

CTRP3/cartducin promotes proliferation and migration of endothelial cells

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Abstract CTRP3/cartducin, a novel secretory protein, is a member of the C1q and tumor necrosis factor (TNF)-related protein (CTRP) superfamily. CTRP3/cartducin gene is transiently up-regulated in a balloon-injured rat carotid artery tissue. In this study, we report a new function of CTRP3/cartducin as a regulator of angiogenic processes. CTRP3/cartducin promoted proliferation and migration of mouse endothelial MSS31 cells in a dose-dependent manner. Further, stimulation of MSS31 by CTRP3/cartducin led to activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK). MAPK/ERK kinase 1/2 (MEK1/2) inhibitor, U0126, and p38 MAPK inhibitor, SB203580, blocked the CTRP3/cartducin-induced cell proliferation, and migration was blocked by U0126, but not the SB203580. Taken together, these results suggest that CTRP3/cartducin may be involved as a novel angiogenic factor in the formation of neointima following angioplasty.

Keywords CTRP3/cartducin · Endothelial cells · Proliferation · Migration · MAPK

Introduction

A new highly conserved family of adiponectin paralogs have been designated C1q and tumor necrosis factor

(TNF)-related proteins (CTRPs). There are seven members in the CTRP family and the proteins in this family exhibit similar structural organization to adiponectin and consist of four distinct domains including a N-terminal signal peptide, a short variable domain, a collagen-like domain, a C-terminal C1q-like globular domain [1, 2]. While structurally related, members of this protein family are functionally diverse. CTRP2 is known to induce the phosphorylation of AMP-activated protein kinase, resulting in increased glycogen accumulation, and fatty acid oxidation [2]. CTRP1 has been reported to be expressed in the vascular wall tissues and to inhibit collagen-induced platelet aggregation by blocking von Willebrand factor binding to collagen [3]. CTRP5 is localized to the lateral and apical membranes of the retinal pigment epithelium and ciliary body, and mutation in CTRP5 gene is associated with late-onset retinal degeneration [4].

CTRP3/cartducin is a growth plate cartilage-derived secretory protein first identified during a search for the genes underlying the induction of chondrocyte differentiation. We showed that this molecule is a novel growth factor that plays important roles in regulating both chondrogenesis and cartilage development [5–7]. Recently, Li et al. [8] examined the temporal patterns of gene expression in a balloon-injured rat carotid artery model using microarray analysis coupled with real time RT-PCR and found transient up-regulation of CTRP3/cartducin gene in injured artery tissue during a period characterized by neointima formation. Neointima formation consists of multiple processes, including not only vascular smooth muscle cell proliferation, but also angiogenesis, vascular remodeling and extracellular matrix deposition [9]. Increased neovascularization has been observed at sites of intimal hyperplasia in models of arterial stenting and angioplasty [10]. In pig coronary arteries, adventitial

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neovascularization correlated with vessel stenosis after balloon injury and stent implantation, and vascular endothelial growth factor expression was reported after stenting [11]. The angiogenic process requires proliferation and migration of endothelial cells, and degradation of the extracellular matrix by these cells under the regulation of several angiogenic factors and angiogenesis inhibitors [12].

In the present study, we hypothesized that CTRP3/cartducin had direct effects on endothelial cells, which would enhance neointima formation after angioplasty. We also examined the possible signaling pathways involved in CTRP3/cartducin action on endothelial cells.

Materials and methods

Reagents

Mouse recombinant CTRP3/cartducin was prepared as described [6]. Anti-ERK1/2, anti-p38 MAPK, and anti-beta-actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Anti-phospho-ERK1/2 (p-ERK1/2), anti-phospho-p38 MAPK (p-p38 MAPK), and anti-phospho-MAPK-activating protein kinase-2 (MAPKAPK-2) (p-MAPKAPK-2) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). MEK1/2 inhibitor U0126 and p38 MAPK inhibitor SB203580 were purchased from Calbiochem (San Diego, CA). Recombinant human fibroblast growth factor-basic (bFGF) was obtained from Sigma-Aldrich.

