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Cardiac Nitric Oxide Synthase-1 Localization Within the Cardiomyocyte is Accompanied by the Adaptor Protein, CAPON

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Abstract

The mechanism(s) regulating nitric oxide synthase-1 (NOS1) localization within the cardiac myocytes in health and disease remains unknown. Here we tested the hypothesis that the PDZ binding-domain interaction between CAPON (*ca*rboxy-terminal *P*DZ ligand of *NOS1*), a NOS1 adaptor protein, contributes to NOS1 localization in specific organelles within cardiomyocytes. Ventricular cardiomyocytes and whole heart homogenates were isolated from sham and post-myocardial infarction (MI) wild-type (C57BL/6) and NOS1$^{-/-}$ female mice for quantification of CAPON protein expression levels. NOS1, CAPON, xanthine oxidoreductase and Dexras1, a CAPON binding partner, were all present and enriched in isolated cardiac sarcoplasmic reticulum (SR) fractions. CAPON co-immunoprecipitated with the mu and alpha isoforms of NOS1 in whole heart lysates, and co-localization of CAPON and NOS1 was demonstrated in the SR and mitochondria with dual immuno-gold electron microscopy. Following MI, CAPON and NOS1 both redistributed to caveolae and co-localized with caveolin-3. In addition, following MI, expression level of CAPON remained unchanged and Dexras1 was reduced, CAPON binding to xanthine oxidoreductase was augmented and the plasma membrane calcium ATPase (PMCA) increased. In NOS1 deficient myocytes, CAPON abundance in the SR was reduced, and redistribution to caveolae and PMCA binding was absent. Together these findings support the hypothesis that NOS1 redistribution in injured myocardium requires the formation of a complex with the PDZ adaptor protein CAPON.

Keywords

Nitric oxide synthase (NOS1); Heart failure; CAPON; PDZ- interaction; PMCA; Dexras1

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Disclosures

None

Introduction

The production of nitric oxide (NO) is governed by three major nitric oxide synthase (NOS) isoforms; neuronal NOS (NOS1), inducible NOS (NOS2) and endothelial NOS (NOS3). In the heart, NOS3 is localized to the plasma membrane, and NOS1 to the sarcoplasmic reticulum and mitochondria, where they exert site specific effects[1]. Of the three isoforms, NOS1 uniquely contains a PDZ domain. Importantly, NOS1 redistributes from the SR to the sarcolemmal membrane following injury and may have functional consequences, protecting the heart by inhibiting calcium overload. It is established that PDZ binding domains are among the most abundant protein interacting domains in organisms [2]. In the brain, the targeting of NOS1 to discrete site is mediated by variety of adaptor proteins[3-7]. An important example of a CNS NOS1 adaptor protein is CAPON (*ca*rboxy-terminal *P*DZ ligand of *NOS1*)[4;8]. Accumulating evidence implicate that PDZ domain-containing proteins function not only as scaffolds but also as mediators of targeting and activity of the associated protein[2;7].

Interest in the cardiac role for CAPON, also known as NOS1AP, is increasing due to several genetic studies that report an association between CAPON gene polymorphisms and QT interval[9-12]. Accordingly, the objective of this study was to test the hypothesis that presence of CAPON in the heart contribute to NOS1 localization in health and disease. Our results reveal that a CAPON-NOS1 complex is present in the sarcoplasmic reticulum, and is part of a macromolecular complex also containing Dexras1 and xanthine oxidoreductase. Following myocardial infarction, NOS1 translocation to caveolae is accompanied by CAPON. In the absence of NOS1, CAPON redistribution after MI does not occur. These results reveal an important role for the adaptor protein regulating NOS1 spatial localization and may have implications for NOS1 localization in states of myocardial injury.

Materials and Methods

Animals

We studied 2-3 months old female C57BL/6 (WT, Jackson Laboratories, Bar Harbor, ME, USA) and transgenic female mice with homozygous deletions for NOS1 bred on a C57BL/6 background (Jackson Laboratories, Bar Harbor, ME, USA). All protocols and experimental procedures were approved by the Institutional Animal Care and Use Committee of The Johns Hopkins University School of Medicine and University of Miami, Miller school of Medicine.

Induction of Myocardial Infarction (MI)

Myocardial infarction was performed as previously described [13], with minor modifications. Briefly, MI was surgically induced by permanent ligation of left anterior descending coronary artery (LAD) 1-2 mm from the tip of the left atrium through open thoracotomy using 7:0 propylene suture (Ethicon; Somerville, NJ). The surgeries were performed under general anesthesia (intraperitoneal injection of 0.02 mg/g etomidate and subcutaneous injection of 12 mg/kg injection buprenorphine) and mechanical ventilation (Minivent 845, Harvard Apparatus). MI was confirmed by blanching as well as a follow up with cardiac ultrasound. All sham models also had open thoracotomy. The needle was passed under LAD without ligation and then chest wall was closed.

