Supplemental Material

Tetrahydrobiopterin Improves Diastolic Dysfunction by Reversing Changes in Myofilament Properties

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Supplemental Methods

S1.1. Analysis of Sarcomeric Protein Phosphorylation by Pro-Q Diamond Phosphoprotein Gel Stain:

Pro-Q Diamond (Invitrogen) gel stain was used to detect changes in phosphorylation states of the proteins. Myofibrils were prepared from DOCA-salt and sham models of the mice hearts, and pellets were solubilized in a non-reducing 2X Laemmli buffer (4% SDS, 20% glycerol, 0.004% bromophenol blue, and 0.125 M Tris HCl pH 6.8) [1]. 25 mM N-ethylmaleimide (NEM) was added to the standard rigor buffer with Trition X-100, the standard rigor wash buffer and the 2X Laemmli buffer. An RC-DC assay (Bio-Rad) was used to determine protein concentrations. Samples were diluted at 1:1 ratio in reducing sample buffer (8 M urea, 2 M thiourea, 0.05 M tris pH 6.8, 75 mM DTT, 3% SDS, and 0.05% bromophenol blue)[2] and approximately 10 µg of protein was loaded on to a 12% resolving 1D SDS-PAGE gel.[3, 4] The gels were stained and destained with Pro-Q Diamond according to the manufacturer's recommendations prior to imaging with a Typhoon 9410 scanner (GE Healthcare). Coomassie R-250 staining was used to normalize protein load to both MLC1 and the whole lane. Optical density of the proteins was determined using ImageQuant TL (GE Healthcare) software and results were exported to Excel for further analysis.

S1.2. Analysis of Sarcomeric Protein Phosphorylation by Western Immunoblotting:

Myofibrils were prepared from DOCA-salt and sham mice hearts with or without BH4 treatment [1] and pellets were solubilized in a reducing 2X Laemmli buffer (4% SDS, 20% glycerol, 0.004% bromophenol blue, 75 mM DTT and 0.125 M Tris HCl pH 6.8). An RC-DC

assay (Bio-Rad) was used to determine protein concentrations. Samples were diluted at 1:1 ratio in reducing sample buffer (8 M urea, 2 M thiourea, 0.05 M tris pH 6.8, 75 mM DTT, 3% SDS, and 0.05% bromophenol blue)[2]. Approximately 10 µg of protein was applied on to a 12% resolving 1D SDS-PAGE gel [3, 4] and transferred onto a 0.2 µm PVDF membrane. The blot was blocked in 5% nonfat dry milk for 1 h. Anti-phospho-ser282-MyBP-C rabbit polyclonal antibody antibody (ENZO) and MyBP-C rabbit antidoby (Santa Cruz) was used at 1:1000 dilution along with anti-rabbit HRP-conjugated secondary antibody (Sigma) at 1:100,000 dilution to detect serine 282 site specific phosphorylation of MyBP-C. Anti-phospho-ser23/24-cTnI rabbit polyclonal antibody (Cell Signaling) was used at 1:1000. Coomassie R-250 staining was used to normalize protein load to both actin and the whole lane. Optical density of the bands was measured with Image J and exported to Excel for further analysis.

References

[1] Layland J, Cave AC, Warren C, Grieve DJ, Sparks E, Kentish JC et al. Protection against endotoxemia-induced contractile dysfunction in mice with cardiac-specific expression of slow skeletal troponin I. FASEB J 2005;19:1137-9.

[2] Yates LD, Greaser ML. Quantitative determination of myosin and actin in rabbit skeletal muscle. J Mol Biol 1983;168:123-41.

[3] Fritz JD, Swartz DR, Greaser ML. Factors affecting polyacrylamide gel electrophoresis and electroblotting of high-molecular-weight myofibrillar proteins. Anal Biochem 1989;180:205-10.

[4] Vahebi S, Kobayashi T, Warren CM, de Tombe PP, Solaro RJ. Functional effects of rho-kinase-dependent phosphorylation of specific sites on cardiac troponin. Circ Res 2005;96:740-7.

Supplemental Figure legend

Figure S1. Phosphorylation levels of myofilaments proteins normalized by total protein. (A-F) Phosphorylation levels of myofilament proteins as assessed by ProQ. (A) MyBP-C, (B) TnT3, (C) TnT4, (D) TnI, (E) MLC2 (F) titin. Data were normalized to total protein and statistically analyzed using JMP statistical software by two-way ANOVA followed by Student's t-test. N=4 mice per group.

