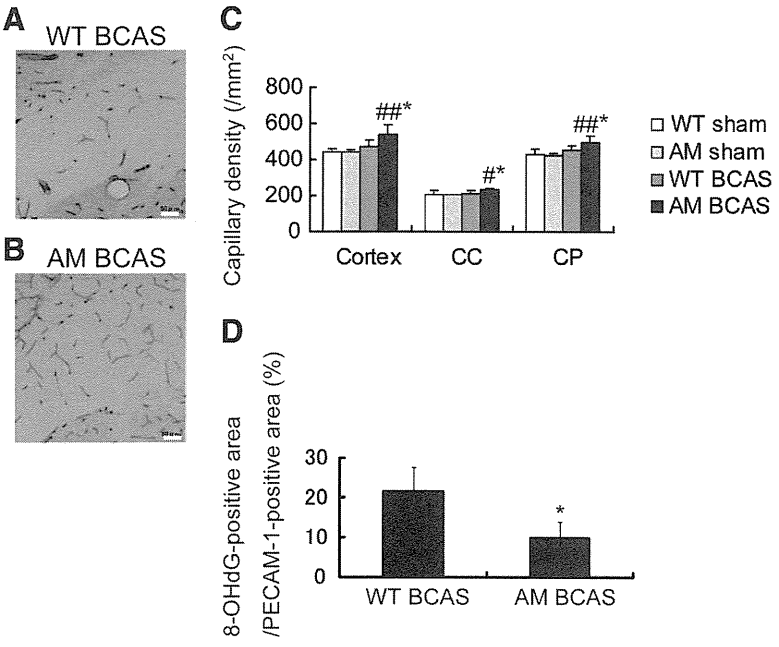

Figure 3. Adrenomedullin (AM) enhances angiogenesis and attenuates oxidative damage in brain microvessels after BCAS. A–B, Representative images of PECAM-1-positive capillaries in corpus callosum sections of a wild-type (WT) or AM-Tg mouse subjected to BCAS operation (WT BCAS or AM BCAS) on Day 7. Scale bar, 50 μm. C, Histogram showing capillary densities of the cortex, corpus callosum (CC), and caudoputamen (CP) of WT or AM-Tg mouse that is subjected to sham or BCAS operation on Day 7 (WT sham, n=4; AM sham, n=4; WT BCAS, n=6; AM BCAS, n=6). Error bars indicate SD. *P<0.05 in AM BCAS vs WT BCAS; #P<0.05, ##P<0.01 vs WT sham. D, To evaluate oxidative damage in cerebral microvessels, double immunofluorescence staining for PECAM-1 and 8-hydroxy-deoxyguanosine (8-OHdG) was performed. Histogram showing oxidative damage in microvessels of the corpus callosum of WT BCAS or AM BCAS (n=4 each). Error bars indicate SD. *P<0.05 vs WT BCAS. BCAS indicates bilateral common carotid artery stenosis; PECAM-1, platelet-endothelial cell adhesion molecule-1; AM-Tg, mice overexpressing circulating AM.

