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(54) **Title**: PROCESS FOR DETECTING ISCHEMIC INJURY

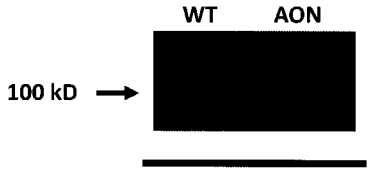

FIGURE 2A

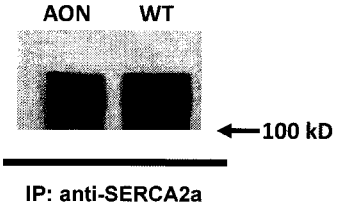

FIGURE 2B

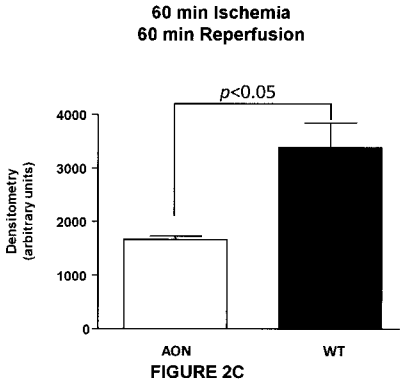

FIGURE 2C

(57) **Abstract**: A process for detecting an ischemic injury comprises determining the presence of nitrotyrosinylated fatty acid binding protein and/or myoglobin. Optionally, the process further comprises measuring the amount of nitrotyrosinylated fatty acid binding protein and/or myoglobin.

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PROCESS FOR DETECTING ISCHEMIC INJURY

[0001] This application claims priority to and the benefit of U.S. Provisional Patent Application Serial No. 61/416,492, filed November 23, 2010. Serial No. 61/416,492 filed November 23, 2010 is incorporated by reference herein in its entirety.

[0002] This invention was made with government support under grant number K08-HL-094703 awarded by National Heart, Lung, and Blood Institute. The government has certain rights in this invention.

BACKGROUND

[0003] The present exemplary embodiment relates to methods for detecting ischemic injuries. It finds particular application in conjunction with detecting myocardial necrosis due to acute coronary syndrome, and will be described with particular reference thereto. However, it is to be appreciated that the present exemplary embodiment is also amenable to other like applications.

[0004] The myocardium is the muscular tissue of the heart wall. An infarct or infarction is the localized death of tissue caused by obstructed inflow of arterial blood. Acute coronary syndrome (ACS) is most frequently caused by several diseases involving coronary arteries. These diseases are ST elevation myocardial infarction, non ST elevation myocardial infarction, and unstable angina.

[0005] Known methods for making an early diagnosis of acute coronary syndrome include examining the absolute levels of fatty acid binding protein or myoglobin. However, it would be desirable to develop other, more sensitive methods for detecting ischemic injury.

BRIEF DESCRIPTION

[0006] The present disclosure relates to methods for detecting ischemic injury. The methods generally comprise determining the presence of nitrotyrosinylated fatty acid binding protein and/or myoglobin.

[0007] Disclosed in embodiments is a method for detecting myocardial injury. The method comprises determining the presence of nitrotyrosinylated fatty acid binding protein and/or myoglobin. Optionally, the method further comprises measuring the amount of nitrotyrosinylated fatty acid binding protein and/or myoglobin.

[0008] The presence of nitrosinylated fatty acid binding protein and/or myoglobin may be determined using an anti-3-nitrotyrosine antibody and a secondary antibody.

[0009] A western blot analysis may be used to determine said presence. The western blot analysis may include the use of a primary anti-3-nitrotyrosine antibody and a secondary antibody. The secondary antibody may be either a horseradish peroxidase conjugated, Cy3- or Cy5- labeled antibody.

[0010] Also disclosed in embodiments is a method for diagnosing ischemic injury in an animal. The method comprises determining the presence of nitrotyrosinylated fatty acid binding protein and/or myoglobin in a protein sample from the animal. Said presence may be determined using a primary anti-3-nitrotyrosine antibody and a secondary antibody. The secondary antibody may be either a horseradish peroxidase conjugated, Cy3- or Cy5- labeled antibody.

[0011] The animal may be a mammal. In some embodiments, the mammal is a mouse or a human.

