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ENPP1-Fc inhibits proliferation of pathological synthetic phenotype vascular smooth muscle cells (VSMCs) in the presence of ATP: the role of ecto-5'-nucleotidase CD73.

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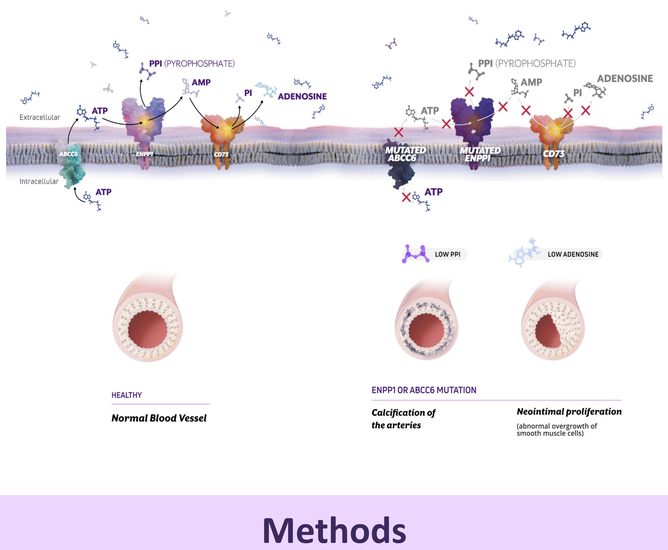
Introduction

Dysregulated proliferation of synthetic vascular smooth muscle cells (VSMCs) results in neointimal hyperplasia. Ectonucleotide pyrophosphatase/ phosphodiesterase 1 (ENPP1) hydrolyzes extracellular ATP to AMP and pyrophosphate, the latter being an inhibitor of mineralization.[1] CD73 in turn, converts AMP to inorganic phosphate and adenosine.[2] Adenosine has been implicated in control of neointimal proliferation.[3] Nitschke et al found that silencing ENPP1 in VSMCs resulted in a tenfold increase in proliferation, and the addition of rhENPP1-Fc (extracellular domain of human ENPP1 fused to Fc domain of human immunoglobulin), AMP, or adenosine attenuated the proliferation.[3] The precise mechanism of ENPP1-Fc anti-proliferative effect has not been fully elucidated. In this experiment we examined the mechanism of ENPP1-Fc anti-proliferative effect on VSMCs, with a particular interest in synthetic VSCM's which demonstrate abnormal proliferative characteristics. Further, we sought to understand the signaling pathway(s) through which AMP/adenosine reduces intimal proliferation



Methods

Human aortic smooth muscle cells isolated from a healthy donor were used in all experiments. Chemiluminescent BrdU incorporation assay was used to study cell proliferation. Concentration of cAMP and VASP phosphorylation were analyzed using HTRF assays. HPLC was used to determine concentrations of Adenosine and Inosine in cell culture media. Pharmacological inhibition of CD73 (AB680), A2A/A2B receptors (AB928) and protein kinase A (KT5720) was used for signaling pathway analysis.

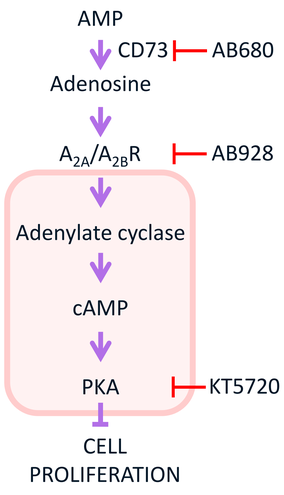


Figure 1. Illustration of the vascular smooth muscle cell proliferation pathway and mechanism of pharmacologic inhibitors. CD39 (not pictured) is a plasma membrane protein that hydrolyzes extracellular ATP and ADP to AMP

Results

Characterization of synthetic and contractile VSMCs

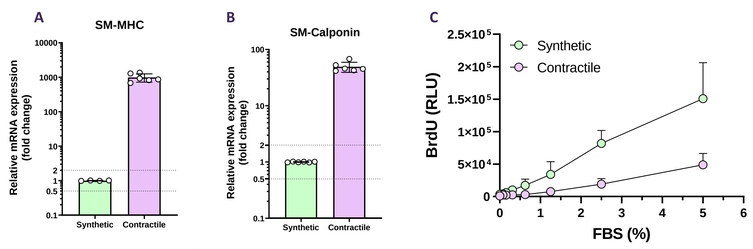


Figure 2. VSMCs were differentiated towards synthetic or contractile phenotypes. Expression of the contractile VSMC biomarkers including smooth muscle myosin heavy chain (SM-MHC) (**A**) and smooth muscle calponin (SM-Calponin) (**B**) was analyzed by qPCR. For cell proliferation assays (**C**) synthetic and contractile VSMCs that had been starved for 24 hours in basal media were cultured in media containing FBS for 3 days. Cell proliferation was evaluated by BrdU incorporation. Values are presented as the mean ± SD.