Echocardiography Measurements

The measurements were performed for 19 selected mice at base line and two weeks follow-up of post- surgery shams and infarcted mice. Using Vevo 770 (visual Sonics, Toronto, Canada) imaging system, long and short axis 2D parasternal views and short axis M mode

views were taken under general anesthesia with isoflurane inhalation through nose mask. The heart rate was kept above 400 bpm with controlled temperature (approx. 37°C). Ejection fraction, contractile parameter, was defined in three long axis and viewed as measure of infarct size and contractility. Left ventricular dimensions at end diastole (LVIDd) and systole (LVIDs) were defined to estimate cardiac dilatation, remodeling and functional damage following MI.

Isolation of Cardiomyocytes

Cardiac myocytes were prepared from mouse hearts as described in detail by Khan *et a*l [14].

Preparation of Cardiac Sarcoplasmic Reticulum (SR)

The preparation of SR was followed by using the modified method of Xu *et al* [15]. Whole hearts from C57BL6 mice (WT, $n = 6$) were pooled and homogenized three times (15 sec each time) by a polytron homogenizer in three volumes of ice-cold lysis buffer (Cell Signaling Technology, Boston, MA) with a protease inhibitor tablet (Roche Applied Science, Indianapolis, IN, USA). Homogenates were centrifuged at 1,000 × *g* for 20 min, the supernatant was collected and centrifuged at 10,000 × *g* for 20 min, and then re-centrifuged again at 200,000 × *g*. The final pellet containing the crude microsomal fraction was suspended in the lysis buffer and further fractionated on a five-step gradient consisting of 44%, 40%, 36%, 32%, and 28% sucrose. The gradients were centrifuged for 6 h at 103,700 × *g*, fractions were collected and diluted in two volumes of 0.4 M KCl, and re-centrifuged for 60 min at 100,000 × *g*. Pellets were resuspended in solubilization buffer and stored at −80°C. Purified SR fractions were resolved electrophoretically and probed with primary antibodies including a polyclonal rabbit antibody detecting C-terminus of CAPON[16] (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-NOS1 (BD Transduction Laboratories, San Diego, Ca, USA) SERCA2a (Affinity Bioreagents, USA), Dexras1 (Millipore) and xanthine oxidase (Neomarker, Fremont, Ca). SERCA2a was chosen as positive SR marker for purified SR fractions.

Co-Immunoprecipitation of NOS1, Dexras1, PMCA and XOR with CAPON

Total heart proteins from whole lysate were immunoprecipitated with either CAPON or NOS1 antibody in the presence of protein A/G-agarose beads. The immunoprecipitated proteins were resolved by electrophoresis. Identified protein bands were probed with anti-CAPON (amino acids 304-503 mapping the C-terminus), anti-NOS1, Dexras1, plasma membrane calcium ATPase (Abcam, USA) or XOR antibody.

Immuno-fluorescent Labeling

Cardiomyocytes were isolated from the left ventricle as previously described [14]. Briefly, freshly isolated cells were fixed in 2% formaldehyde in phosphate-buffered saline (PBS; pH 7.4, 10 min), rinsed in PBS, the unreacted aldehyde groups were quenched in 100 mM glycine in PBS (10 min) followed by rinsing in PBS. The cells were permeabilized for 10 min in 0.025% Triton X-100 in PBS, rinsed and blocked with 10% normal donkey serum. Cells were simultaneously dual labeled with anti-goat caveolin-3 (1:200, Santa Cruz Biotechnology), and/or anti-rabbit CAPON (1:200, Santa Cruz Biotechnology). Primary antibodies were diluted either 1:50 or 1:200 in buffer containing 18 mM sodium citrate buffer, 2% normal goat serum, 1% BSA, and 0.02% sodium azide (pH 7.4) were added and incubated overnight on an orbital shaker. After washing in PBS, the cells were incubated for 2 h with a fluorescent (Alexa 488, green fluorescence and/or Alexa 546, red fluorescence) secondary antibody (1:500 dilution, Molecular Probes, Oregon). After washing, fluoromount-G mounting media was added to a small volume of cell suspension, mixed thoroughly and mounted on glass slides, cover slipped and stored inverted in the dark at 4

°C. The slides were viewed on a Zeiss Axiovert 200 confocal microscope with 510-Meta confocal laser scanning module and digital images were obtained. The excitation/emission wavelengths for the donkey anti-goat antibody were 543/560 and for the donkey anti-rabbit antibody were 488/505.

Immuno-gold Labeling

Immuno-gold electron microscopy was performed by standard procedures[17]. Briefly, adult WT and NOS1$^{-/-}$ sham and MI operated mice were deeply anesthetized, hearts were harvested and perfused with 4% formaldehyde in PBS and fixed overnight at 4°C. Fixed tissue was cryo-preserved in 2.3 M sucrose with 20% polyvinylpyrrolidone and frozen in liquid nitrogen. Ultrathin (70 to 90nm) cryo-sections were cut with a Leica Ultracut UCT microtome and placed on formvar film, carbon coated, glow discharged nickel grids. The cryo-sections were labeled with 10 ug/ml of polyclonal anti-CAPON, monoclonal anti NOS1 and monoclonal anti-caveolin-3 primary antibody followed by goat anti-rabbit or mouse colloidal gold (6 or 12 nm) at a 1:20 dilution, (Jackson Immuno Research.). The grids were then contrasted and stained by the PVA-embedding method[17] Cryo-sections were observed with a Hitachi 7600 transmission electron microscope operated at 80kV. Digital images were taken with an AMT CCD 1K by 1K camera with AMTv542 software.

