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Selective inhibition of plasma membrane calcium ATPase 4 improves angiogenesis and vascular reperfusion

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ABSTRACT

Aims: Ischaemic cardiovascular disease is a major cause of morbidity and mortality worldwide. Despite promising results from pre-clinical animal models, VEGF-based strategies for therapeutic angiogenesis have yet to achieve successful reperfusion of ischaemic tissues in patients. Failure to restore efficient VEGF activity in the ischaemic organ remains a major problem in current pro-angiogenic therapeutic approaches. Plasma membrane calcium ATPase 4 (PMCA4) negatively regulates VEGF-activated angiogenesis *via* inhibition of the calcineurin/NFAT signalling pathway. PMCA4 activity is inhibited by the small molecule aurintricarboxylic acid (ATA). We hypothesize that inhibition of PMCA4 with ATA might enhance VEGF-induced angiogenesis.

Methods and results: We show that inhibition of PMCA4 with ATA in endothelial cells triggers a marked increase in VEGF-activated calcineurin/NFAT signalling that translates into a strong increase in endothelial cell motility and blood vessel formation. ATA enhances VEGF-induced calcineurin signalling by disrupting the interaction between PMCA4 and calcineurin at the endothelial-cell membrane. ATA concentrations at the nanomolar range, that efficiently inhibit PMCA4, had no deleterious effect on endothelial-cell viability or zebrafish embryonic development. However, high ATA concentrations at the micromolar level impaired endothelial cell viability and tubular morphogenesis, and were associated with toxicity in zebrafish embryos. In mice undergoing experimentally-induced hindlimb ischaemia, ATA treatment significantly increased the reperfusion of post-ischaemic limbs.

Conclusions: Our study provides evidence for the therapeutic potential of targeting PMCA4 to improve VEGF-based pro-angiogenic interventions. This goal will require the development of refined, highly selective versions of ATA, or the identification of novel PMCA4 inhibitors.

1. Introduction

Ischaemic cardiovascular diseases (including ischaemic heart disease, peripheral arterial disease, and stroke) constitute a leading cause of morbidity and mortality worldwide [28]. In some patients, bypass surgery and interventional endovascular procedures can successfully restore blood flow to the ischaemic tissue [1,33]. However, the

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distribution and diffuseness of arterial occlusions preclude surgical revascularization in a high proportion of patients [1]. Therapeutic strategies to promote the formation of new blood vessels in the ischaemic organ (referred to as therapeutic angiogenesis) constitute a promising alternative for these patients [1,11,42].

Angiogenesis, the formation of new blood vessels from pre-existing ones, is a tightly regulated process involving the participation of several pro- and anti-angiogenic factors [10]. One of the critical pro-angiogenic factors is vascular endothelial growth factor (VEGF) [38]. A number of gene-based and protein-based approaches have been developed to deliver exogenous VEGF to ischaemic tissues [42]. Data obtained in animal models of myocardial and limb ischaemic disease demonstrated that VEGF-based pro-angiogenic therapies induce successful reperfusion of the ischaemic organ [40]. Unfortunately, to date clinical translation of these procedures has produced only limited patient benefit [12]. The reasons for the lack of clinical success with therapeutic angiogenesis approaches are complex, but one central problem in current approaches is failure to restore efficient VEGF activity in the ischaemic organ [12,40,42]. Design of new, more effective treatments requires a deep understanding of the molecular and biochemical processes governing VEGF-induced angiogenesis.

We recently reported a novel role for plasma membrane calcium ATPase 4 (PMCA4) as a negative regulator of VEGF-activated angiogenesis [4]. PMCAs are enzymatic high-affinity systems that export calcium from the cytosol to the extracellular environment [37]. There are four PMCA isoforms (PMCA1–4) encoded by four distinct genes [37]. PMCA1 and 4 are expressed ubiquitously, whereas PMCA2 and 3 are restricted to highly specialised cells and tissues [37]. PMCA4 is the major isoform present in endothelial cells [30]. Our previous work showed that PMCA4 attenuates VEGF-induced angiogenesis by establishing an inhibitory interaction with the signalling protein calcineurin [4]. Calcineurin is a serine/threonine phosphatase activated by increases in cytosolic calcium concentration [20]. Activated calcineurin mediates dephosphorylation of NFAT (nuclear factor of activated T cells) transcription factors, promoting their translocation to the nucleus and the subsequent expression of NFAT-target genes [15]. The calcineurin/NFAT signalling axis plays a critical role in VEGF-induced angiogenesis [2,14]. PMCA4 is thought to downregulate calcineurin activity by tethering it to low-calcium micro-domains created by the pump's calcium expulsion action and, in so doing, impair VEGF-activated pro-angiogenic signalling [4]. We therefore hypothesize that pharmacological blockade of PMCA4 function, and thus suppression of the negative effect of PMCA4 on angiogenesis, might potentiate the efficiency of therapeutic strategies involving VEGF. Supporting this possibility, we previously reported more efficient limb reperfusion in *PMCA4*$^{-/-}$ knockout mice undergoing femoral artery ligation than in their wild-type littermates [4].