Cell lines and cell culture

The mouse endothelial cell line MSS31 was first established from newborn mouse spleens [13]. It was kindly provided by Dr. Yasufumi Sato (Tohoku University) and cultured in alpha-minimal essential medium (α -MEM) (Sigma-Aldrich) supplemented with 10% FCS (PAA Laboratories, Linz, Austria) at 37°C in a humidified atmosphere containing 5% CO₂. To investigate the effects of cartducin on the MAPK signaling pathways, the cells were seeded at a density of 1×10^4 cells/well in 24-well plates and grown for 48 h. The cells were then washed and cultured for 24 h in the medium without serum. Subsequently, 5 μ g/ml of CTRP3/cartducin was added to the medium for 5, 15, 30, and 60 min. For experiments with protein kinase inhibitors, cells were pretreated with specific inhibitors for 1 h prior to CTRP3/cartducin treatment. In the control experiments, 50 mM NaH₂PO₄ (pH 8.0) containing 1 mM EDTA and/or 0.4% dimethyl sulfoxide (DMSO) was added to the culture.

Measurement of DNA synthesis and cell number

To determine the growth-stimulatory effect of CTRP3/cartducin on MSS31 cells, bromodeoxyuridine (BrdU) assay was performed as described previously [6]. In brief, MSS31 cells were seeded at a density of 1×10^4 cells/well in 96-well plates and grown for 24 h. The medium was then replaced with α -MEM containing 0.1% FCS for 24 h. Subsequently, 5 μ g/ml of CTRP3/cartducin was added to the medium, incubated for 24 h, and labeled with BrdU during the last 3 h of incubation. The cell number was measured as described previously [6]. Briefly, MSS31 cells maintained in α -MEM containing 5% FCS were treated with 5 μ g/ml of CTRP3/cartducin for three days.

Measurement of cell migration

The motility response of MSS31 cells to CTRP3/cartducin was assayed by using a modified Boyden chamber technique. MSS31 cells were trypsinized, washed and resuspended in serum-free α -MEM containing 0.25% bovine serum albumin (BSA). The cell suspension (100 μ l, 5×10^4 cells/well) was added to the transwell inserts (8.0- μ m pore size, Corning, Inc., Corning, NY) and the insert was then incubated for 2 h to allow cell attachment. Then, 500 μ l of serum-free α -MEM containing 0.25% BSA with or without CTRP3/cartducin (5 μ g/ml) was added to the lower chamber and incubated for 5 h. Non-migrating cells on the top of the membrane were removed by scraping and migrated cells on the lower surface of the membrane were fixed with ethanol and stained with hematoxylin. The number of cells migrating through the membrane was counted in five random fields per well under a microscope ($\times 400$ magnification). All assays were performed in triplicate.

Western blot analysis

Protein immunoblotting was performed as previously described [7]. Briefly, total cellular proteins were prepared by lysing cells in CellLytic lysis buffer (Sigma-Aldrich) containing protease inhibitor (Sigma-Aldrich) and phosphatase inhibitor (Sigma-Aldrich) cocktails, separated by SDS-PAGE, and transferred to PVDF membranes. The membranes were blocked for 30 min at room temperature and then incubated with first antibodies directed against ERK1/2, p-ERK1/2, p38 MAPK, p-p38 MAPK, MAPKAPK-2, and beta-actin for 18 h at 4°C. The detection of bound antibodies was performed by the WesternBreeze Chromogenic Detection System (Invitrogen, Carlsbad, CA) using alkaline phosphatase (AP)-conjugated donkey anti-rabbit IgG antibody (Promega, Madison WI).

Statistical analysis

The unpaired Student's *t*-test was used for statistical analysis of the experiments. Error bars represent SD, and $P < 0.05$ was taken as the level of significance.