Western-Blot Analysis

This analysis was performed to compare expression levels of CAPON and other proteins from heart tissue of WT and NOS1$^{-/-}$ sham and MI operated mice. Isolated myocytes or whole hearts were homogenized in cold cell lysis buffer (Cell Signaling) with one tablet of complete protease inhibitors (Roche Diagnostics). Total protein concentration was quantified with the bicinchoninic acid assay (Pierce), then 30-50ug of total protein was separated in 7%, 10% Bis-Tris SDS-polyacrylamide gels (Invitrogen). The proteins were transferred onto PVDF membranes and blocked for 1hr with 5% nonfat dry milk. After blocking, the membranes were incubated with primary antibody overnight at 4°C. Further the membranes were incubated for 2 hours with Horseradish Peroxidase-conjugated secondary antibody. Immuno-blots were detected using enhanced chemiluminescent reagents (SuperSignal, Pierce) and analyzed with a digital densitometer (XRS, Bio-Rad). The membranes were then stripped off from the antibodies and reprobed for monoclonal anti-GAPDH antibodies (Research Diagnostics Inc.). Mouse brain tissue homogenate was used as a positive control for NOS1.

Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM). Data between groups were compared using Student *t*-test. The criterion of statistical significance was $*P<0.05$.

Results

Myocardial infarction

MI was induced by LAD ligation, and subsequent LV dysfunction was confirmed by echocardiography. Infarcted mice exhibited reduced ejection fractions ($P<0.001$, n=10, figure S1) while ejection fraction (EF) in sham operated mice remained unchanged (n=9). Infarcted mice showed considerable dilatation and remodeling with left ventricular diameter increasing from baseline of 3.21±0.16mm to 4.13±0.19 ($P<0.001$) at end-diastole (Supplement Figure S1), which is a measure of substantial infarction compared to sham operated mice (Supplement Fig.1).

Co-expression of CAPON with NOS1 in the SR

We used several immuno-blotting strategies to test the prediction that CAPON is localized within the heart in association with NOS1. We first assessed the subcellular localization of the peptide in WT sham and MI mice. Indeed, we demonstrated that CAPON is present in the main SR membrane fraction (fraction 1&2) as confirmed by detection of the marker protein SERCA2, which was also consistent with co-localization of NOS1 in the SR (Fig. 1a). The four fractions relate to the SR sections collected from sucrose gradient preparation. Fractions 1-4 are 28%, 32%, 36% and 40%, respectively. The purified SR fractions were collected at 28 (#1) and 32% (#2) sucrose gradient, the fractions collected at 36 and 40% were mixed with junctional terminal cisternae. Protein concentration of each fraction was determined prior to Western-blot experiments.

As previously shown by Xu *et al*[15], NOS1 in brain homogenate (alpha subtype, 155 KDa) has a slightly lower molecular weight than in the heart (mu subtype, 160 KDa) (Fig. 1a). A protein-protein interaction between CAPON and NOS1 was demonstrated by separate co-IP techniques. For this purpose NOS1 was first immuno-precipitated, the blot was then reprobed to identify CAPON by two separate anti-CAPON antibodies[16], one identifying the polypeptide at its C- (Fig. 1c) and the other at its N-terminus (not shown). In control experiments, NOS1 did not co-IP with CAPON in NOS1 deficient mice (Fig. 1b).

Absence of CAPON in plasma membrane, Presence of CAPON in the SR

Further evidence for the subcellular localization of CAPON was sought using fluorescence and electron microscopy. Dual fluorescent labeling of cardiomyocytes with CAPON and caveolin-3 antibody was visualized with laser confocal microscopy. CAPON exhibited a staining consistent with a t-tubular pattern. However, CAPON did not co-localize with caveolin-3 (Fig. 2). Immuno-electron microscopy demonstrated CAPON and NOS1 co-localized in the Z-line of the SR (Fig. 3a) while only caveolin-3 was found in sarcolemmal caveolae (Fig. 3b). Importantly, CAPON and NOS1 were both visualized in the mitochondria, another site of NOS1 localization (Supplementary Figure S2).

Expression and Translocation of CAPON post MI

An important issue regarding NOS1 in the heart is the loss of its localization following cardiac injury[18;19]. Accordingly, we first examined CAPON abundance in tissues following MI (Fig. 4a). CAPON abundance was not altered post-MI in WT whole heart (Fig. 4a) or isolated myocytes (data not shown), whereas CAPON abundance decreased in $NOS1^{-/-}$ mice post-MI (Fig. 4a). Importantly following MI, NOS1 localization was redistributed and this was accompanied by CAPON. Dual immuno-gold electron microscopy demonstrated the presence of NOS1 and CAPON in sarcolemmal caveolae of WT mice two weeks post-MI (Fig. 3c&d) co-localized with caveolin-3. There was no residual binding from either of the secondary gold-labeling particles (data not shown).

