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Regulation of vascular function by *RCAN1* (*ADAPT78*)

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Abstract

RCAN1 (Adapt78) functions mainly, if not exclusively, as a regulator of calcineurin, a phosphatase that mediates many cellular responses to calcium. Identification of this regulatory activity has led to a surge of interest in RCAN1, since calcineurin is involved in many cellular and tissue functions, and its abnormal expression is associated with multiple pathologies. Recent studies have implicated RCAN1 as a regulator of angiogenesis. To more fully investigate the role of RCAN1 in vascular function, we first extended previous studies by assessing RCAN1 response in cultured endothelial cells to various vascular agonists. Strong induction of isoform 4 but not isoform 1 was observed in human umbilical vein- and bovine pulmonary aortic-endothelial cells in response to VEGF, thrombin, and ATP but not other agonists. Inductions were both calcium and calcineurin dependent, with the relative effect of each agonist cell-type dependent. Ectopic RCAN1 expression also inhibited calcineurin signaling in the HUVEC cells. Based on these strong RCAN1 responses and a lack of RCAN1-associated vascular studies beyond angiogenesis, we investigated the potential role of RCAN1 in vascular tone using whole mounted mesenteric artery. RCAN1 knockout mice exhibited an attenuated mesenteric vasoconstriction to phenylephrine as compared with wild-type. Overall contractility was unaffected, suggesting that this component of smooth muscle action is similar in the two mouse strains. Constriction in the knockout artery appeared to be potentiated by the addition of the nitric oxide synthase (NOS) inhibitor L-NAME, suggesting that elevated nitric oxide (NO) production occurs in the knockout vasculature and contributes to the weakened vasoconstriction. Our results reveal a newly identified vascular role for RCAN1, and a potential new target for treating vascular- and calcineurin-related disorders.

Keywords: Calcineurin; RCAN1; Adapt78; Vascular; Endothelial cells; Isoform 1; Isoform 4; Calcium

RCAN1, initially referred to as *DSCR1* and *ADAPT78*, was first identified as a Down syndrome critical region-localized gene on human chromosome 21 [1]. It encodes two major isoforms, 1 and 4. RCAN1 isoform 1 exhibits little modulation, whereas isoform 4 has been shown to be inducible by multiple stresses, especially calcium, and to be cytoprotective when overexpressed in hamster HA-1 cells [2–4] or neuronal cells [5]. RCAN1 protein inhibits calcineurin, an intracellular phosphatase that mediates many cellular responses to calcium [6–9]. This observation has led to increased interest in RCAN1, since calcineurin is involved in many cellular and tissue functions, and its abnormal expression is associated with multiple pathologies [10–13]. Recently, RCAN1 (Regulator of Calcineurin 1) has become the official designation for this protein previously referred to by multiple names including DSCR1, Adapt78, MCIP1, and calcipressin [14].

RCAN1 is expressed in multiple tissues, and most abundantly in skeletal muscle, heart, and brain [15]. Recent studies indicate that it also plays an important role in endothelial cells. For example, endothelial cells in culture treated with VEGF or thrombin exhibit elevated expression of RCAN1 isoform 4. This has been reported mainly at the mRNA level and in some cases, also at the protein level. Specifically, Lizuka et al. [16], Qin et al. [17], and

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Hesser et al. [18] have demonstrated RCAN1 protein expression in HUVEC cells after stimulation with VEGF; Minami et al. [19] have reported thrombin induction of RCAN1 in HUVEC cells; and Yao et al. [20] have reported VEGF induction of RCAN1 in both HUVEC and human retinal endothelial cells (HRECs) cells.

Recently, RCAN1 has also been implicated in angiogenesis, although its precise role here is still being investigated. Several groups have reported antiangiogenic RCAN1 activity [16,17,19,20]. For example, overexpression of RCAN1 in endothelial cell resulted in a decreased vascular density on a matrigel plug assays and reduced melanoma tumor size [19]. Conversely, matrigel implantation into the subcutaneous tissue of mice in the presence of antisense RCAN1 RNA showed an inhibition of VEGF-mediated angiogenesis, suggesting a pro-angiogeneic function for RCAN1 [16]. Another study reports that both RCAN1 isoforms affect angiogenesis, but with opposite actions; i.e., isoform 1 stimulates angiogenesis, while isoform 4 inhibits [17].