Expression of ecto-nucleotidases by synthetic and contractile VSMCs

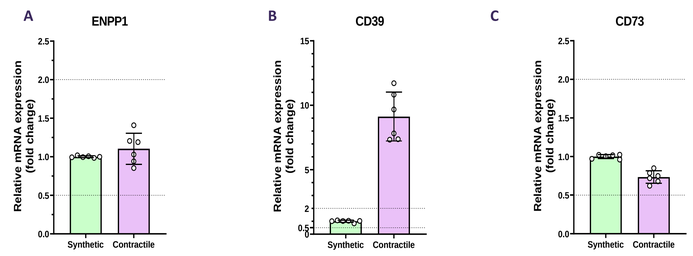


Figure 3. VSMCs were differentiated towards synthetic or contractile phenotypes. Expression of mRNA encoding ENPP1 (**A**), ATP/ADP-specific ecto-nucleotidase CD39 (**B**) and AMP-specific ecto-nucleotidase CD73 (**C**) was analyzed using qPCR. Values represent relative mRNA expression and are presented as the mean ± SD.

Expression of adenosine receptors by synthetic and contractile VSMCs

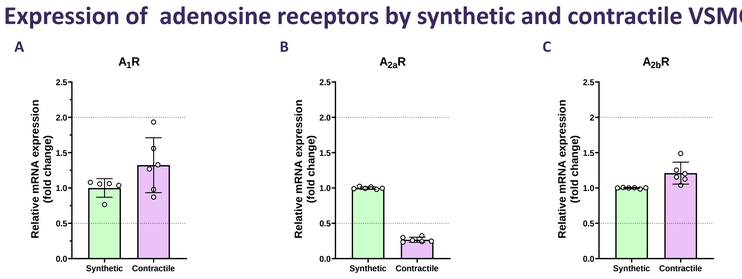


Figure 4. VSMCs were differentiated towards synthetic or contractile phenotypes. Expression of mRNA encoding A_1R (**A**), $A_{2a}R$ (**B**) and $A_{2b}R$ (**C**) was analyzed using qPCR. Values represent relative mRNA expression and are presented as the mean ± SD.

Effect of ENPP1-Fc/ATP, AMP, Adenosine and C-Adenosine on proliferation of synthetic VSMC

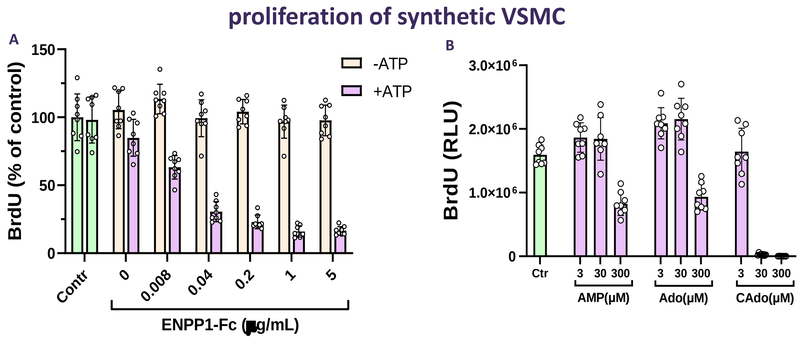


Figure 5. Synthetic VSMCs were starved for 24 hours in basal media. Cells were then cultured for 3 days in FBS containing media to which had been added ENPP1-Fc/ATP (**A**), ATP alone (300 μM, **A**), AMP (3-300 μM, **B**), Adenosine (Ado) (3-300 μM, **B**), or the non-hydrolysable Ado analog 2-Chloro-Adenosine (CAdo) (3-300 μM) **B**). Cell proliferation was evaluated by BrdU incorporation. Values are presented as the mean ± SD.

VSMCs metabolize AMP produced from ENPP1-Fc/ATP co-treatment to Adenosine and Inosine

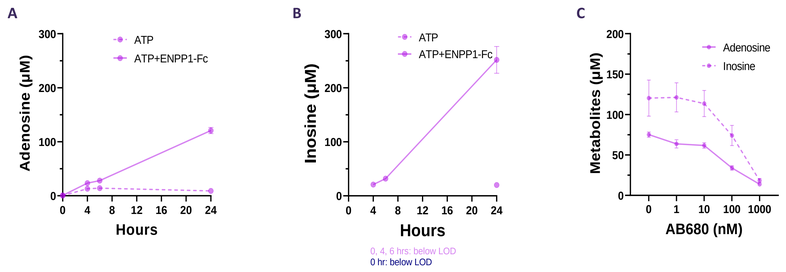


Figure 6. Synthetic VSMCs were incubated in basal media supplemented with 300 μM ATP (**A**, **B**) or with ENPP1-Fc (0.2 μg/ml) and ATP (**A**,**B**,**C**) in the presence or absence of CD73 inhibitor AB680 (**C**). Concentrations of Adenosine and Inosine in culture media were determined by HPLC. Values are presented as the mean ± SD.