[0012] The protein sample may comprise any mammal sample. In some embodiments, the sample comprises a myocardial lysate.

[0013] The ischemic injury may be acute coronary syndrome. The ischemic injury may also be an ischemia-reperfusion injury.

[0014] Further disclosed is a method for diagnosing acute coronary syndrome in an animal. The method comprises rehydrating an isoelectric focusing strip using a protein sample from the animal; electrophoresing the strip on a gel; and performing a western analysis on the gel to determine the presence of nitrotyrosinylated fatty acid and/or myoglobin in the protein sample.

[0015] The protein sample may comprise any mammal sample. In some embodiments, the sample comprises a myocardial lysate.

[0016] The western analysis may use a primary anti-3-nitrotyrosine antibody and a secondary antibody. The secondary antibody may be a either a horseradish peroxidase conjugated, Cy3- or Cy5- labeled antibody.

[0017] The animal may be a human.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] FIG. 1 illustrates gel electrophoresis pictures of myocardial lysates processed according to an example of the present disclosure.

[0019] FIG. 2 illustrates gel electrophoresis pictures of myocardial lysates processed according to another example of the present disclosure.

DETAILED DESCRIPTION

[0020] The present disclosure generally relates to methods for detecting ischemic injury. The methods generally comprise determining the presence of nitrotyrosinylated fatty acid binding protein and/or myoglobin.

[0021] Disclosed in embodiments is a method for detecting myocardial injury. The method comprises determining the presence of nitrotyrosinylated fatty acid binding protein and/or myoglobin. Optionally, the method further comprises measuring the amount of nitrotyrosinylated fatty acid binding protein and/or myoglobin.

[0022] The phrase "and/or" includes each element individually or the combination of elements. For example, "determining the presence of nitrotyrosinylated fatty acid binding protein and/or myoglobin" includes: determining the presence of nitrotyrosinylated fatty acid binding protein; determining the presence of nitrotyrosinylated myoglobin; or determining the presence of both nitrotyrosinylated fatty acid binding protein and nitrotyrosinylated myoglobin.

[0023] The presence of nitrosinylated fatty acid binding protein and/or myoglobin may be determined using an anti-3-nitrotyrosine antibody and a secondary antibody.

[0024] A western blot analysis may be used to determine said presence. The western blot analysis may include the use of a primary anti-3-nitrotyrosine antibody and a secondary antibody. The secondary antibody may be either a horseradish peroxidase conjugated, Cy3- or Cy5- labeled antibody.

[0025] Also disclosed in embodiments is a method for diagnosing ischemic injury in an animal. The method comprises determining the presence of nitrotyrosinylated fatty acid binding protein and/or myoglobin in a protein sample from the animal. Said presence may be determined using a primary anti-3-nitrotyrosine antibody and a secondary antibody. The secondary antibody may be either a horseradish peroxidase conjugated, Cy3- or Cy5- labeled antibody.

[0026] The animal may be a mammal. In some embodiments, the mammal is a mouse or a human.

[0027] The protein sample may comprise any mammal sample. In some embodiments, the sample comprises a myocardial lysate.

[0028] The ischemic injury may be acute coronary syndrome. The ischemic injury may also be an ischemia-reperfusion injury.

[0029] Further disclosed is a method for diagnosing acute coronary syndrome in an animal. The method comprises rehydrating an isoelectric focusing strip using a protein sample from the animal; electrophoresing the strip on a gel; and performing a western analysis on the gel to determine the presence of nitrotyrosinylated fatty acid and/or myoglobin in the protein sample. Optionally, the amount of nitrotyrosinylated fatty acid and/or myoglobin may be measured.

[0030] An ischemia-reperfusion injury generally involves tissue damage caused by a blood supply returning to a tissue that has experienced a time period of ischemia or lack of oxygen and nutrients. The return of blood flow following ischemia typically causes inflammation and oxidative damage instead of merely restoring the tissue to its normal function. The inflammation and oxidative damage result from the induction of oxidative stress. More specifically, ischemia-reperfusion injury involves the generation of reactive oxygen species and reacting nitrogen species. Said generation is influenced by cellular protective mediators such as superoxide dismutase (SOD), catalase, and glutathione peroxidase. Abnormal calcium ion (Ca^{2+}) handling also plays a role. In particular, the accumulation of calcium and the generation of reactive species, including nitric oxide (NO); superoxide ($O_2^{\bullet -}$); and peroxynitrite ($ONOO^-$), are believed to be important.