CAPON localization in $NOS1^{-/-}$

To test whether redistribution of CAPON required formation of a complex with NOS1, we performed MI in NOS1 deficient mice. CAPON abundance was reduced in $NOS1^{-/-}$ (Fig. 4b) but found to be localized to the SR by immuno-EM (Fig. 3e&f). Following MI, CAPON did not redistribute to caveolae (Fig. 3g&h).

NOS1-CAPON binding partners

Next we tested for additional binding partners of NOS1 and CAPON in the heart. Dexras1, a binding partner of CAPON in the brain, was shown to be present in the heart. Subcellular fractionation of heart tissues demonstrated both Dexras1 and xanthine oxidoreductase

(XOR) in the main SR fractions (Fig. 5a). XOR and Dexras1 (not shown) both co-immunoprecipitated with NOS1-CAPON complex showing augmented level of XOR post-MI (Fig. 5b). In addition following MI, Dexras1 abundance declined (Fig. 4b).

Binding to PMCA

PMCA is another reported sarcolemmal ion pump to which NOS1 binds. Accordingly we tested whether CAPON is involved in this interaction. Indeed, co-IP experiments demonstrated that CAPON and PMCA exhibit protein-protein interaction. Interestingly, post MI this interaction increases consistent with a redistribution of the CAPON-NOS1 complex following injury (Fig. 5 c & d). Here, the co-immunoprecipitation of PMCA with CAPON was evaluated based on the main PMCA protein band at 140-KDa, other detected PMCA bands at 180 and 90 KDa were not analyzed as they are reported by Hammes *et al*[20] to be aggregation and proteolytic products of the protein (Fig. 5 c & d).

Discussion

The major new finding reported here is that the PDZ containing adaptor protein, CAPON, participates in neuronal NO synthase (NOS1) localization in the mammalian heart. CAPON is bound to NOS1, and the protein-protein complex is found in the SR and mitochondria of the normal heart. Following injury, CAPON showed to accompany NOS1 in its redistribution within the myocyte to sites at the cell membrane. Additionally, other adaptor proteins such as Dexras1 are also part of the NOS1 macromolecular complex within the heart. These finding offer novel insights into the mechanisms underlying NOS1 following cardiac injury.

Among all three mammalian NOS isoforms, NOS1 is the only isoform containing an extended PDZ-domain that mediates protein binding [21]. In the brain, the adaptor protein CAPON regulates NOS1 stability, changes in sub-cellular localization and possibly its expression during synapse formation [22-24]. Segalat *et al* [22] demonstrated the elevated expression profile of CAPON-NOS1 in mouse muscle to be related to muscle growth, injury and repair, as in muscular dystrophy. Chang *et al* [16] have revealed the presence of CAPON in the heart and its over expression in cardiomyocytes caused inhibition of L-type calcium channel. Heretofore, a role for CAPON in regulating NOS1 localization in the heart has not yet been established. This issue has become of significant importance due to several reports that NOS1 redistributes following cardiac injury on one hand, and another series of work that describes a potential cardiac role for CAPON within the heart.

NOS1 redistribution following injury

Following cardiac injury or in cardiomyopathy, NOS1 translocates from SR to plasma membrane[18;19]. This redistribution is of functional significance as increased NOS1 at the membrane can inhibit L-type Ca^{2+} currents and protect from increased intracellular calcium levels following cardiac injury, a process that is regulated by nitrosylation of the channel[25]. Chang *et al* [16], by using an over-expression model detect CAPON in the cytoplasm of cardiomyocytes, supporting the notion that excess production of CAPON could lead to altered subcellular compartmentation. Initially, association of CAPON to NOS1 was established in the brain[8;23]. Here, we demonstrate the use of several techniques confirming that CAPON is indeed co-localized with NOS1 in the SR of mammalian heart. Our results reveal that under physiologic conditions, the NOS1-CAPON complex is found within the SR and mitochondria, two sites previously documented as sites for NOS1 localization and activity[1;18]. Following MI, while CAPON abundance is not altered, its localization is changed in a manner parallel to that of NOS1. In this regard NOS1 and CAPON were found in association with caveolin-3[26] and the plasma membrane calcium

pump (PMCA) [26-28]. As previously reported NOS1 interacts with the PDZ domain of PMCA4b in the membrane[27]. The specific requirement for the NOS1-CAPON complex in this redistribution within the cell was established by the lack of CAPON translocation in NOS1$^{-/-}$ mice. Not only was there no redistribution, but CAPON abundance decreased in the NOS1 hearts, thus strongly supporting the importance of this interaction in stabilizing the NOS1 translocation in MI hearts. This key finding strongly supports the idea that routing NOS1 from the SR to plasma membrane requires the PDZ-binding interaction of CAPON. Consequently, our finding is in full agreement with the established notion that PDZ domain-interactions function in the clustering, targeting, routing and probably regulating the activity of associating proteins[2]. As shown by using multiple techniques, CAPON's primary physiologic location in the cardiomyocyte is the SR associated with NOS1.

