ONLINE SUPPLEMENT

TRYPTASE/PAR-2 INTERACTIONS INDUCE SELECTIVE MAPK SIGNALING AND COLLAGEN SYNTHESIS BY CARDIAC FIBROBLASTS.

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Short Title: Tryptase/PAR-2 and MAPK signaling in fibroblasts

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Collagen Production by Isolated Cardiac Fibroblasts.
Briefly, 6N HCL was added to all media samples in a 1:1 ratio and samples were maintained at 108^{o}C overnight, filtered, and subjected to vacuum centrifugation. The samples were reconstituted in citrate buffer (0.2 M) and reacted with chloramine-T for 20 min, followed by incubation with aldehyde for 20 minutes, before cooling on ice. The absorbance was read at 550 nm and compared to hydroxyproline standards ranging from 0 to 10 μg/mL.

Western Blot Analysis.
Total protein was extracted from isolated adult rat cardiac fibroblasts by homogenization of the cells with buffer containing protease inhibitor cocktail (Pierce). Protein concentrations were determined by Bio-Rad Protein Assay. 35 μg of total protein was then loaded and separated on 10% SDS-PAGE gel and transferred onto nitrocellulose membrane (BioRad). Ponceau-S staining was used to confirm equal loading and accurate transfer of proteins from the SDS-PAGE gel to the nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in TBS-0.01 % Tween for 2 hours at room temperature. For detection of PAR-2, the blots were probed with mouse anti-PAR-2 antibody (1:500, Santa Cruz Biotechnology) for 2 hours at room temperature, and then goat anti-mouse secondary antibody (1:1000, Santa Cruz Biotechnology) for 2 hours at room temperature. For α-smooth muscle actin (α-SMA), the blots were incubated with mouse anti-α-SMA antibody (1:2000, Santa Cruz Biotechnology) for 2 hours at room temperature. For ED-A fibronectin, the blots were incubated with mouse anti-ED-A fibronectin antibody (1:1000, Abcam) overnight at 4^{o}C. The blots were subsequently incubated in goat anti-mouse secondary antibody as stated previously. Blots were also probed for GAPDH, which served as a loading control [(Primary Antibody: mouse anti-GAPDH, 1:3000 at room temperature for 2 hours, Santa Cruz Biotechnology); (Secondary Antibody: goat anti-mouse IgG_{2a}, 1:5000 at room temperature for 2 hours, Santa Cruz Biotechnology)]. Immunoreaction signals were visualized with enhanced chemiluminescence (Pierce) by exposure to hyperfilm (Phenix Research Products). Densitometry analysis was performed with the BioRad GS-800 Calibrated Densitometer. PAR-2, αSMA, and ED-A fibronectin were quantified as a ratio to GAPDH.

MAPK Activation.
Total ERK and phosphorylated ERK were determined using commercially available ELISA kits from Assay Designs (Ann Arbor, MI). Total p38 and phosphorylated p38 were determined by commercially available ELISA kits from Enzo Life Science (Plymouth Meeting, PA). Total SAPK/JNK and phosphorylated SAPK/JNK were determined by commercially available ELISA Kits from Cell Signaling Technology (Beverly, MA). All determinations were performed using manufactures' protocols.