[0031] In ischemia-reperfusion injuries and other cardiovascular disease states, increased protein tyrosine nitration, an irreversible detrimental modification of proteins mediated by peroxynitrite, is observed. Tyrosine nitration leads to decreased activity of several myocardial proteins including mitochondrial and calcium regulating proteins.

[0032] Reactive oxygen species (ROS), including superoxide and hydroxyl radicals, as well as reactive nitrogen species (RNS) such as peroxynitrite, formed either by the reaction of hydrogen peroxide with nitrite or the reaction of the free radical superoxide with the free radical nitric oxide, are involved in the pathogenesis of myocardial ischemic reperfusion injury. Myocardial cells are protected from oxidant challenge by an antioxidant network (AON) which includes enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSHPx-1). Superoxide anions are readily dismutated by superoxide dismutase (SOD) into

hydrogen peroxide, which can then be converted into hydroxyl radicals by a Fenton-type reaction or scavenged by either catalase or glutathione peroxidase. Three SOD isoenzymes have been identified in mammalian cells: copper and zinc-containing SOD (Cu/Zn SOD, SOD1), expressed as dimers in the cytoplasm; manganese-containing SOD (Mn SOD, SOD2), expressed as tetramers in the mitochondria; and the tetrameric extracellular SOD (EC SOD, SOD3). Glutathione peroxidase 1 (GSHPx-1) is localized in both the cytosol as well as the mitochondrial matrix and can utilize either lipid peroxides or H_2O_2 as substrate. Studies suggest that hearts from SOD1 and GSH-Px knockout mice are more susceptible to ischemia-reperfusion injury and that over-expression of either SOD1, SOD3, or GSHPx-1 renders the heart more resistant to ex vivo myocardial ischemia-reperfusion injury. However, in a study exploring the in vivo efficacy of over-expression of individual antioxidant enzymes, neither overexpression of GSHPx-1 nor over-expression of SOD1 was found to be cardioprotective. One possibility is that neither enzyme alone is capable of full cardioprotective efficacy. Indeed, prior work has suggested that manipulation of the expression of one enzyme may lead to an imbalance in the antioxidant system.

[0033] The methods for detecting myocardial injury may be used with any suitable detection apparatus. A non-limiting example of such an apparatus is a biomarker assay system.

[0034] The following examples are for purposes of further illustrating the present disclosure. The examples are merely illustrative and are not intended to limit the systems and methods of the present disclosure to the materials, conditions, or process parameters set forth herein.

EXAMPLES

[0035] Methods.

[0036] The investigations described conform to the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health, and were approved by The Ohio State University Medical Center Institutional Animal Care and Use Committee.

[0037] *Transgenic Mice.*

[0038] The generation of antioxidant network-overexpressing (AON) triple transgenic mice has been described previously. Constructs were prepared by cloning tagged cDNAs for human antioxidant enzymes (SOD1, SOD3 and GSHPx-1) into a vector containing the mouse H-2K^b promoter. AON mice were backcrossed for more than 10 generations onto the C57BL6 background.

[0039] Myocardial ischemia-reperfusion protocols

[0040] Ex vivo myocardial ischemia-reperfusion injury.

[0041] Hearts isolated from AON mice or wild-type (WT) littermate controls were perfused using a Langendorff system and subjected to global ischemia of 30 minutes and reperfusion of 60 minutes. At the end of reperfusion, the heart was processed for subsequent analysis of myocardial infarct size, ROS generation and protein modification as described below. Ex vivo hearts were trimmed of all atria and fat while still cannulated, and perfused with 2 ml 1.5% 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C. Hearts were frozen, sectioned in 1 mm sections, fixed in 10% formalin, and weighed. To calculate infarct size/weight, both sides of each section were digitally photographed and contoured with MetaVue software to delineate ischemic (red), and infarct (white) tissue. Infarcts were reported as a percentage of total left ventricular (LV) area multiplied by the total weight of that section.