Inhibition of CD73 reverses anti-proliferative effect of ENPP1-Fc/ATP and AMP

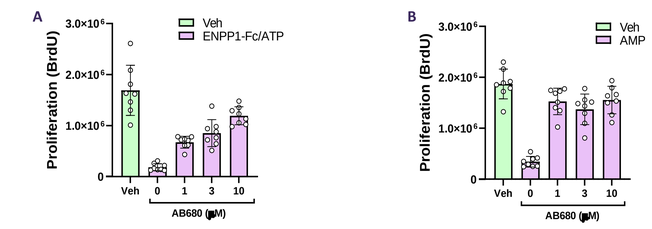


Figure 7. Synthetic VSMCs were starved for 24 hours in serum free media. Then cells were cultured in a media supplemented with heat-inactivated FBS (5%) and containing AB680 (0-10 μM) and, either (**A**) ENPP1-Fc (0.2 μg/ml) and ATP (300 μM) or (**B**) AMP (300 μM). After 3 days, cell proliferation was evaluated by BrdU incorporation. Values are presented as the mean ± SD.

Inhibition of CD73 or dual inhibition of Adenosine 2a and 2b receptors attenuates AMP-induced cAMP synthesis

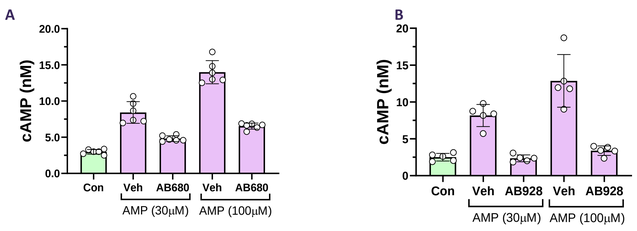


Figure 8. Synthetic VSMCs were pretreated for 18 hours with either (**A**) CD73 inhibitor AB680 (1 μM) or (**B**) dual $A_{2a}R/A_{2b}R$ antagonist AB928 (0.3 μM). Cells were washed with basal media and stimulated for 4 hours with AMP (30, 100 μM). Total cAMP was measured using HTRF assay. Values are presented as the mean ± SD.

Treatment with AMP activates Protein Kinase A in VSMCs

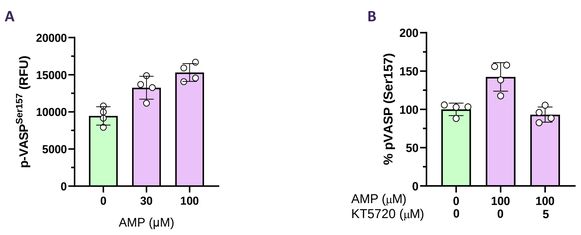


Figure 8. Synthetic VSMCs were preincubated for 30 min in the basal media supplemented with 0.25% FBS and stimulated for 30 min with AMP in the absence (**A**) or presence (**B**) of PKA inhibitor KT5720. Phosphorylation of VASP at Ser157 was measured using HTRF assay. Values are presented as the mean ± SD.

Conclusions

- Expression of the ATP/ADP-specific ecto-nucleotidase CD39 was decreased and expression of $A_{2a}R$ was increased in synthetic VSMCs when compared to contractile VSMCs.
- Treatment with AMP, Adenosine, C-Adenosine or an ENPP1-Fc/ATP combination inhibited VSMC proliferation.
- Synthetic VSMCs metabolized AMP to Adenosine and Inosine in CD73 dependent manner.
- Inhibition of CD73 reversed the anti-proliferative effects of AMP or ENPP1-Fc/ATP treatments and attenuated AMP-mediated cAMP synthesis.
- Adenosine generated from extracellular AMP induced activation of Gs-coupled adenosine receptors and the PKA signaling pathway, implicated in vasodilation and cellular proliferation.
- These findings support pre-clinical and clinical studies on VSMC function modulation in diseases associated with neointimal proliferation, which include Generalized Arterial Calcification of Infancy (GACI).

References

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3. Nitschke Y, et al. *Experimental & Molecular Medicine*. 2018;50(10):1-12.

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