Results

CTRP3/cartducin induces proliferation of MSS31 cells

To investigate whether CTRP3/cartducin has the ability as an angiogenic stimulus, we first examined the effect of CTRP3/cartducin on MSS31 cell proliferation. A dose-dependent increase in BrdU incorporation into the DNA was observed in MSS31 cells. A maximum of over 4-fold stimulation in DNA synthesis occurred in the presence of 5 $\mu\text{g/ml}$ of CTRP3/cartducin (Fig. 1A). We also compared the effect of CTRP3/cartducin with that of bFGF (15 ng/ml) which is known to stimulate MSS31 cell proliferation [14], and found that the effect of CTRP3/cartducin on endothelial cell proliferation was less potent than that of bFGF (Fig. 1A). We next determined the effect of CTRP3/cartducin on the cell number of MSS31 cells. CTRP3/cartducin significantly increased the number of MSS31 cells to about 1.6-fold that of the control (Fig. 1B).

CTRP3/cartducin induces migration of MSS31 cells

Since endothelial proliferation and migration are critical steps in angiogenesis, we next examined whether CTRP3/cartducin could affect endothelial cell migration. CTRP3/cartducin significantly increased MSS31 cell migration in a dose-dependent manner. A maximum of over 2-fold stimulation in migration occurred in the presence of 5 $\mu\text{g/ml}$ of CTRP3/cartducin. We also compared the effect of CTRP3/cartducin with that of bFGF (15 ng/ml) which is known to stimulate MSS31 cell migration [14], and found that this effect of CTRP3/cartducin was more potent than that of bFGF for MSS31 migration (Fig. 2).

CTRP3/cartducin activates the ERK1/2 and p38 MAPK pathways in MSS31 cells

The MAPK and PI3K/Akt pathways respond to angiogenic factors. Therefore, we analyzed the effects of CTRP3/cartducin on the phosphorylation of three groups of MAPKs, such as ERK, JNK, and p38 MAPK, or Akt in MSS31 cells. Western blot analysis detected increased ERK1/2 phosphorylation in MSS31 cells treated with 5 $\mu\text{g/ml}$ of CTRP3/cartducin after 5 min, with the maximal increase occurring after 15 min of treatment, and decreasing after 1 h (Fig. 3A). Increased p38 MAPK

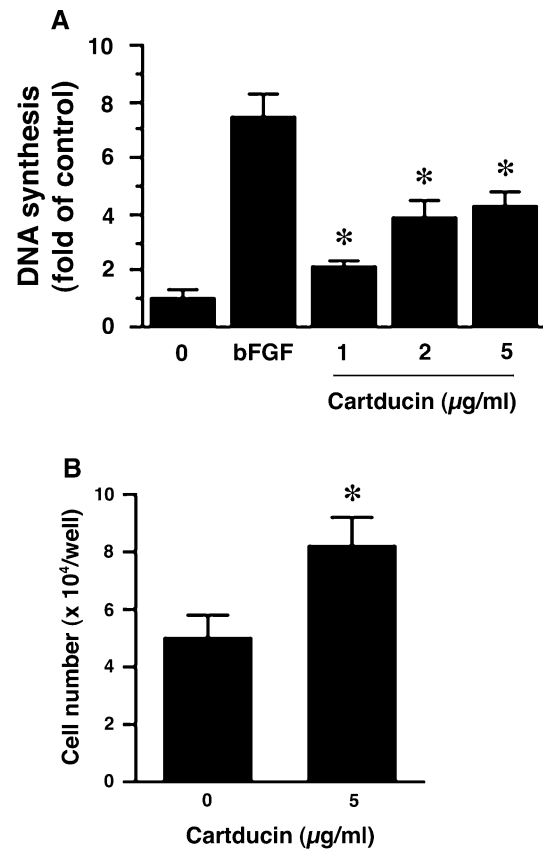


Fig. 1 CTRP3/cartducin induces endothelial cell proliferation. (A) Effects of CTRP3/cartducin and bFGF on DNA synthesis in MSS31 cells. The cells were treated with the indicated concentrations of CTRP3/cartducin or 15 ng/ml of bFGF for 24 h, and BrdU incorporation was measured. (B) Effect of CTRP3/cartducin on MSS31 cell numbers. The cells were treated with 5 $\mu\text{g/ml}$ of CTRP3/cartducin in a medium containing 5% FCS for three days. Data are represented as the mean \pm SD of triplicate determinations. Similar results were obtained from two independent experiments. * $P < 0.05$