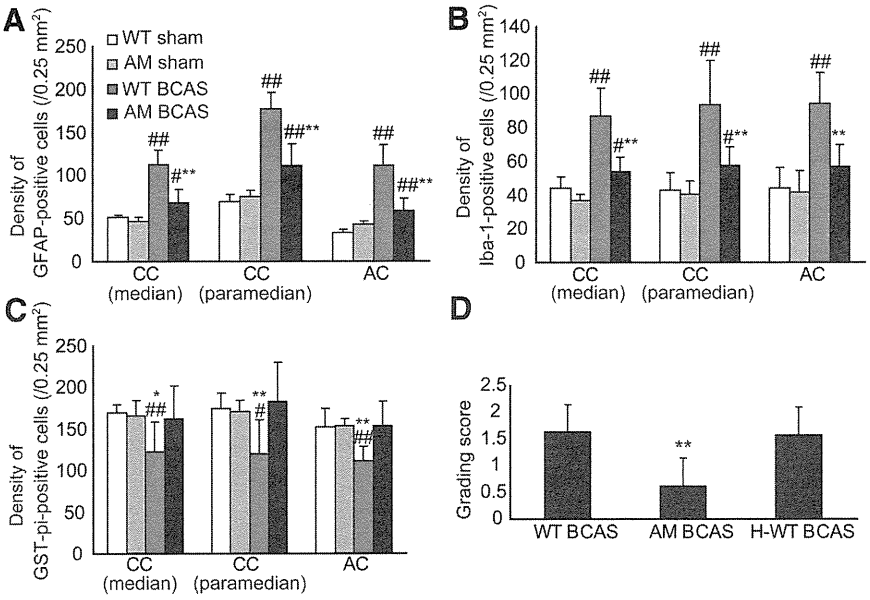

Figure 4. Adrenomedullin (AM) restores white matter integrity after BCAS. Histograms showing the density of cells immunoreactive for GFAP (A), Iba-1 (B), or GST-pi (C) in the medial and paramedial portions of the corpus callosum (CC) and the anterior commissure (AC) of a wild-type (WT) or AM-Tg mouse subjected to sham or BCAS operation on Day 28 (WT sham, n=4; AM sham, n=4; WT BCAS, n=8; AM BCAS, n=8). Error bars indicate SD. *$P<0.05$, **$P<0.01$ in AM BCAS vs WT BCAS; #$P<0.05$, ##$P<0.01$ vs WT sham. D, Histograms showing grading score of the CC of WT BCAS (n=14), AM BCAS (n=8), and hydralazine-treated WT BCAS (H-WT BCAS; n=7) on Day 28 post-BCAS. Error bars indicate SD. **$P<0.01$ in AM BCAS vs WT BCAS and H-WT BCAS. BCAS indicates bilateral common carotid artery stenosis; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium binding adapter molecule 1; GST-pi, glutathione-S-transferase-pi; AM-Tg, mice overexpressing circulating AM.

mice on Day 28 (Figure 4A–C; Supplemental Figure IIA, a–c, e–g, and i–k). In BCAS-operated AM-Tg mice, by contrast, the density of astrocytes and microglia significantly decreased and that of mature oligodendrocytes significantly increased compared with BCAS-operated WT mice (Figure 4A–C; Supplement Figure IIA, d, h, and l). Similarly, BCAS-operated AM-Tg mice on Day 7 showed significantly decreased density of microglia and increased density of mature oligodendrocytes but no difference in the density of astrocytes compared with BCAS-operated WT mice (Supplemental Figure IIB–D).

Klüver–Barrera staining on Day 28 revealed that BCAS-induced WM lesions were predominant in the corpus callosum and the caudoputamen in WT mice (Supplemental Figure IIA, m–o). In AM-Tg mice, such WM lesions became far less severe (Supplemental Figure IIAp). In the hydralazine-treated WT mice, BCAS-induced WM lesions were as severe as those in WT mice, suggesting that such positive effects of AM may be independent of the hypotensive effect of AM (Figure 4D).

Thus, BCAS-induced WM lesions were restored in the AM-Tg mice in parallel with inhibition of glial activation and preservation of mature oligodendrocytes.

Adrenomedullin Prevents Working Memory Deficits After BCAS

To evaluate working memory, we examined a Y maze test and an 8-arm radial maze test. The Y maze test was performed 1 month after the surgery. Alternations of entries in the arms of the Y maze were significantly increased in BCAS-operated AM-Tg mice (64.6%±7.5%) compared with BCAS-operated WT mice (58.2%±8.0%), although alternations of entries were not significantly different between WT and AM-Tg mice after sham operation (Figure 5A). Spontaneous activity was not significantly different among the 4 groups of mice (Figure 5B). In another set of mice, the 8-arm