[0042] In vivo myocardial ischemia-reperfusion.

[0043] Mice were anesthetized with ketamine (55 mg/kg) plus xylazine (15 mg/kg). Atropine (0.05 mg SC) was administered to reduce airway secretions. Animals were intubated and ventilated with room air (tidal volume 250 µl, 120 breaths/min) with a mouse respirator (Harvard Apparatus, Holliston, MA). Rectal temperatures were maintained at 37°C by a thermo-regulated heating pad. Following thoracotomy, an 8-0 silk suture was placed around the left anterior descending (LAD) coronary artery for ligation. After either a 20 or 60 minute duration of ischemia the occlusion was released and reperfusion was confirmed visually. At 60 min of reperfusion (MDA and protein analysis) or 24 hours of reperfusion (infarct analysis) the mice were reanesthetized, intubated and ventilated as outlined above. The chest

was reopened along the previous incision line to expose the heart and the left main coronary artery was religated in the same location as before. The heart was excised and the aorta cannulated. Three ml of 10% Phthalo Blue (Heubach Inc.) was slowly injected directly into aorta to stain the heart for delineation of the ischemic zone from the non-ischemic zone. The area of the myocardium that does not stain with Phthalo Blue is defined as the area-at-risk (AAR). Serial, short axis 1-mm-thick sections were cut and incubated in 1.0% 2,3,5-triphenyltetrazolium chloride (TTC) for 15 minutes at 37°C for demarcation of the viable and nonviable myocardium within the AAR. After TTC staining, the area of infarction was pale, whereas the viable myocardium was red. Each of the 1-mm-thick myocardial slices was placed in 10% formalin overnight then weighed and the areas of infarction, AAR, and nonischemic left ventricle assessed. To calculate infarct size/weight, both sides of each of the myocardial slices were photographed with a high resolution digital camera, and contoured with a planimeter (Adobe PhotoShop 5.0) to delineate the borders of the entire heart, the non-ischemic area, and the infarcted area. The sizes of the nonischemic area, AAR, and infarct size area (IS) were calculated as percentages of the total left ventricle (LV) area multiplied by the total weight of that slice.

[0044] Protein analysis.

[0045] Western analysis.

[0046] Western blot analysis is an analytical technique used to detect specific proteins in a tissue sample. The analysis uses gel electrophoresis to separate native or denatured proteins by the polypeptide length or three-dimensional structure of the protein. The proteins are then further probed using antibodies to detect the presence of a target protein.

[0047] Hearts were homogenized in buffer containing 50 mM Tris (pH=7.4), 150 mM NaCl, 0.5% NP-40, 1 mM sodium pyrophosphate, 5 mM sodium vanadate, 1 mM benzamidine, and 1 mM sodium fluoride with protease inhibitor cocktail (Sigma) for three 10 second cycles. After 30 min of protein solubulization, samples were centrifuged at 13,000 rpm for 10 min and 50% glycerol was added to each supernatant to a final concentration of 10%. Supernatants were vortexed, aliquoted, and frozen at -80°C. All protein processing was carried out at 4°C. Equal amounts of protein, verified with Coomassie staining, were loaded into BioRad SDS-PAGE gels

and electrophoresed. After transfer onto nitrocellulose, membranes were washed in 0.05% Tween in Tris buffered saline (pH 7.5), blocked in 5% milk, and probed with primary antibody (anti-human GSHPx, rabbit polyclonal, Cell Signaling, Danvers, MA; anti-human-SOD1, rabbit polyclonal, Cell Signaling, Danvers, MA; anti-human-SOD3, rabbit polyclonal , Abcam, Cambridge, MA; anti-SERCA, custom rabbit polyclonal, Invitrogen, Carlsbad, CA; anti-Phospholamban, custom rabbit polyclonal, Invitrogen, Carlsbad, CA; anti-Ryanodine Receptor, mouse monoclonal IgG1, Affinity Bioreagents Golden, CO; anti-Calsequestrin, rabbit polyclonal IgG, Affinity Bioreagents, Golden, CO; anti-GAPDH, rabbit IgG, Cell Signaling, Danvers, MA; Anti-3-NT, mouse monoconal IgG2, Millipore, Temecula, CA). Membranes were washed in 0.05% Tween in TBS, incubated with the appropriate secondary antibodies conjugated to HRP (KPL), and Supersignal (Pierce) used to visualize proteins. The blots were imaged and quantified in a BioRad ChemiDoc utilizing Quantity One software.