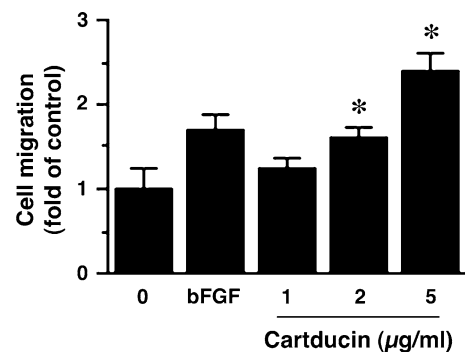


Fig. 2 CTRP3/cartducin induces endothelial cell migration. The cells were treated with the indicated concentrations of CTRP3/cartducin or 15 ng/ml of bFGF for 5 h, and the motility response of the cells to CTRP3/cartducin was assayed by a modified Boyden chamber technique. Data are represented as the mean \pm SD of triplicate determinations. Similar results were obtained from two independent experiments. * $P < 0.05$

phosphorylation was also detected after 15 min, decreasing after 1 h (Fig. 3A). In contrast, CTRP3/cartducin had no effect on the activities of JNK1/2 and Akt, and none of their phosphorylated forms were detected (data not shown). ERK1/2 have been shown to be activated by their upstream activators, MEK1/2. We next examined the action of the specific MEK1/2 inhibitor, U0126, on ERK1/2 pathway activation. The phosphorylation of ERK1/2 induced by CTRP3/cartducin was completely inhibited by pretreatment with U0126 (10 μ M) (Fig. 3B). The action of the specific p38 MAPK inhibitor, SB203580, on phosphorylation of p38 MAPK down stream target was also examined. The phosphorylation of MAPKAPK-2 induced