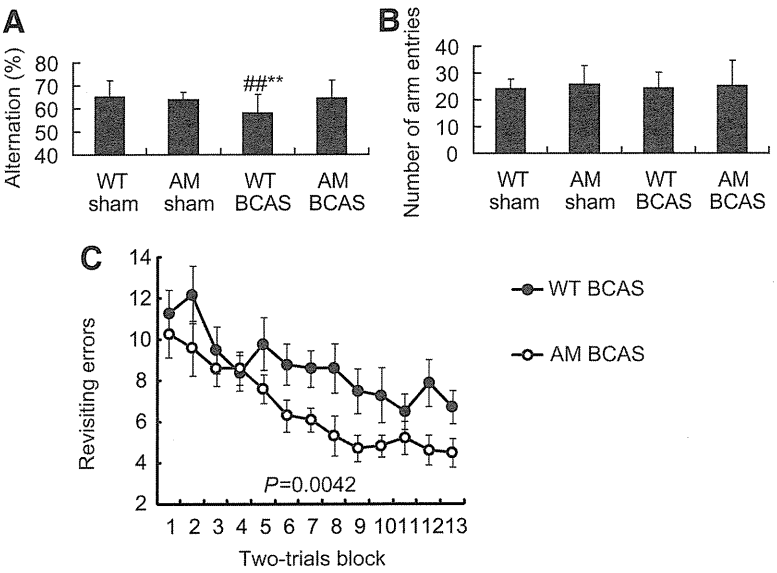

Figure 5. Adrenomedullin (AM) prevents working memory deficits after BCAS. A, Histogram showing spontaneous alternation in the Y maze test of wild-type (WT) or AM-Tg mice at 1 month after sham or BCAS operation (WT sham, n=17; AM sham, n=10; WT BCAS, n=26; AM BCAS, n=17). Error bars indicate SD. **$P<0.01$ in AM BCAS vs WT BCAS; ##$P<0.01$ vs WT sham. B, Histogram showing spontaneous activity in the Y maze test. Error bars indicate SD. C, The number of revisiting errors in the 8-arm radial maze test at 1 month after BCAS of WT or AM-Tg mouse (WT BCAS, n=19; AM BCAS, n=17). Values are expressed as means±SEM. BCAS indicates bilateral common carotid artery stenosis; AM-Tg, mice overexpressing circulating AM; AM, adrenomedullin.

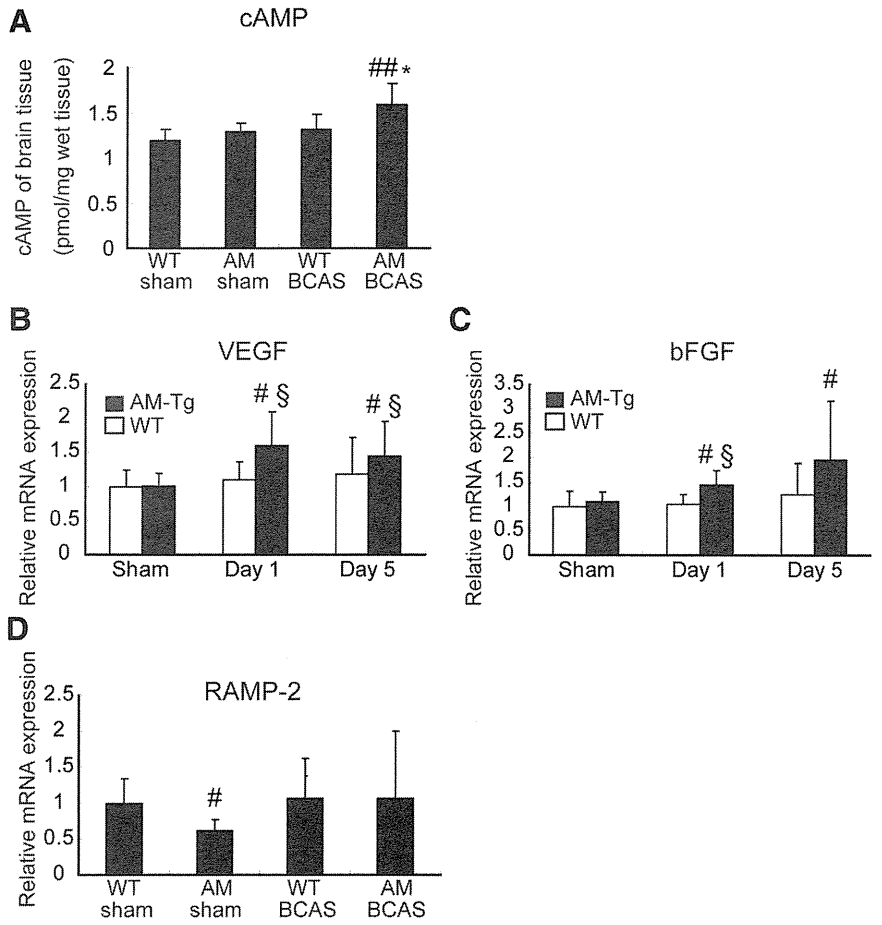

Figure 6. Adrenomedullin (AM) increases brain cAMP, VEGF, and bFGF levels after BCAS. A, Histogram showing brain cAMP levels in wild-type (WT) or AM-Tg mice on Day 5 after sham or BCAS operation (WT sham, n=4; AM sham, n=5; WT BCAS, n=5; AM BCAS, n=5). Error bars indicate SD. *P<0.05 in AM BCAS vs WT BCAS; ##P<0.01 vs WT sham. B–C, Histogram showing mRNA levels of VEGF (B) and bFGF (C) in the brains of WT or AM-Tg mice on Days 1 and 5 (n=5 to 7 each). Error bars indicate SD. #P<0.05 vs WT sham; §P<0.05 in AM sham vs AM BCAS. D, Histograms showing mRNA levels of AM receptor, RAMP2, in the brains of WT or AM-Tg mice subjected to sham or BCAS operation (WT sham, n=7; AM sham, n=5; WT BCAS, n=7; AM BCAS, n=7) on Day 5. The mRNA levels are presented as ratios relative to levels of 18S rRNA. Error bars indicate SD. #P<0.05 vs WT sham. VEGF indicates vascular endothelial growth factor; bFGF, basic fibroblast growth factor; BCAS, bilateral common carotid artery stenosis; AM-Tg, mice overexpressing circulating AM; AM, adrenomedullin; RAMP2, receptor activity-modifying protein-2.

radial maze test was started 1 month after BCAS. BCAS-operated AM-Tg mice showed a significant reduction in the number of revisiting errors compared with BCAS-operated WT mice (Figure 5C). We have found a significant correlation of the averaged number of revisiting errors 1 month after BCAS with CBF on Days 7, 14, and 28, but not with CBF immediately after BCAS, or on Days 1 and 3 (Supplemental Figure III).

Taken together, these results suggest that AM restores working memory deficits induced by BCAS.

Adrenomedullin Increases cAMP Level in the Forebrain After BCAS

The restorative effect of AM described led to the investigation of the underlying mechanisms behind angio-/arteriogenesis and antioxidative activity. The brain level of cAMP, a second messenger known to associate with AM, was therefore measured. A significant increase in the brain level of cAMP was found in AM-Tg mice after BCAS (AM BCAS, 1.6±0.2 pmol/mg wet tissue) compared with WT mice after BCAS (WT BCAS, 1.3±0.2 pmol/mg wet tissue) on Day 5, although the level of cAMP was not different between WT and AM-Tg mice after sham operation (Figure 6A).

These results suggest that chronic ischemic stress induced AM-mediated elevation of cAMP in the brain.

Adrenomedullin Increases mRNA and Protein Levels of Vascular Endothelial Growth Factor and Basic Fibroblast Growth Factor in the Forebrain After BCAS

The reasons behind the apparent AM-initiated signaling pathway-led arteriogenesis and angiogenesis were next examined. Brain levels of vascular growth factors, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), were therefore measured. The mRNA and protein levels of brain VEGF and bFGF were significantly increased in AM-Tg mice on Days 1 and 5 post-BCAS compared with sham-operated WT mice (Figure 6B–C; Supplemental Figure IV).

Chronic Ischemic Insult Upregulates Brain mRNA Level of Adrenomedullin and Abolishes Receptor Activity-Modifying Protein-2 Suppression Induced by Adrenomedullin

The status of mouse AM, high-affinity AM receptors, calcitonin receptor-like receptors, and Subtypes 2 and 3 of a family of receptor activity-modifying proteins (RAMP2 or 3) were then measured on Day 5. The brain mRNA level of mouse AM was significantly increased (3.1-fold) in BCAS-operated WT mice compared with sham-operated WT mice. In addition, brain RAMP2 mRNA level was significantly lower (0.6-fold) in sham-operated AM-Tg mice compared with sham-operated WT mice. Such downregulation of RAMP2 mRNA

was abolished after the AM mice were subjected to BCAS operation, suggesting that feedback inhibition is a plausible cause for the downregulation (Figure 6D).