Calcineurin is activated by elevated calcium, leading to the dephosphorylation of target substrates such as NFAT, tau, CREB, BAD, and others via calcineurin phosphatase activity [10]. Endothelial nitric oxide synthase (eNOS), which is the source of the vasodilator nitric oxide in endothelial cells, is also a target of calcineurin. Dephosphorylation of two eNOS residues (threonine 495/497 and serine 116) by calcineurin contributes to eNOS activation [21,22]. Given RCAN1's known inhibition of calcineurin and calcineurin's ability to induce eNOS activation, we hypothesize that RCAN1 regulates nitric oxide levels through its modulatory effects on calcineurin.

Here, we detail endothelial cell RCAN1 response to various agonists. Combined with reported angiogenic effects of RCAN1, we further investigate whether RCAN1 can also affect vascular tone. These studies also allowed us to test our hypothesis that RCAN1 can regulate nitric oxide levels.

Materials and methods

Cell cultures

Human umbilical vein endothelial cells (HUVEC) were maintained in M-199 media (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS), endothelial cell growth factor (15 mg), and heparin (10 μg/ml). Bovine pulmonary aortic endothelial (BPAE) cells were cultured in 10% FBS and also supplemented with heparin (100 μg/ml). All cultures contained 50–100 U/ml penicillin and 50–100 μg/ml streptomycin, and were maintained in a humidified incubator atmosphere of 95% air and 5% CO_2 at 37 °C.

Cell culture treatments

For cell culture treatments, HUVEC and BPAE monolayer cells were placed in serum-free media and, after overnight incubation, treated with the appropriate agents (see Table 1), then placed back in the incubator for the indicated times. For HUVEC cells treatment with VEGF and cyclosporin, cells were pretreated with or without 20 μM BAPTA-AM or 200 nM cyclosporin for 30 min prior to agonist addition. At the designated times, the cells were washed with cold PBS and lysed by direct addition of 2× Laemmli SDS sample buffer [23] followed by trituration and boiling.

Table 1
Relative effect of agonists on HUVEC RCAN1-4 induction

Agonist	Concentration	General response*
VEGF	40 ng/ml	++++
Thrombin	0.125 U/ml	++++
ATP	100 μM	++
TNFα	25 ng/ml	+
Transferrin	20–50 ng/ml	−
TGFβ	625 pg/ml	+
Insulin	500 nM	−
EGF	50 ng/ml	+
PDGF	0.92 U/ml	−
Angiotensin II	100 nM	−

* $N \geqslant 2$ for all agonists tested.

For HUVEC infection studies, Adenoviral *RCAN1* was constructed by subcloning *RCAN1-4* cDNA into a shuttle vector and contransforming into *Escherichia coli* strain BJ5183 with the Adenoviral backbone plasmid pAdEasy-1 as described [24]. Recombinant Adenoviral plasmids were selected on kanamycin, confirmed by restriction digest, and transfected into AD-293 cells for virus particle packaging. This construct also contain GFP as a separate loci to track the success of infection.

Western blot analyses

Equal amounts of boiled cell lysates and aortic ring extracts were electrophoresed on an (usually 12.5%) SDS–polyacrylamide gel, electroblotted to nitrocellulose, and incubated with primary antibody followed by peroxidase-conjugated secondary antibody and signal development with Western light chemiluminescent substrate (Perkin-Elmer, Boston, MA). All signals were captured on film and quantified using the ImageJ program. The RCAN1 antibody used was a mouse monoclonal antibody directed against the C-terminal region of human RCAN1 (generously provided by Dr. Sandra Ryeom and Dr. Frank McKeon, Harvard Medical School).

Animal-related studies

For animal-related studies, wild-type and knockout mice were sacrificed by CO_2 inhalation and decapitation, and mesenteric arteries carefully harvested. All animal procedures were performed according to the NIH guide for animal care and as approved by the Albany Medical College animal care and use committee. The mesentery was removed and placed in ice-cold PSS solution. Arterial segments (~1.5 mm long, 200–300 μm OD) were dissected free of fat and connective tissue and mounted on glass cannulae in a pressure myograph (Living Systems Inc., Burlington, VT). The segments were pressurized to 60 mm Hg and bathed in circulating, heated (37 °C), oxygenated PSS. The myograph was mounted on the stage of an inverted microscope and changes in vascular diameter monitored with a video dimension analyser coupled with Chart software (AD instruments). Following a brief equilibration period, arteries were challenged with 60 mM KCl to test patency; poorly contracting arteries were discarded. After washout of KCl and re-equilibration, arteries were contracted with cumulative concentrations of phenylephrine (PE). Maximal contraction was determined by the addition of high KCl (95 mM) to arteries contracted with the highest concentration of PE. Arteries treated with L-NAME (NG-nitro-L-arginine methyl ester) were incubated for 20 min and arteries were contracted in the continued presence of the agent.