NOS1 is reported by us and others to be critically important post-MI, promoting survival, anti-remodeling, and offsetting pro-arrhythmic increases in L-type calcium entry into the myocytes[13;29;30]. These effects are likely to be associated with translocation of NOS1 from the SR to the cell membrane. Saraiva *et al* [13] have described a probable protective role of intact NOS1 activity in the heart after MI. Subsequently, NOS1 deficiency in knockout mice has shown to cause adverse effects in states of myocardial injury[13].

Dexras1, XOR and PMCA, binding partners of CAPON-NOS1

Dexras1 is a member of Ras family that binds via the N-terminal of CAPON to NOS1 forming a ternary complex[31]. In the central nervous system, the Dexras1 proximity to NOS1, which is mediated by CAPON, is required for Dexras1 nitrosylation, activation, and subsequent down-stream signaling to regulate iron uptake into the cell [32]. Our results confirm the presence of Dexras1 in the heart as also shown by Cheah *et al*[32]. Therefore, here we show that Dexras1 also participates in the cardiac macromolecular complex containing NOS1 and CAPON. After injury, Dexras1 abundance fell suggesting that this effector molecule does not participate in NOS1-CAPON redistribution within the myocyte. Future studies are required to delineate the downstream signaling role of Dexras1 in the heart.

Xanthine oxidoreductase (XOR) is a major source of reactive oxygen species in the heart [33]. In normal tissue the enzyme exist predominantly as xanthine dehydrogenase (150 KDa) as well as proteolytically cleaved monomers of 130 and 85 KDa. We have previously reported that XOR has a direct protein-protein interaction with NOS1 in the sarcoplasmic reticulum[13;34]. Deficiency or translocation of NOS1 increases XOR activity, which in turn impairs excitation-contraction coupling and myofilament calcium sensitivity[33]. As predicted by these previous findings, XOR co-immunoprecipitated with CAPON and showed increased level post-MI.

Another important subcellular localization for NOS1 is in association with the plasma membrane calcium pump, PMCA4b, a PDZ domain containing protein [27]. PMCA4b is localized in caveolae [35] and is known to participate in modifying growth[36], differentiation[20], apoptosis[37] and hypertrophic stimuli[35]. Following MI, we noted increased CAPON binding to PMCA4b suggesting a novel translocation site for the CAPON-NOS1 complex in settings of cardiac injury. This observation supports further the concept that CAPON participates in NOS1 subcellular localization in health and disease.

An additional line of evidence implicates CAPON (NOS1AP) in cardiovascular disease. A recent study showed that presence of CAPON accelerates cardiac repolarization by inhibition of L-type calcium channel in guinea pig heart [16]. Their finding together with the earlier studies of Arking *et al* [10], Aarnoudse *et al*[9] and as well as recent studies[11;12] on gene polymorphisms of CAPON (NOS1AP) raises the notion that CAPON and NOS1 affect

not only cardiac contractility, but also tend to mediate ventricular arrhythmias through effects on the ryanodine receptor and calcium handling, a process in part regulated by nitric oxide.

Limitation

While we were able to bolster our conclusions using tissue from the NOS1 knockout mouse before and after MI, a CAPON knockout mouse is not presently available. In the future genetic manipulation of CAPON levels in the cardiomyocyte could provide further insights into the observations reported here. As mentioned above, Chang *et al* [16] report that CAPON over-expression in cardiomyocytes results in localization of CAPON in cell membrane, adding support to the idea that alterations in the amounts of CAPON and/or NOS1 could have physiologic impact on myocyte function.

In conclusion, here we demonstrate the presence of NOS1 binding partners CAPON and Dexras1 in the cardiac sarcoplasmic reticulum. CAPON, an adaptor protein that exerts a PDZ binding interaction exhibits protein-protein associations with NOS1. With cardiac injury, CAPON abundance remains unchanged and accompanies translocation of NOS1 to the plasma membrane, while lack of NOS1 in $NOS1^{-/-}$ heart tissues did not cause translocation of CAPON to the plasma membrane. Due to this redistribution the PMCA binding levels associated with CAPON was augmented post-MI. Thus, formation of an NOS1-CAPON complex stabilizes NOS1 localization within the heart. These findings offer novel insights into the role of CAPON, newly appreciated as a genetic marker for QT duration, in the heart as a participant in NOS1 signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

NOS1	neuronal nitric oxide synthase
SR	sarcoplasmic reticulum
MI	myocardial infarction

co-IP	co-immunoprecipitation
PMCA	plasma membrane calcium ATPase
XOR	xanthine oxidoreductase

