Myocardial Calcification and Fibrosis in Dystrophic Mice is Reduced by RhoA Inactivation

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Introduction: Myocardial calcification refers to the excessive deposition of calcium in the cardiac muscle and is usually observed in the aging population as well as long-term survivors of substantial myocardial infarctions (1-2). Myocardial calcification could either be the result of chronic degeneration or reflect ongoing pathologic processes (2); however, the cellular and molecular mechanisms leading to myocardial calcification remains largely unknown. We have recently reported the observation of extensive skeletal muscle calcification/heterotopic ossification in the dystrophin/utrophin double knockout (dKO) mouse model of Duchenne muscular dystrophy (3), which could be mediated by the over-activation of the RhoA signaling in muscle stem cells (MSCs) (4). RhoA is a small GTPase protein that regulates cell morphology and migration in response to extracellular signaling and stresses. RhoA activation in MSCs induces their osteogenesis potential, inhibits their adipogenic potential, mediates BMP-induced signaling, and promotes osteoblastic cell survival (5). The involvement of RhoA in mediating inflammatory processes and myocardial fibrosis has previously been described (6). In addition, previous studies have indicated that the sustained activation of the RhoA pathway can block the differentiation of muscle cells by inhibiting myoblast fusion (7). Cardiac involvement is the leading cause of early death in DMD patients, and the current study was performed to elucidate the role of RhoA in mediating fibrosis and calcification in the cardiac muscle of dystrophic mice.

Methods: 1. Animals: Wild-type (WT, C57BL/10J) mice were purchased from Jackson Laboratory. The mdx and dKO (mdx; utrn/-/-) mice are maintained in our in house colony. Compared to mdx mice which feature a normal life span (~2 years), dKO mice undergo early death (~8 weeks) and have a more severe phenotype that more closely resembles the DMD pathology. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh.
2. RhoA inactivation with Y-27632: dKO mice from 3 weeks of age received either an intraperitoneal (IP) injection of Y-27632 [5mM in Phosphate Buffered Saline (PBS), 10mg/kg per mouse], which is a systematic inhibitor of RhoA signaling or PBS only (control). IP injections were conducted 3 times a week for 4 weeks.
3. Histology: Mice were sacrificed and 10µm cryostat sections were prepared from the cardiac muscles of the mice. Alizarin red stained was conducted to stain calcium deposition in the cardiac muscle.
4. Statistics: N =6 for each group. Student’s T-test was used to evaluate for significance.

Results: 1. Cardiac muscle of dKO mice featured increased fibrosis and calcification when compared to mdx and WT mice. Trichrome staining of the cardiac muscles from 8-week old WT, mdx, and dKO mice was conducted to characterize ECM collagen deposition, which revealed that fibrosis formation was generally severe in the dKO mice, mild in the mdx mice, and absent in WT mice (Figure 1A). Alizarin red staining of the cardiac muscle revealed that calcification occurred in the dKO mice (Figure 1B), but not in the WT or mdx mice.
2. Up-regulation of RhoA in the cardiac muscle of the dKO mice. Semi-quantitative PCR showed that, compared to the WT and mdx mice, the expression of RhoA and the inflammation signaling genes, TNF-α and IL-6, was up-regulated in the dKO cardiac muscle, while the expression of the anti-inflammatory gene Klotho was down-regulated (Figure 1C). We suggest that the activation of RhoA and inflammatory mediators are involved in the cardiac fibrosis and calcification seen in dKO mice.
3. Systemic RhoA inactivation via intraperitoneal injection (IP) of Y-27632 reduced fibrosis and HO in dKO cardiac muscle. We hypothesized that RhoA inactivation could reduce fibrosis and calcification in the cardiac muscles of the dKO mice. To confirm this hypothesis, Y-27632 was injected intraperitoneally (IP) in 3-week old dKO mice. As expected, after 4 weeks of continuous injection. Semi-quantitative PCR revealed that the expression of RhoA, TNF-α and IL-6 was down-regulated with Y-27632 administration, while the expression of Klotho was up-regulated (Figure 2A). Meanwhile, fibrosis and calcification in the cardiac muscles of dKO mice was found to be decreased compared to non-treated mice (Figure 2B-E).

Discussion: Both calcification and fibrosis in cardiac muscle is typically the result of a pathologic process. Our current results revealed that the RhoA signaling pathway may mediate the calcification and fibrosis processes in the cardiac muscles of dKO mice. RhoAs seems to be co-activated with inflammatory signaling in severely dystrophic cardiac muscle, and the inactivation of RhoA signaling could repress this signaling. Therefore, our results indicate that the involvement of RhoA signaling in the therapeutic prevention of calcification and fibrosis in cardiac muscle should be further investigated as a potential target for treating DMD patients and other pathologic conditions of the heart.
**Significance:** Our data reveals the involvement of RhoA signaling in regulating calcification and fibrosis of cardiac muscle, and indicates RhoA may serve as a potential target for repressing injury-induced and congenital cardiac muscle calcification and fibrosis in humans.

**Acknowledgments:** We would acknowledge the funding support from DOD and editing helps from James Cummins.

**References:**
Figure 1

A
Trichrome stain (8W)

B
Alizarin Red (8W)

C
mRNA of cardiac muscle (4W)