Annexin 2 plays a role in flow-induced PGE2 release, is phosphorylated during flow and colocalizes with raft-like structures in osteoblasts

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Introduction: Biophysical signals induced by mechanical loading elicit a variety of cellular responses in bone cells, however, there is no consensus regarding the underlying mechanotransduction mechanism. Members of the annexin family, a group of structurally related Ca2+-binding proteins, have been implicated as potential players in the mechanotransduction signaling cascade (1, 2). In particular, annexin 2 (ANXA2) has been shown to play a role in mechanical stress-induced increases in proliferation in osteoblasts (2). ANXA2, like all annexins, associates with phospholipid membranes in a Ca2+-dependent manner. Interestingly, ANXA2, has also been found in association with lipid rafts (3), potential sites of signal transduction and membrane trafficking. In this study we determined the effect of oscillating fluid flow (OFF) on ANXA2 phosphorylation, the effects of ANXA2 gene knockout on OFF-induced prostaglandin E2 (PGE2) responses and the localization of ANXA2 in relation to lipid rafts in osteoblasts.

Materials and Methods: Cell culture: MC3T3-E1 osteoblastic cells were maintained in alpha-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. siRNA targeting mouse ANXA2 was stably introduced into MC3T3-E1 cells using pSIREN-RetroQ (BD Biosciences) retroviral transduction and puromycin selection. Cells were cloned by limiting dilution and a line designated MCA2-C3 was chosen based on the magnitude of annexin 2 expression knockdown. Control cells were stably transduced with empty retroviruses and growth-selected in puromycin without cloning.

Oscillating fluid flow: Cells were exposed to fluid flow as previously described (1). Briefly, cells cultured on glass slides were mounted in a parallel plate flow chamber attached to a custom designed flow delivery device. Cells were exposed to sinusoidally oscillating flow at 1 Hz and a peak shear stress of 15 dynes/cm2. Flow media was α-MEM with 2% FBS. Immunoprecipitation: 50 μg total protein was immunoprecipitated with 2μg monoclonal mouse anti-annexin II antibody (BD Transduction laboratories) using Catch and Release® v2.0 spin columns according to the manufacturer (Upstate). After incubation overnight at 4°C, precipitated proteins were eluted with denaturing buffer and levels of protein and tyrosine-phosphorylation were determined by Western blotting. PGE2 release: Media PGE2 levels were determined using a commercially available enzyme immunoassay system (Amersham) and normalized to total protein for each sample. Fluorescence microscopy: To visualize ANXA2, cells were transfected with 4.4 μg ANXA2-YFP DNA vector and 4ul Lipofectamine 2000 (Invitrogen) in 1.5ml α-MEM without serum for 5 hours. After transfection the cells were grown in complete medium before subculturing. Transfected cells on glass slides were fixed and stained with Vybrant® Alexa Fluor® 555 Lipid Raft Labelling Kit (Molecular Probes) and DAPI.

Results: 30min of OFF stimulated tyrosine phosphorylation of ANXA2 in osteoblastic cells, figure 1.

Figure 1. Representative western blot of phospho-tyrosine levels (Tyr-P) in anti-ANXA2 immunoprecipitates from MC3T3-E1 cells exposed to static conditions or OFF for 10, 30 or 60min.

OFF-induced increases in PGE2 release were inhibited in ANXA2 deficient cells, Figure 2.

Figure 2. Effects of OFF on PGE2 levels in MC3T3-E1 cells and cells deficient in ANXA2 (ANXA2 KO). Cells were exposed to 15 dynes/cm2, 1Hz OFF for 2h. PGE2 release into the media was measured after a further 1h incubation period under static conditions. Bars represent mean PGE2 levels +/- SE. *represents a statistically significant difference, p<0.05, n=6.

ANXA2 was found to disperse throughout the cell with a significant proportion colocalizing with raft-like structures at the plasma membrane, Figure 3.

Figure 3. Fluorescent staining of raft-like structures (red; cholera toxin B/plasma membrane ganglioside GM1), ANXA2 (green; ANXA2-YFP) and the nucleus (blue; DAPI) in an MC3T3-E1 cell. Co-localization of ANXA2 with raft-like structures at the plasma membrane appears yellow.

Discussion: Attenuation of the OFF-induced PGE2 response in ANXA2 deficient cells suggests an important role for this annexin in cellular mechanotransduction. Furthermore, our data suggest that tyrosine phosphorylation during flow may be required for ANXA2 to function in this capacity. Interestingly, ANXA2 is a known substrate for protein kinases (4) and phosphorylation appears to affect its lipid binding characteristics (5). We also demonstrate co-localization of ANXA2 with lipid raft domains. Further studies will elucidate the specific role of ANXA2 in mechanosignaling and the possibility that the mechanosensor and signaling complex are located in raft-like structures.

References: