SUPPLEMENTAL DATA

Generation and validation of a Ser-150 phosphorylation specific cTnI antibody. As a tool to detect the specific cTnI Ser-150 phosphorylation we developed a rabbit polyclonal antibody directed against the human cTnI peptide TLRRVRI[pS]ADAMMQAC including the phosphorylated Ser-150 residue (pTnI 150; 21st Century Biochemicals). Raw serum was immunodepleted against the non-phosphate containing peptide followed by affinity purification against the cTnI Ser-150 phosphate containing peptide of the same sequence. To demonstrate the specificity of this antibody we conducted both 1-D and 2-D IEF Western blot of non-phosphorylated recombinant human cTn (Control Tn) and cTn treated with PAK (PAK Tx Tn), an enzyme known to phosphorylate cTnI at residue Ser-150 (1). One dimensional SDS-PAGE of control and PAK treated cTn was subjected to Western blot probed with the pTnI 150 antibody, detected by a Horseradish Peroxidase conjugated rabbit secondary and developed by ECL Plus. The results in Supplemental Figure 1A demonstrate the antibody specifically recognized only PAK treated cTn without cross reaction to non-phosphorylated cTnI. Similar protein loading for cTn was verified by stripping the membrane and subsequently re-probing the same membrane with a mouse cTnI antibody detected using an Alkaline Phosphatase conjugated mouse secondary developed with BCIP/NBT detection. The use of these differential detection methods avoids cross recognition of the pTnI 150 antibody signal in the total cTnI blot. Similar PAK treated recombinant human cTn and untreated cTn were mixed, subjected to 2-D IEF Western blot and differentially detected as described above. The results in Supplemental Figure 1B demonstrate the pTnI 150 antibody only recognizes the 2 acidic, PAK phosphorylated cTnI species (P1 and P2) of recombinant cTn without cross reaction to the unphosphorylated cTnI species (U) identified by alignment of the same blot stripped and probed with an cTnI antibody.

Further specificity of the pTnI 150 antibody was demonstrated by treating unphosphorylated recombinant human cTn (Control Tn) or cTn containing cTnI with Ser-150 mutated to the non-phosphorylatable Ala residue (Tn S150A) with the AMPK holoenzyme. Supplemental Figure 2 demonstrates that unlike Control Tn, Tn containing the cTnI S150A mutation was not detected by the pTnI 150 antibody following increasing incubation time with the AMPK holoenzyme. These results further demonstrate the specificity of the pTnI 150 antibody for phosphorylated Ser-150 without cross reaction to non-Ser-150 phosphorylated cTnI. Taken together these findings demonstrate the pTnI 150 antibody does not recognize non-phosphorylated cTnI and therefore is a suitable tool to identify cTnI Ser-150 phosphorylation.

REFERENCES

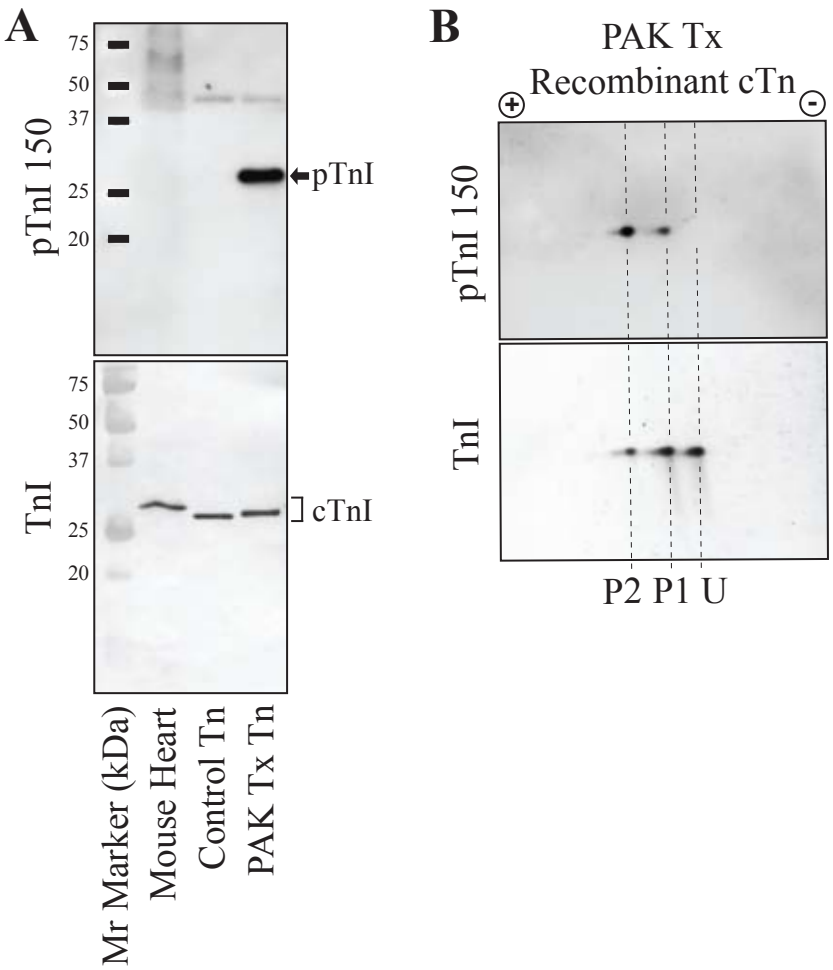
1. Buscemi, N., Foster, D. B., Neverova, I., and Van Eyk, J. E. (2002) *Circ Res* **91**, 509-516