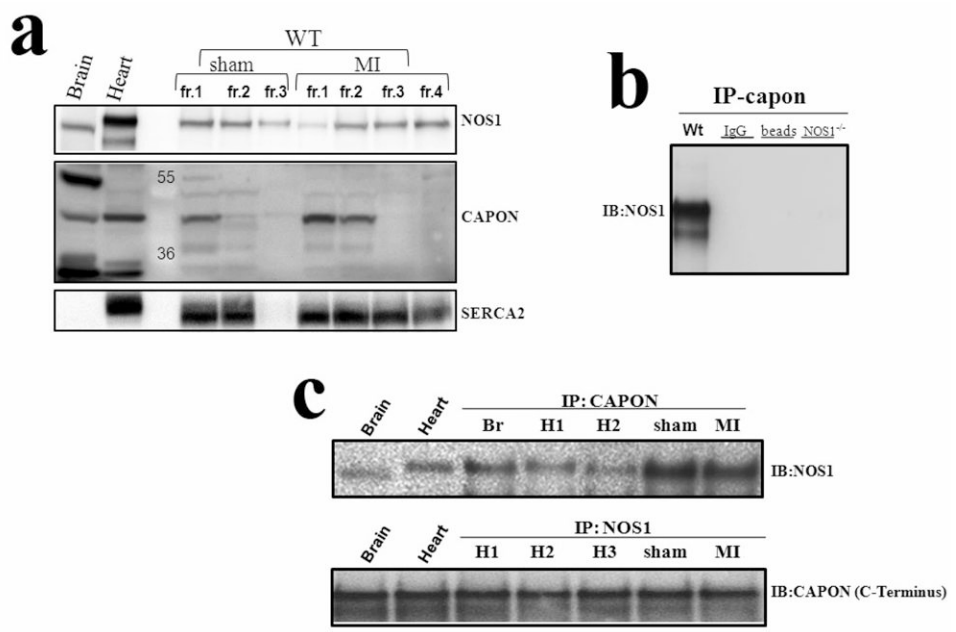

Figure 1. Detection of CAPON and NOS1 in preparations of SR fractions and by co-immunoprecipitation (co-IP) techniques in WT mice hearts

a) SR fractions (1-4) prepared by sucrose gradient (28, 32, 36, 40%), CAPON and NOS1 in subcellular SR fractions from WT sham and MI of whole heart homogenate, fraction (fr) #1 is heavy SR, # 2 light SR, # 3 &4 light SR with junctional terminal cisternae, number of pooled hearts = 6. Heart and brain lysate are positive controls. **b**) IP of CAPON and Co- IP of NOS1 from WT and NOS1$^{-/-}$ hearts, rabbit IgG and beads are negative controls. **c**) Upper panel; IP of CAPON and co-IP of NOS1.

Lower panel; IP of NOS1 and co-IP of CAPON detected by anti-CAPON antibody detecting the C-terminus epitope. In all the images; the WT whole heart lysate (Heart) and brain extracts lysate (Brain) are positive controls. H1 and H2: WT normal control heart lysates, sham and MI: operated hearts. All applied CAPON antibody detects C-terminus epitope, unless otherwise stated. Results are representative of minimum 3-5 separate experiments.

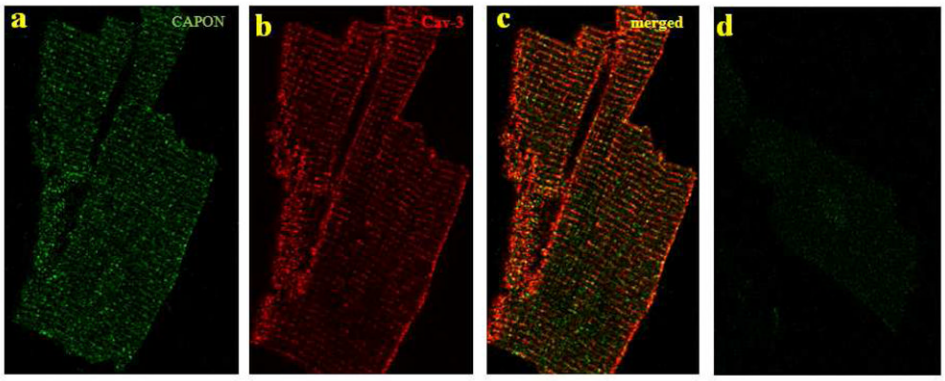

Figure 2. Dual immuno-fluorescent labeling WT cardiomyocytes for CAPON and caveolin-3 visualized by confocal microscopy

(**a**), CAPON (Alexa 488, green fluorescence), (**b**), caveolin-3, (Alexa 546, red fluorescence) and (**c**), overlay image of a and b showing the non-colocalized CAPON with caveolin-3 in the plasma membrane. (**d**), Alexa green fluorescent antibody labeling of CAPON on $NOS1^{-/-}$ cardiomyocytes. The images are representative of 3-4 experiments.