Discussion

Three major conclusions may be drawn from the present study. First, it was demonstrated that increased levels of circulating AM restored cerebral hemodynamics, promoted arteriogenesis as well as angiogenesis, alleviated oxidative damage in the cerebral microvessels, and preserved WM integrity; this subsequently attenuated working memory deficits in a mouse model of chronic cerebral hypoperfusion. Second, AM selectively upregulated brain levels of cAMP, VEGF, and bFGF in the hypoperfused brain but not in the normoperfused brain. Finally, it was found that such proangiogenic/arteriogenic changes did not occur in sham-operated AM-Tg mice in which the expression of AM receptor component RAMP2 was significantly suppressed, possibly through feedback inhibition.

We have found a significant correlation in the averaged number of revisiting errors at 1 month after BCAS with CBF on Days 7, 14, and 28, but not with CBF immediately after BCAS or on Days 1 and 3. Therefore, the recovery of CBF is one of the substrates for the functional improvements, whereas several phenomena other than CBF recovery may play roles in the pathophysiology of this BCAS model. In fact, we demonstrated that AM induced not only CBF recovery as a result of arteriogenesis, but also angiogenesis (not associated with CBF recovery), antioxidative activity in the microvessels, and attenuation of microglial inflammatory responses. The other effects of AM, including antiapoptotic effects and regulation of endothelial permeability or the blood–brain barrier,[3] need further investigation. Positive effects of AM may be mediated by multiple pathways.

Previous reports showed that the AM/cAMP/PKA cascade blocks oxidative damage in ischemic injury[8] and promotes angiogenic effects of the endothelial cells in vitro.[9] We found that chronic ischemic insult and circulating AM are both required to raise cAMP levels in the brain; this may be associated with alleviating oxidative damage and promoting angiogenesis.

The elevation of VEGF is consistent with the previous report that AM administration upregulates the expression of VEGF in both in vitro and in vivo hindlimb ischemia models.[6] Although no previous studies have reported that AM enhances the expression of bFGF after ischemia, we demonstrated the AM-induced upregulation of bFGF after BCAS in vivo. AM was also found to upregulate bFGF as well as VEGF in the cultured endothelial cells (unpublished data). Previous reports have demonstrated that combined gene delivery of VEGF and bFGF produces additive or synergistic effects on angiogenesis or collateral development, probably due to the protective effects of bFGF against VEGF-induced fluid leakage.[10] Thus, AM-induced elevation of bFGF may be associated with the development of functional vessels.

AM acts through 2 subtypes of receptor (AM1 and AM2), which derive from the interaction of the calcitonin receptor-like receptors with RAMP2 or 3.[11] Interestingly, RAMP2 mRNA level in sham-operated AM-Tg mice was significantly decreased compared with sham-operated WT mice but nearly reached normalization after BCAS. This may explain why arteriogenesis and angiogenesis were significantly promoted in AM-Tg mice only after ischemic insult. Shindo et al have reported that RAMP2 rather than RAMP3 is a key determinant of the effects of AM on the vasculature and is essential for angiogenesis and vascular integrity.[11] These results suggest that the AM-initiated signaling pathway is suppressed by downregulation of RAMP2 in the normoperfused brain but that such suppression is abolished by chronic ischemic stress, leading to AM-induced arteriogenesis and angiogenesis. Such tissue selectivity could be an advantage for clinical application of AM in patients with subcortical vascular dementia.

Recently, the concept of an "oligovascular niche" has been proposed, in which crosstalk between endothelial cells and oligodendrocytes, mediated by an exchange of soluble signals such as fibroblast growth factor, are thought to play an important role in sustaining oligodendrocyte homeostasis and WM integrity.[12] Because cerebral endothelial cells contribute to numerous signaling cascades that help regulate brain homeostasis and function,[13] angio-/arteriogenesis and inhibition of oxidative damage in the cerebral endothelial cells induced by AM might lead to oligovascular protection—namely, successful vascular growth and vasoprotection and preservation of white matter/oligodendrocyte integrity—and prevention of cognitive decline after chronic cerebral hypoperfusion in mice.

