Al collegio docenti del Dottorato in Medicina Molecolare

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INTRODUCTION

Tubular aggregates myopathy (TAM) is a rare variant or myopathy that can have dominant or recessive transmission. It is a progressive disease with symptoms like loss of strength, muscle pains, cramps and at cellular levels the presence of tubular aggregates (TAs) inside muscle fibers. TAMs usually are caused by defects in SOCE activation. SOCE (Store-Operated Calcium Entry) is an essential pathway for the uptake of calcium from the extracellular space. The mechanism is of notable importance in muscle cells (and fibers) to fill sarcoplasmic reticulum of Ca2+, needed for the signalling and muscular contraction. SOCE is composed mainly of 2 proteins: stromal interaction molecule 1 (STIM1) and Calcium release-activated calcium channel protein 1 (ORAI1). STIM1 is sensible to Ca2+ level inside the SR thanks to EF-hand luminal domain and has the role of initiator of SOCE mechanism following the depletion of calcium storage. When calcium ions no longer bind STIM1 luminal domain this protein lengthens the cytoplasmic domain and take contact with ORAI1on the cellular membrane, this conformation with STIM1 extended towards the membrane and ORAI1 recruited and activated is called "puncta". The result of this activation is the influx of calcium from the extracellular space inside the cytoplasm. Calsequestrin (CASQ) is another crucial protein in calcium regulation and homeostasis, weighing 45 kDa is localized inside the lumen of SR. His role is to acts as a buffer of calcium, binding it with low affinity but high avidity, allowing the storage in high quantity. CASQ has a role in the regulation of SOCE, interacting with STIM1 when calcium concentration decrease.

My research aims to understand the mechanism behind the pathological mutations of CASQ1 and their role in the development of TAM. In particular we are investigating 4 pathological mutations isolated from TAM (and Vacuolar myopathy) patients.

METHODS

For the transfection of mutations in mice we used DNA plasmids prepared and purified from E.Coli amplification cultures.

Mice were transfected 1 week before the experiment with 10ng of plasmid in immediate proximity of the FDB muscle. First 2 mutations tested were p.Gly103Asp and p.Asp44Asn of CASQ1 protein. The mutated CASQ1 was also tagged with GFP to identify the transfected fibers. As control we transfected CASQ1-WT tagged with GFP. All the transfection and experiments were conducted on mice KO for CASQ1 (CASQ-Null). As negative control all the experiments were also performed on CASQ-Null mice not transfected.

Muscle fibers were isolated from FDB muscles of mice and plated on Lab-Tek™ II previously incubated with laminin. To evaluate SR calcium storage and SOCE activation all the models undergo two different experiments, both performed using the fluorescent calcium indicator Fura-2 and a fluorescence microscope.

SOCE activation was tested using the method of "Manganese quenching", depleting the SR of FDB fibers from calcium with a cocktail of 15μM CPA and 1μM Thapsigargin during the loading phase (1h) with the calcium indicator Fura-2 AM in a calcium-free solution. After the recording of basal level of fluorescence (at 360nm to avoid interference by calcium movements) fibers were treated with a solution containing 0.5mM MnCl2 and the fluorescence decay (quench) was recorded for at least 6'.
The quenching of Fura-2 fluorescence is directly proportional to the SOCE activation, due to manganese using SOCE as channel for cell entry. After normalization with basal levels SOCE activation was expressed as ΔF/t (quench of fluorescence over time).

SR calcium storage (the primary role of CASQ1) was assessed again using Fura-2 as calcium indicator, this time recording the fluorescence at 340nm and 380nm, and using the ratio to calculate the cytoplasmic levels of calcium. This time fibers were kept in a calcium-containing medium until the start of the recording, were after the basal level was registered, fibers were treated with a depletion cocktail containing 10uM ionomycin and 30uM CPA, in Ca2+ free conditions. Calculating the AUC of the resulting release curve, over 6 mins, we can evaluate the total calcium storage of that fibre.

RESULTS

The fibers stay vital and responsive until 24h after being plated. Significant differences in both SOCE activation and total calcium content are clearly visible between CASQ1-WT and CASQ1-KO fibers, and also between CASQ1-WT GFP fibers (Casq-Null mice that were transfected with the WT CASQ1(tagged protein) and KO fibers, thus confirming the role of CASQ1 as buffer of calcium in the SR and modulator of SOCE activation.
Regarding the transfection of p.Gly103Asp and p.Asp44Asn mutations experiment are still in place but we have already assessed a difference in the protein diffusion inside the RS, probably due to higher tendency to form aggregates, as the histological analysis of the patients would suggest.

PUBLICATIONS

Lazzerini Pietro Enea, Acampa Maurizio, CUPELLI Michael, Gamberucci Alessandra, Srivastava Ujala, NANNI Claudio , Bertolozzi Iacopo , Vanni Francesca , FROSALI Alessandro, CANTORE Anna, Cartocci Alessandra, D'Errico Antonio, SALVINI Viola , ACCIOLI Riccar, B. M. (2021) 'Unravelling atrioventricular block risk in inflammatory diseases: systemic inflammation acutely delays atrioventricular conduction via a cytokine-mediated inhibition of connexin43 expression', *JAHA: Journal of the American Heart Association*, JAHA/2021/. (manuscript accepted for publication)

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