Figure S2. Phosphorylation levels of myofilaments proteins normalized by MLC1. (A-F) Phosphorylation levels of myofilament proteins as assessed by ProQ. (A) MyBP-C, (B) TnT3, (C) TnT4, (D) TnI, (E) MLC2 (F) titin. Data were normalized to MLC1 and statistically analyzed using JMP statistical software by two-way ANOVA followed by Student's t-test. N=4 mice per group.

Figure S3. Phosphorylation levels of MyBP-C and cTnI. (A) Phosphorylation levels of myofilament proteins as assessed by Western blotting against specific antibodies, Phospho-ser282-MyBP-C, MyBP-C, phospho-Ser23/24-cTnI, and cTnI. (B) SDS-PAGE. Densitometry of Western blotting using phospho-Ser282-MyBP-C (C) and phospho-Ser23/24-TnI (D) normalized to actin. (E) Phosphorylation levels from TnI and MyBP-C were correlated. Data were normalized to actin and statistically analyzed using JMP statistical software by two-way ANOVA followed by Student's t-test. N=4 mice per group.

Figure S4. Glutathionylation levels of MyBP-C. Whole blot image of MyBP-C glutathionylation level against anti-glutathione antibody.

Supplemental Figures

Figure S1

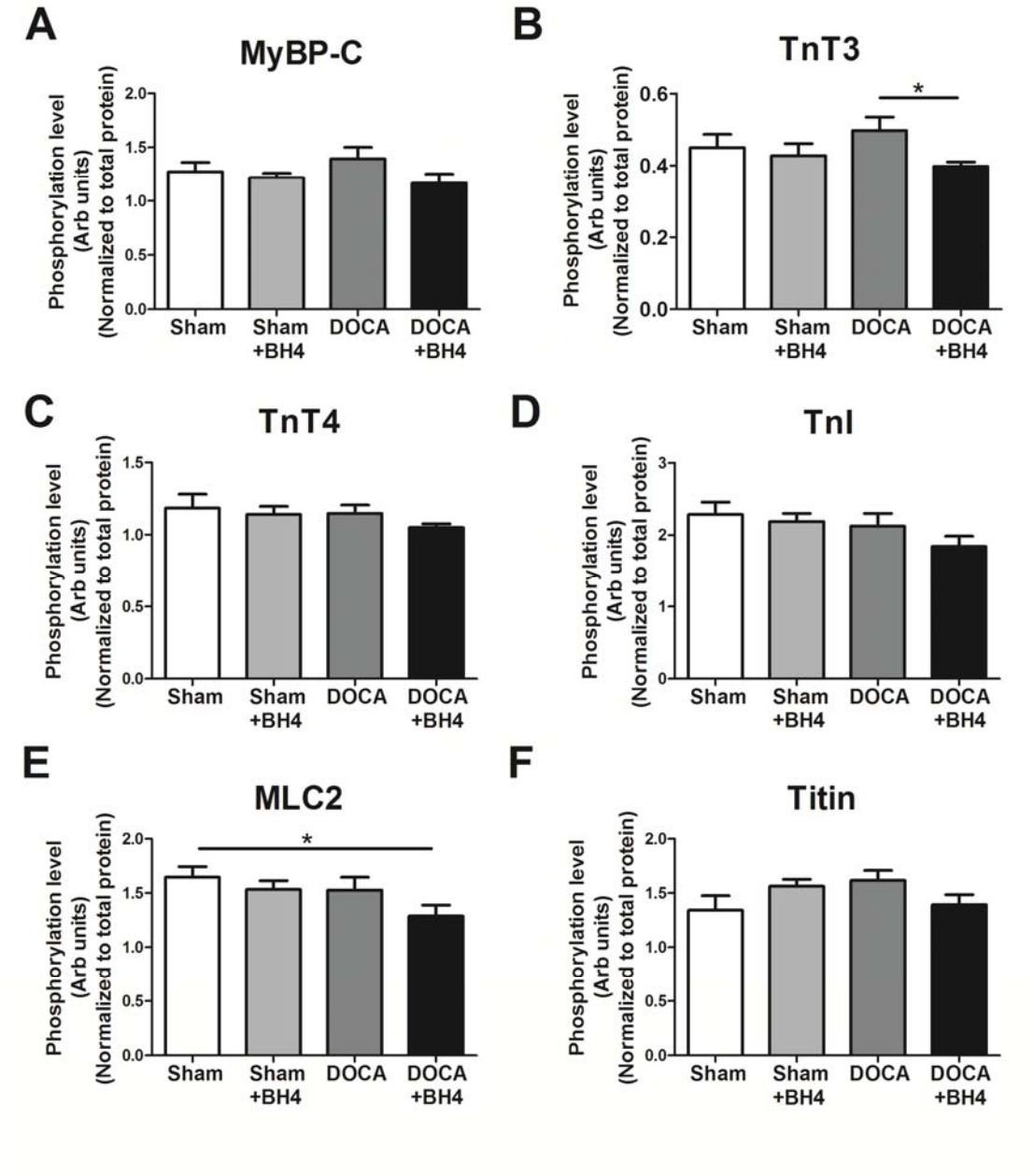


Figure S2

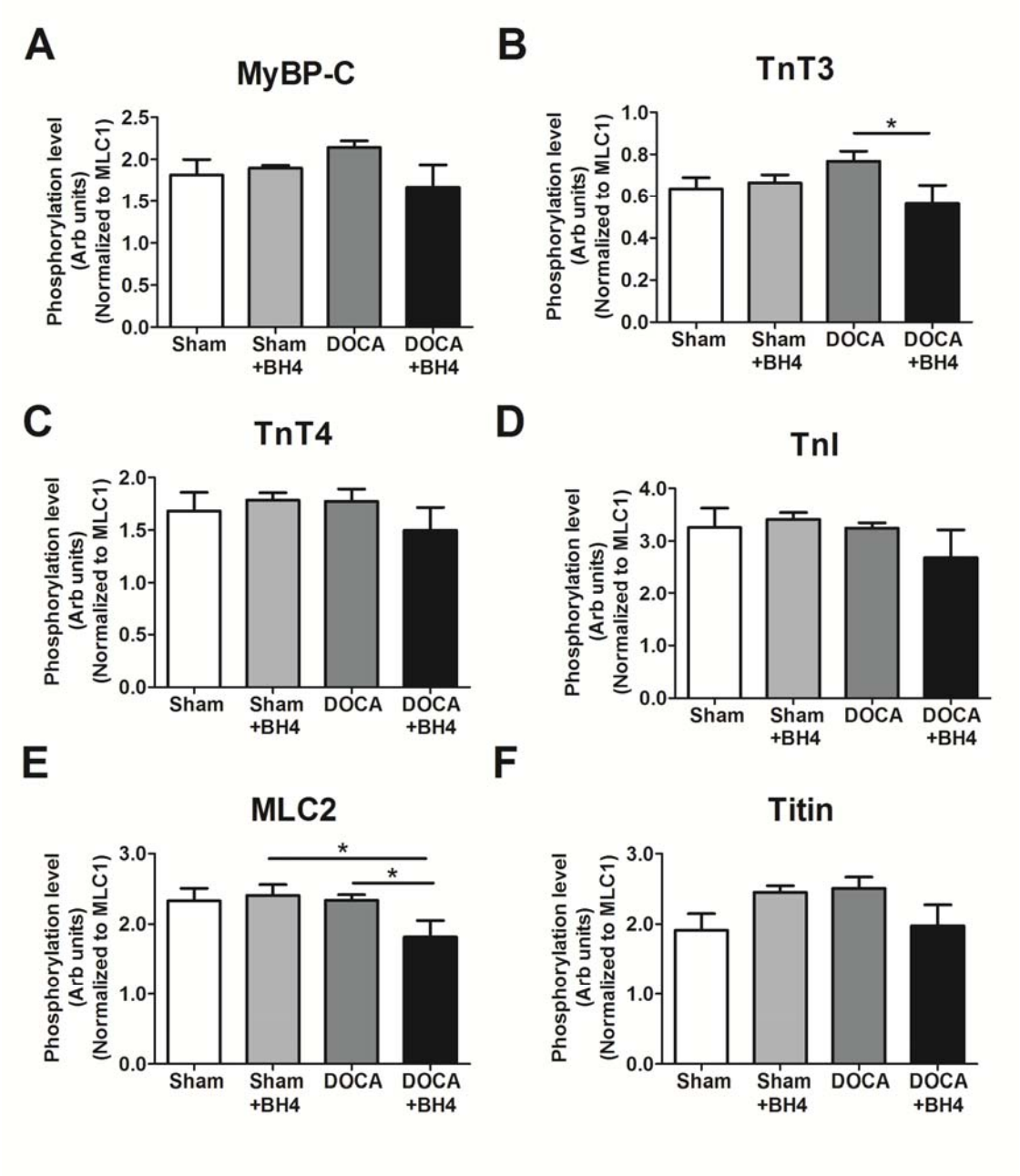

A
MyBP-C
Phosphorylation level (Arb units) (Normalized to MLC1)
2.5
2.0
1.5
1.0
0.5
0.0
Sham
Sham +BH4
DOCA
DOCA +BH4
B
TnT3
Phosphorylation level (Arb units) (Normalized to MLC1)
1.0
0.8
0.6
0.4
0.2
0.0
*
Sham
Sham +BH4
DOCA
DOCA +BH4
C
TnT4
Phosphorylation level (Arb units) (Normalized to MLC1)
2.0
1.5
1.0
0.5
0.0
Sham
Sham +BH4
DOCA
DOCA +BH4
D
TnI
Phosphorylation level (Arb units) (Normalized to MLC1)
4.0
3.0
2.0
1.0
0.0
Sham
Sham +BH4
DOCA
DOCA +BH4
E
MLC2
Phosphorylation level (Arb units) (Normalized to MLC1)
3.0
2.0
1.0
0.0
*
*
Sham
Sham +BH4
DOCA
DOCA +BH4
F
Titin
Phosphorylation level (Arb units) (Normalized to MLC1)
3.0
2.0
1.0
0.0
Sham
Sham +BH4
DOCA
DOCA +BH4