In conclusion, this study demonstrates that circulating AM is a highly potent and effective modality for restoring perfusion, promoting arteriogenesis and angiogenesis in the chronically ischemic brain, inhibiting oxidative damage in the cerebral microvessels, preserving ischemic WM integrity, and attenuating working memory deficits in a mouse model of subcortical vascular dementia. Future clinical studies are required to evaluate and confirm the efficacy of AM in chronic cerebral vascular diseases, especially subcortical vascular dementia.

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Disclosures

None.

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ONLINE SUPPLEMENT

Supplemental Methods

Mice

A generation of transgenic mice overexpressing human adrenomedullin (AM-Tg mice) has been described in a previous study.[1] Because the product is secreted from the liver, it mimics intravenous administration of the agent. All procedures were performed according to the guidelines of the Animal Use and Care Committee of Kyoto University. The adrenomedullin (AM) gene contains coding regions for not only AM but also proadrenomedullin N-terminal 20 peptide (PAMP), a different vasodilating peptide. Amidation at their carboxyl terminals after their synthesis is needed for both AM and PAMP to exert their biological activity. The bioactive amidated forms are known as mature AM and mature PAMP, respectively. To identify the specific effects of AM, we generated a transgene construct with a point mutation on the PAMP amidation signal in the full-length AM gene cDNA. Guanine was substituted for cytosine on the 3' end of the PAMP coding region so that glycine on the C terminal of the PAMP product was replaced with alanine. In this way, amidation and maturation of PAMP by peptidylglycine α-hydroxylase and α-hydroxyglycine N-C lyase were inhibited. The mutant AM gene cDNA was then inserted into a plasmid containing the human serum amyloid P component promoter, which is widely used to target gene expression specific to the liver. When the product is secreted from the liver, it mimics intravenous administration of the agent. The *Hin*dIII-*Xho*I fragment of the plasmid was microinjected into the pronucleus of fertilized C57BL/6J mice eggs. Comparisons were then performed between AM-Tg mice and wild-type (WT) littermates. Plasma concentrations of human total AM were measured with a commercially available immunoradiometric assay (Shionogi). Blood pressure (BP) was measured by the tail cuff method (Softron). The mice were housed in a room with a 12-hour light/dark cycle (lights on at 7:00 a.m.) with access to food and water *ad libitum*.

Induction of chronic cerebral hypoperfusion

Adult C57BL/6J male mice (10–12 weeks old, 22–29 g; Shizuoka Laboratory Animal Center) were subjected to bilateral common carotid artery stenosis (BCAS) using microcoils with an internal diameter of 0.18 mm, as previously described.[2-4] Sham-operated mice underwent the same surgical procedure without using microcoils. Anesthesia was induced with 4% halothane and maintained with 1.5% halothane in 80% nitrous oxide and 20% oxygen. Rectal temperature was maintained between 36.5°C and 37.5°C.

Exogenous administration of hydralazine

To **evaluate** the possibility that the lower BP observed in AM-Tg mice caused beneficial effects on BCAS, we further analyzed BP-matched mice by administration of low-dose hydralazine (0.1 mmol/L) in drinking water.[1]

Exogenous administration of AM

Recombinant human mature AM dissolved in 0.9% saline was exogenously administrated to C57BL/6J WT mice by means of osmotic pumps (Alzet Model 1002) as previously described.[1, 5] The AM groups received a continuous intraperitoneal injection of recombinant human AM at a rate of 50 ng/h for 2 weeks beginning on day 1 post-BCAS. The vehicle groups received 0.9% saline only.

Measurement of cerebral blood flow

The regions of interest corresponded with the regions around Heubner's anastomoses connecting the dorsal branches of the anterior cerebral artery (ACA) and the middle cerebral artery (MCA). The baseline cerebral blood flow (CBF) recordings were obtained immediately before and after the operation, as well as 1, 3, 7, 14, and 28 days after surgery. The CBF values were averaged between bilateral sides and expressed as a percentage of the baseline value. Changes in cerebral surface blood