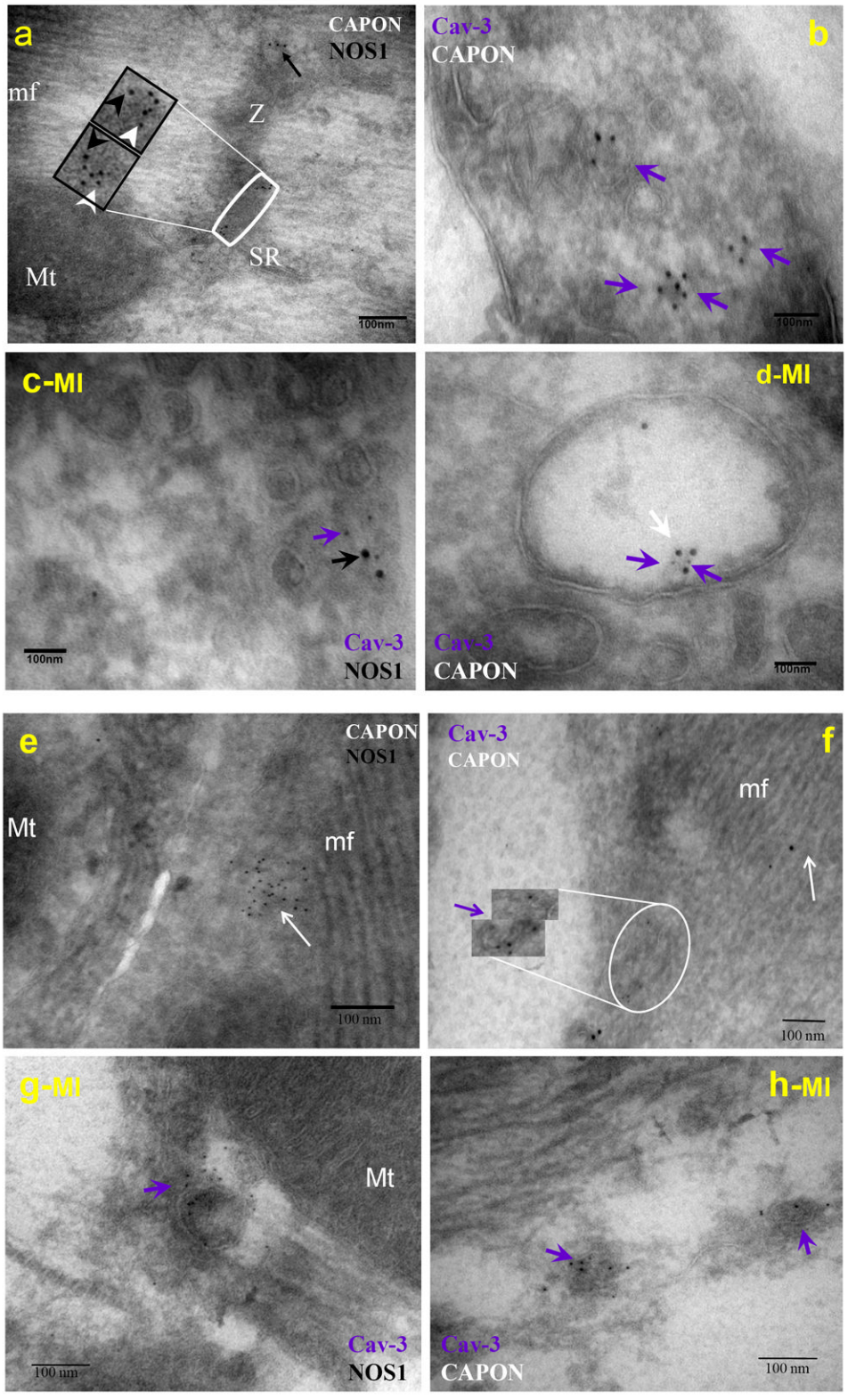

Figure 3. Detection of CAPON and NOS1 by dual immuno-gold labeling visualized by transmission electron microscopy of WT (a, b,c, and d) and NOS1$^{-/-}$ (e, f, g and h) mouse heart tissue

a) co-localization of CAPON (6-nm, white) and NOS1 (12-nm, black) using gold-conjugated secondary antibody shown in Z line and SR conjunction where t-tubule is associated to form dyad structure for CAPON and NOS1. **b)** localization of caveolin-3 (purple) in caveolae. **c, d)** depicts two weeks post-MI co-localization of CAPON and NOS1 with caveolin-3 to caveolae plasma membrane in WT, respectively. Labels; Caveolin-3 (6-nm, purple arrow), CAPON and NOS1 (12-nm, white and black arrow, respectively). In $NOS1^{-/-}$; **e)** shows presence of CAPON (6-nm-white) and absence of NOS1 (12-nm, black) in SR conjunction. **f)** presence of caveolin-3 (6-nm, purple arrow) in the caveolae in magnified squares and CAPON (12-nm, white arrow) in the SR. **g** and **h)** depicts 2 weeks post-MI, the absence of CAPON and NOS1 (12-nm) in caveolae associated to caveolin-3 (6-nm) in $NOS1^{-/-}$ mice, respectively. Image magnification: 150,000-200,000. Mt: mitochondria, mf: myofilaments, Z: Z-line, SR: sarcoplasmatic reticulum. There were no detected residual binding from any of the secondary gold-labeling particles (data not shown). Each set of images are representative from 3 individual mice tissues.