Figure S3

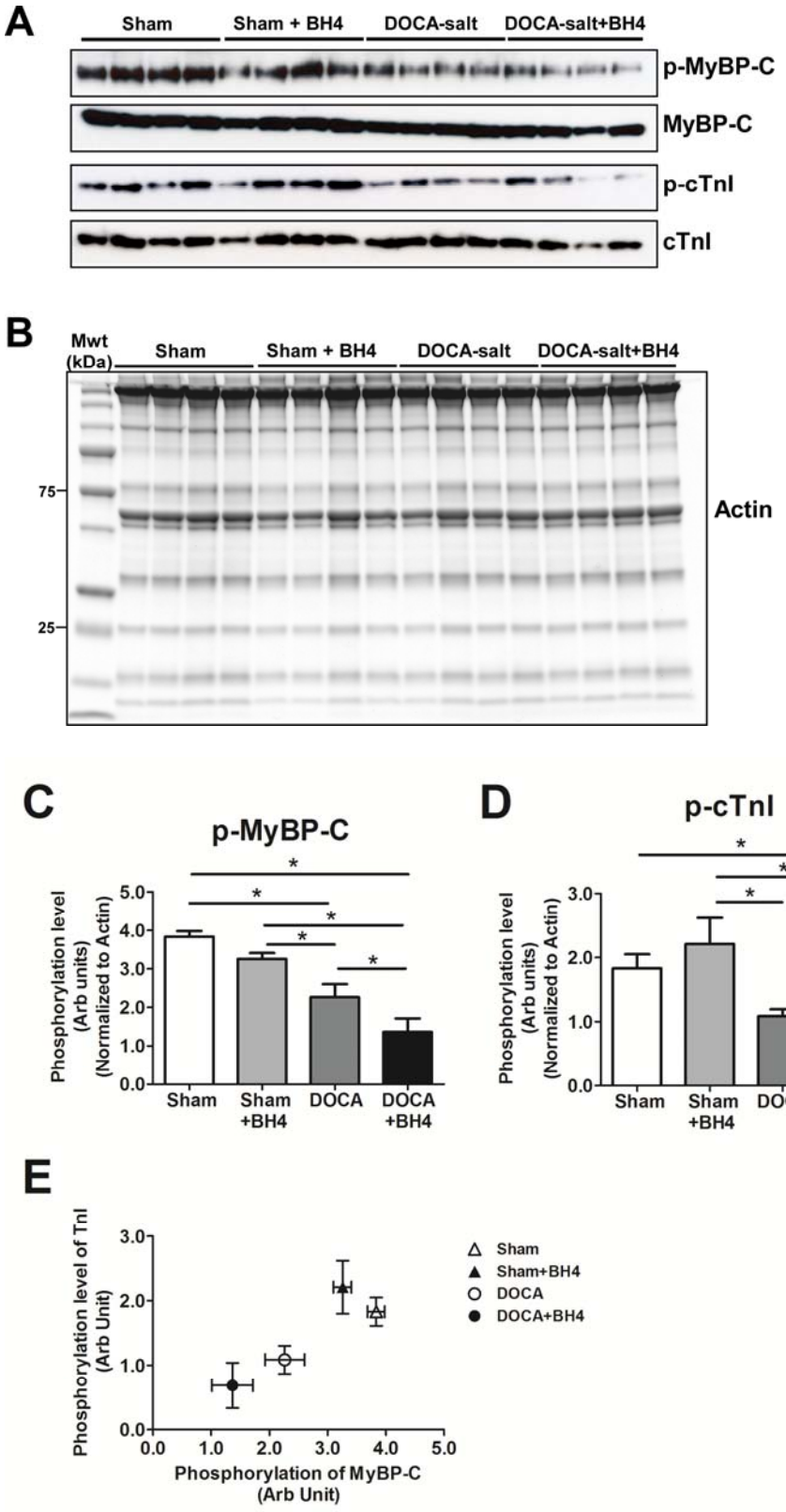
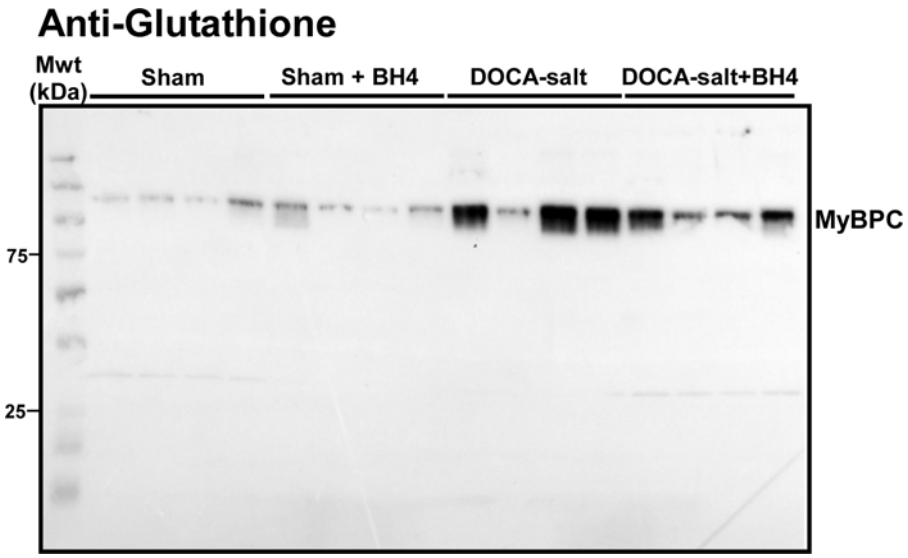

A
Sham
Sham + BH4
DOCA-salt
DOCA-salt+BH4
p-MyBP-C
MyBP-C
p-cTnI
cTnI
B
Mwt (kDa)
Sham
Sham + BH4
DOCA-salt
DOCA-salt+BH4
75
25
Actin
C
p-MyBP-C
Phosphorylation level (Arb units) (Normalized to Actin)
Sham
Sham +BH4
DOCA
DOCA +BH4
D
p-cTnI
Phosphorylation level (Arb units) (Normalized to Actin)
Sham
Sham +BH4
DOCA
DOCA +BH4
E
Phosphorylation level of TnI (Arb Unit)
Phosphorylation of MyBP-C (Arb Unit)
Sham
Sham+BH4
DOCA
DOCA+BH4

Figure S4


Anti-Glutathione
Mwt
(kDa)
Sham
Sham + BH4
DOCA-salt
DOCA-salt+BH4
75
25
MyBPC