A recent study showed that low concentrations of the small molecule aurintricarboxylic acid (ATA) strongly inhibit the calcium extrusion activity of PMCA4 without affecting the activity of PMCA1 or other major calcium pumps such as SERCA and the Na^+/K^+ ATPase [26]. This finding prompted us to examine whether inhibition of PMCA4 with ATA enhances VEGF-induced angiogenesis.

Here, we show that treatment of primary endothelial cells with low concentrations of ATA results in a remarkable increase in VEGF-mediated activation of the calcineurin/NFAT pathway and in the expression of the VEGF-induced, NFAT-dependent, pro-angiogenic protein RCAN1.4. Inhibition of PMCA4 with ATA also enhances endothelial cell motility and tubular morphogenesis, with no harmful effects on the cells. These results highlight the clinical potential of targeting PMCA4 to improve VEGF-based therapeutic interventions that promote blood vessel formation in patients suffering from ischaemic cardiovascular disease.

2. Materials and methods

2.1. Cells and cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from TCS Cellworks and cultured in tissue culture flasks pre-coated with 0.1% gelatin in endothelial cell growth medium (ECGM, PromoCell) supplemented with ECGM-supplement mix and 1% penicillin/streptomycin/amphotericin B (Sigma-Aldrich). HUVECs were used at passages 6–8.

Mouse lung endothelial cells (MLEC) were purified from wild-type or PMCA4-null mice as described [4]. Generation and characterization of PMCA4 knockout mice has been previously reported [34].

2.2. Luciferase reporter assay

HUVECs were infected with Ad-NFAT-Luc (a replication-deficient adenovirus harbouring an NFAT-dependent, luciferase-based reporter system) at a multiplicity of infection (MOI) of 50. Generation of Ad-NFAT-Luc-infected particles was as described [4]. Infected cells were incubated in ECGM containing 0.5% fetal calf serum for 16 h and then stimulated with VEGF (25 ng/ml) for 6 h. Luciferase activity was determined as described [17].

2.3. siRNA gene knock-down

siRNA-mediated knock-down of *PMCA4* gene expression was performed using "ON-TARGET-plus SMART pool human ATP2B4" (Thermo Scientific). "ON-TARGET-plus non-targeting pool control duplexes" (Thermo Scientific) was used as a control.

For siRNA transfection, HUVECs were plated in 0.1% gelatin pre-coated 6-well tissue culture plates (3×10^5 cells/well) and incubated overnight. The following morning, cells were washed with PBS and incubated in serum-free, antibiotic-free OPTIMEM medium for 1 h. Then, 100 pmol of siRNA/well were transfected using 5 μl of Lipofectamine 2000 (Thermo Scientific). Transfection medium was removed after incubation for 6 h, and substituted by ECGM supplemented with ECGM-supplement mix and 1% penicillin/streptomycin/amphotericin B (Sigma-Aldrich). Cells were incubated for 72 h and then used for further experiments.

2.4. Quantitative Real-Time PCR

Total RNA from HUVECs was extracted using the "Total RNA purification kit" (Norgen) and cDNA synthesis was performed with 0.5 μg of total RNA using the "High capacity cDNA reverse transcription kit" (Applied Biosystems). *PMCA4* RNA expression was determined by qRT-PCR using TaqMan Gene Expression Assay (Hs00608066_m1) in a 7500 Fast Real-Time PCR System (Applied Biosystems, UK). PCR cycling conditions consisted of an initial enzyme activation step at 95 °C for 10 min, followed by denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min for 40 cycles. Ct value was normalised using the Ct values for the housekeeping gene *Hprt-1* (TaqMan Gene Expression Assay, Hs99999909_m1). Analysis of data was carried out using the comparative $2^{-\Delta\Delta CT}$ method.

2.5. Western blot

Total proteins were isolated by direct lysis of HUVECs, treated as indicated, in NuPAGE LDS sample buffer containing 0.05% β-mercaptoethanol. Membrane-associated proteins were isolated using the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem) as reported [3].

We have noticed that repeated and prolonged boiling degrades endothelial PMCA4; therefore we minimised repeated cycles of freeze-thawing-boiling. Protein samples were boiled for 1 min and then