SUPPLEMENTAL FIGURES

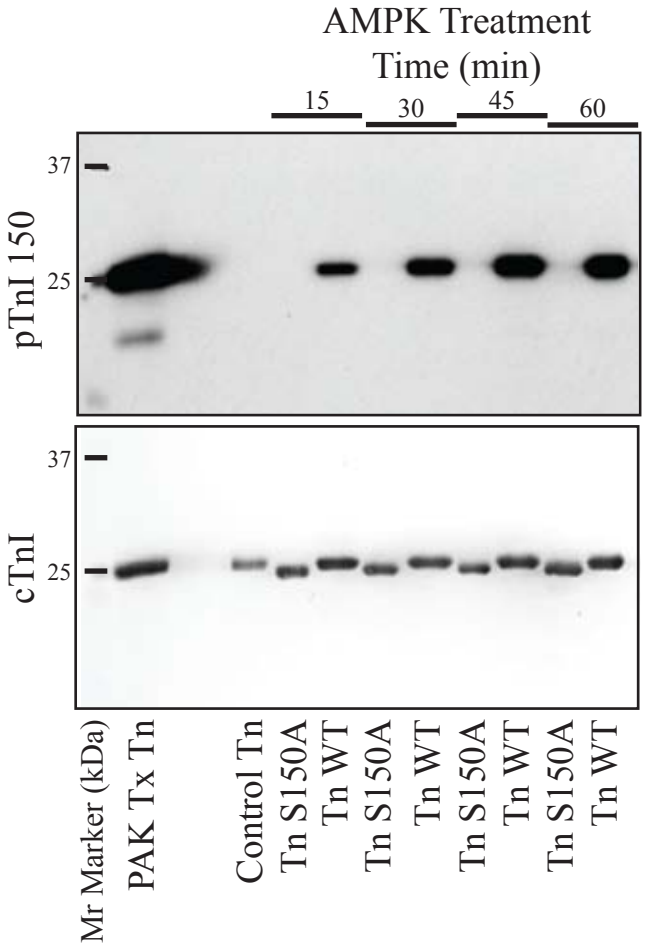
Supplemental Figure 1. The AMPK pTnI 150 antibody is specifically to Ser-150 phsophorylated cTnI. (A) Un-phosphorylated recombinant human cTn (Control Tn) and PAK treated human cTn (PAK Tx Tn) containing cTnI Ser-150 phosphorylation were separated by 1-D SDS-PAGE and subjected to Western blot with our pTnI 150 antibody. Results demonstrate the pTnI 150 antibody only recognizes the PAK Tx Tn sample in the absence of cross reaction to the non-phosphorylated Control Tn at similar cTnI loading as demonstrated by cTnI detection. (B) The

Control and PAK Tx Tn were mixed, separated by 2-D IEF and subjected to Western blot with the pTnI 150 antibody. Results demonstrate the pTnI 150 antibody only recognizes the 2 most acidic, phosphorylated cTnI species (P1 and P2) without cross reaction to the un-phosphorylated cTnI species (U) identified by differential cTnI detection and alignment.

Supplemental Figure 2. The pTnI 150 antibody does not recognize AMPK treated cTnI containing Ser-150 mutated to Ala. Un-phosphorylated recombinant human cTn (Control Tn) or cTn containing the non-phosphorylatable cTnI Ser-150 to Ala mutation (Tn S150A) were incubated in the presence of the AMPK holoenzyme and the reaction stopped at 15 min intervals. Following SDS-PAGE, Western blot with the pTnI 150 antibody only recognized Tn WT in the absence of cross reaction to the non-phosphorylated Tn S150A. These results demonstrate the specificity of the pTnI 150 antibody for Ser-150 phosphorylation.



Supplemental Figure 1



Supplemental Figure 2