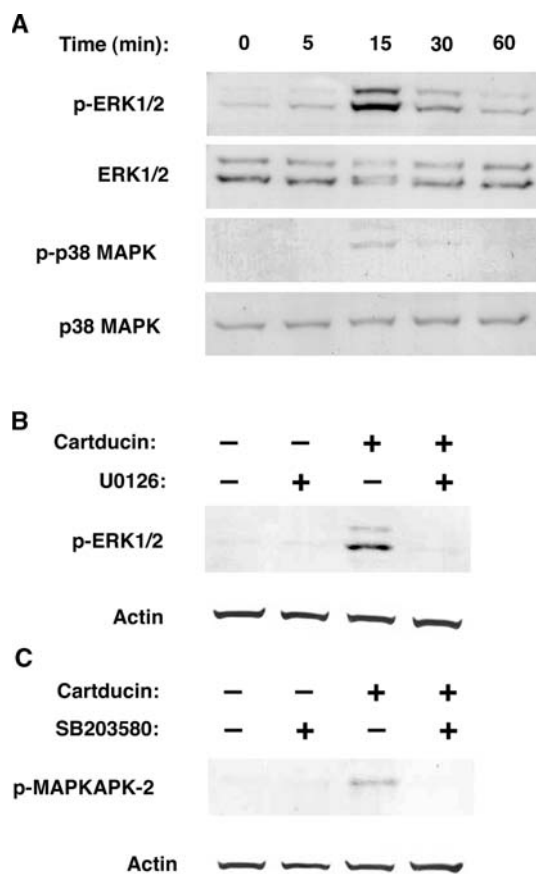


Fig. 3 Effects of CTRP3/cartducin or MAPK inhibitors on activation of ERK1/2 and p38 MAPK signaling pathways in MSS31 cells. (A) Subconfluent cultures of MSS31 cells stimulated with 5 μ g/ml of CTRP3/cartducin for the indicated times were analyzed by Western blot for phosphorylated ERK1/2 (p-ERK1/2) and phosphorylated p38 MAPK (p-p38 MAPK). (B) MSS31 cells were pretreated with a MEK1/2 inhibitor, U0126 (10 μ M), for 1 h, then stimulated with 5 μ g/ml of CTRP3/cartducin for 15 min, then analyzed for phosphorylated ERK1/2 (p-ERK1/2). (C) MSS31 cells were pretreated with a p38 MAPK inhibitor, SB203580 (10 μ M), for 1 h, then stimulated with 5 μ g/ml of CTRP3/cartducin for 15 min, then analyzed for phosphorylated MAPKAPK-2 (p-MAPKAPK-2)

by CTRP3/cartducin was completely inhibited by pretreatment with SB203580 (10 μ M) (Fig. 3C).

ERK1/2 and p38 MAPK pathways are involved in CTRP3/cartducin-induced proliferation of MSS31 cells

Since, Western blot analysis confirmed CTRP3/cartducin-induced ERK1/2 and p38 MAPK pathways activation in MSS31 cells, we next determined, whether, CTRP3/cartducin-induced endothelial cell proliferation is mediated through activation of these pathways. U0126 and SB203580 alone had no effect on proliferation, and no toxicity at the concentration used was observed. U0126, as well as SB203580, blocked CTRP3/cartducin-induced MSS31 cell proliferation in a dose-dependent manner (Fig. 4). Thus, both ERK1/2 and p38 MAPK pathways are required for CTRP3/cartducin-induced proliferation of endothelial cells.

The ERK1/2 pathway is involved in CTRP3/cartducin-induced migration of MSS31 cells

We further determined whether CTRP3/cartducin-induced endothelial cell migration is mediated through activation of ERK1/2 or p38 MAPK pathways. U0126 and SB203580 alone had no effect on migration. SB203580 did not affect CTRP3/cartducin-induced MSS31 cell migration. On the other hand, U0126 significantly reduced the migration of these cells after CTRP3/cartducin stimulation, suggesting the involvement of the ERK1/2 pathway in the ability of CTRP3/cartducin to stimulate migration of endothelial cells (Fig. 5).

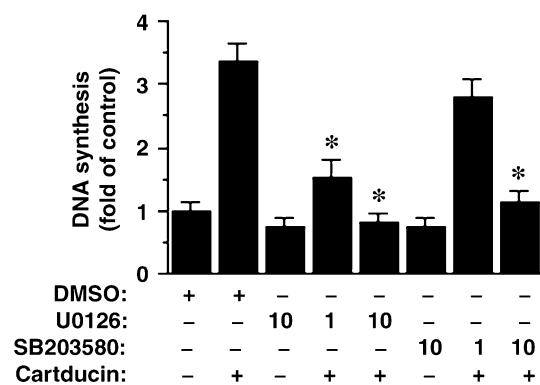


Fig. 4 Effects of MAPK inhibitors on proliferation of MSS31 cells. Subconfluent cultures of MSS31 cells were serum-starved and preincubated with the indicated concentrations (1–10 μ M) of U0126 or SB203580 for 1 h before CTRP3/cartducin treatment (5 μ g/ml), and BrdU incorporation was measured. Data are represented as the mean \pm SD of triplicate determinations. Similar results were obtained from two independent experiments, * P < 0.05 versus CTRP3/cartducin-treated vehicle control

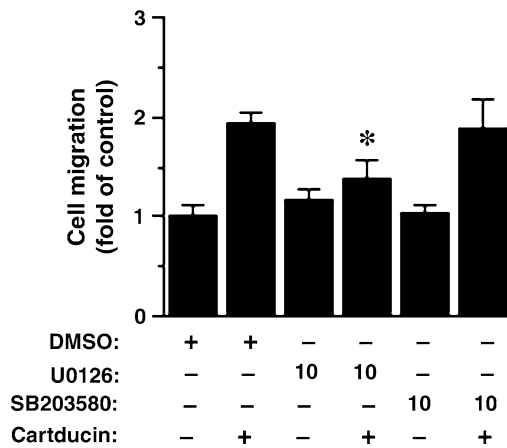


Fig. 5 Effects of MAPK inhibitors on migration of MSS31 cells. Subconfluent cultures of MSS31 cells were preincubated with 10 μ M of U0126 or SB203580 for 1 h before CTRP3/cartducin treatment (5 μ g/ml), and cell migration was measured. Data are represented as the mean \pm SD of triplicate determinations. Similar results were obtained from two independent experiments. * $P < 0.05$ versus CTRP3/cartducin-treated vehicle control

Discussion

CTRP3/cartducin and adiponectin have a highly homologous structure characteristic for the family of CTRP consisting of seven members. CTRP3/cartducin gene was recently reported to be transiently up-regulated in a balloon-injured rat carotid artery tissue [8]. Although CTRP3/cartducin is known to play important roles in regulating both chondrogenesis and cartilage development, little is known about its other biological activities. In the present study, we have demonstrated direct effects of CTRP3/cartducin on endothelial cells. Indeed, we showed, using the mouse endothelial cell line MSS31, that CTRP3/cartducin was able to promote endothelial cell proliferation and migration in a dose-dependent manner.

In contrast to adiponectin [15], no CTRP3/cartducin-specific receptor has yet been identified and cloned. However, both the ERK1/2 and PI3K/Akt pathways are known to be activated by CTRP3/cartducin stimulation in mesenchymal chondrogenitor cells [7]. Regulation of endothelial behavior during angiogenesis is the result of a very complex network of intracellular signaling systems, and both the MAPK and PI3K/Akt pathways are critical regulators of the proliferation, migration, and survival of endothelial cells [16]. In this study, we found that ERK1/2 and p38 MAPK signaling pathways were activated in CTRP3/cartducin-treated MSS31 cells. Using specific kinase inhibitors, we were able to study the role of these signaling pathways and dissected the pathways leading to proliferation and migration in these cells. CTRP3/cartducin-induced DNA synthesis of MSS31 cells was inhibited

by both U0126 and SB203580, suggesting that both ERK1/2 and p38 MAPK pathways are required for CTRP3/cartducin-induced proliferation of endothelial cells. Indeed, both the ERK1/2 and p38 MAPK pathways are known to play an important role in the proliferation of MSS31 cells stimulated by bFGF [14].

The ERK1/2 pathway is reported to be required for endothelial cell migration stimulated by bFGF [17], whereas, vascular endothelial growth factor (VEGF)-induced endothelial cell migration is independent of ERK1/2 [18]. Similarly, the p38 MAPK pathway is known to be associated with bFGF- and VEGF-stimulated endothelial cell migration [14, 19], whereas p38 MAPK is dispensable for endothelial cell migration stimulated by some factors, such as monocyte chemoattractant protein-1 (MCP-1) [20] and hypoxia-induced mitogenic factor (HIMF) [21]. Here, we showed that in spite of the evidence that CTRP3/cartducin induces the simultaneous activation of several signal cascades, their impact on cell migration could be different. CTRP3/cartducin-induced migration of MSS31 cells was inhibited by U0126, but not SB203580, suggesting that ERK1/2, but not the p38 MAPK, pathway is required for CTRP3/cartducin-induced endothelial cell migration.

In summary, we have demonstrated for the first time that CTRP3/cartducin positively regulates two key steps in angiogenesis, i.e., proliferation and migration of endothelial cells, via distinct MAPK signaling pathways. These results suggest that CTRP3/cartducin may play an important role in the pathophysiology of neointimal hyperplasia and restenosis following angioplasty.

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