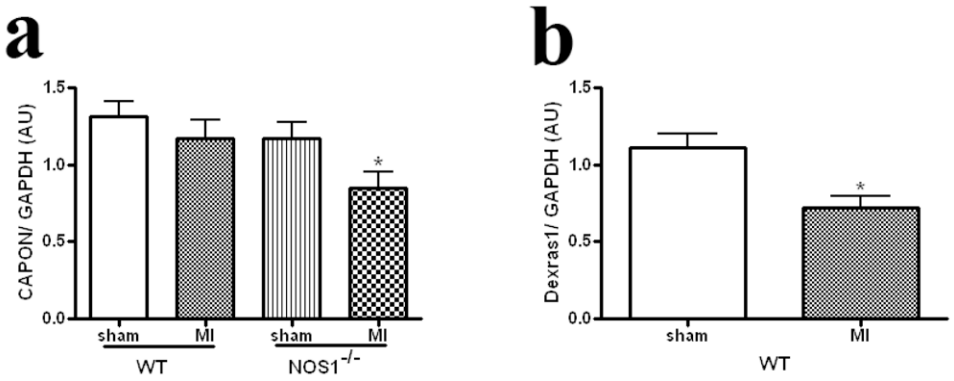

Figure 4. Protein expression level of CAPON and Dexras1 in mice hearts

a) Western-blot analysis of expression level of CAPON in WT compared to NOS1$^{-/-}$ homogenates, n=5-6. **b**) Expression level of Dexras1 in Wt heart homogenates shows significant decrease post-MI, n=5-7. *P<0.05.

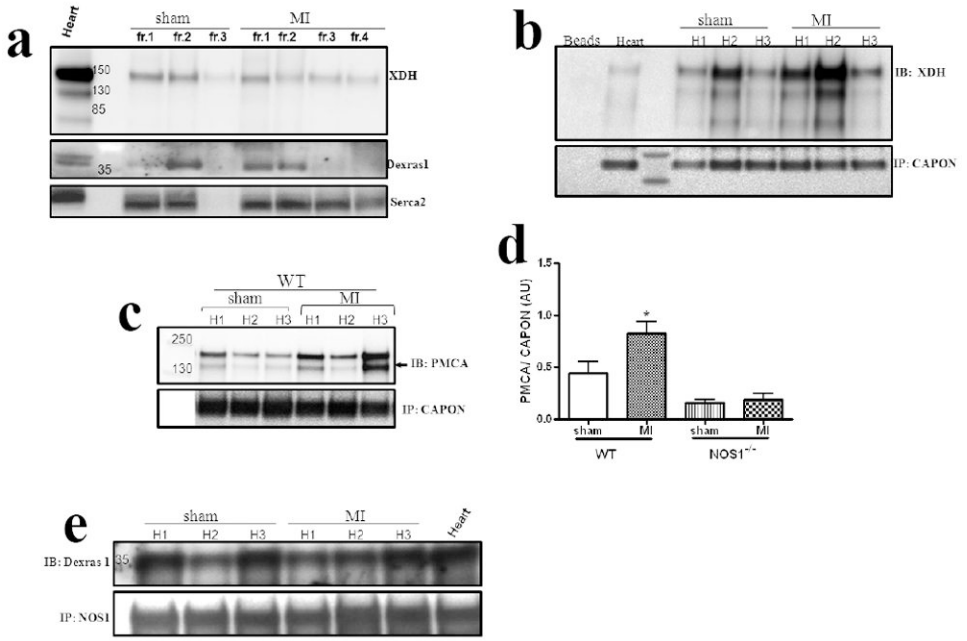

Figure 5. Detection of XOR, Dexras1 and PMCA in heart homogenates of WT mice

a) xanthine dehydrogenase (XDH is predominant form, 150KDa) and Dexras1 in subcellular SR fractions prepared from WT heart lysate, no protein was measured in fraction #4 of sham hearts. SR samples fractions are as prepared in figure 1A and Serca2 is used as SR maker protein. **b**) IP of CAPON and co-IP (IB; immunoblot) of XOR (XDH: 150KDa band is predominant, lower two bands at 130 and 85 KDa are proteolytically cleaved forms of the enzyme). **c**) IP of CAPON and co-IP (IB) of PMCA (140 KDa), protein bands at 180 and 90 are proteolytic products of the protein. **d**) Bar graph demonstrating the binding of PMCA to CAPON as performed by co-immuno-precipitation in (**c**), **P*<0.05, n=5-7. **e**) IP of NOS1 and co-IP of Dexras1. H1, H2, H3 and H4 are individual heart homogenate. Heart; WT control heart and bead is used as negative control for IgG. All western-blot images are representative of 3 separate experiments.