[0048] Glutathione peroxidase activity.

[0049] For GSHPx activity, ventricles were homogenized in buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 1 mM DDT, centrifuged at 13,000 rpm for 15 min at 4°C and supernatants stored at -80°C. Assays were conducted according to the manufacturer's protocol (GPX kit; Cayman Chemical Company, Ann Arbor, MI). In brief, the assay measures GSHPx activity indirectly by a coupled reaction with glutathione reductase. Oxidized glutathione (GSSG), produced upon reduction of an organic hydroperoxide by GSHPx, is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the A340 is directly proportional to the GSHPx activity in the sample. Final activity was expressed per mg protein.

[0050] Superoxide dismutase activity.

[0051] For *SOD1/3* activity, ventricles were homogenized in buffer containing 20 mM HEPES (pH 7.2), 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose, centrifuged at 1,500g for 5 min at 4°C and supernatants stored at -80°C. Assays were conducted according to the manufacturer's protocol (SOD kit 706002, Cayman

Chemical Company, Ann Arbor, MI). SOD activity is assessed by measuring the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine. Superoxide radical formation is measured via a tetrazolium salt conversion to a formazan dye by monitoring absorbance at 450 nm. Final activity was expressed per mg protein.

[0052] Immunoprecipitation.

[0053] Hearts were rapidly excised, cannulated through the aorta, perfused with 3 mL of ice-cold saline and weighed. Ventricles were homogenized in buffer containing 50 mM Tris (pH7.4), 150 mM NaCl, 0.5% NP-40, 1 mM sodium pyrophosphate, 5 mM sodium vanadate, 1 mM benzamidine, and 1 mM sodium fluoride with protease inhibitor cocktail (Sigma) for 10 sec x 3 cycles. After 30 min of protein solubulization, samples were centrifuged at 13,000 rpm for 10 min and 50% glycerol was added to each supernatant to a final concentration of 10%. Supernatants were vortexed, aliquoted, and frozen at -80°C. 500 micrograms of total protein homogenate with protease inhibitor cocktail (Sigma) were added to 10 ug of 3-NT (Millipore) or SERCA2a (Invitrogen) antibody and agitated for 1 hour at 4°C. Each sample/antibody complex was incubated with Protein A and G bead slurry (Calbiochem) for 3 hours and centrifuged at 10,000 x g for 30 sec. Supernatants were removed and beads washed 3 times with lysis buffer containing 50 mM Tris-HCl (pH 8.0). 150 mM NaCl, and 1 % NP-40. Laemmli buffer was added to each bead pellet, vortexed, boiled at 99°C for 10 min. Resulting samples were spun at 10,000 x g for 5 min, supernatants loaded onto 4-20% SDS-PAGE gels, subjected to electrophoretic separation and transferred to nitrocellulose membranes for subsequent Western protocol as outlined above.

[0054] 2-Dimensional gel electrophoresis.

[0055] Three replicates of control and transgenic heart homogenates, processed by the Western protocol, were pooled in equal protein amounts. The samples were centrifuged again at 13,000 rpm for 10 min to remove sediment and supernatants treated with RIPA buffer containing 40mM Tris-Cl (pH 8.8), 7M Urea, 2M thiourea, 0.25% NP-40, 0.25% ASB-14 and protease inhibitors (Roche)for 30 min at 4°C. Samples were sonicated on ice and centrifuged at 13,000 rpm for 30 min.

Supernatants were treated with 20% TCA, incubated on ice for 30 min, washed with 1 ml ice-cold acetone and air-dried.

[0056] 3-Nitrotyrosine detection

[0057] For 3-NT detection, 100 ug protein of each pooled sample was used to rehydrate a 3-10 pH 24cm isoelectric focusing (IEF) strip overnight (GE IPGphor). The IEF strip was focused using the manufacturer's protocol then electrophoresed on a 12% SDS-PAGE gel. The gel was removed, transferred to nitrocellulose then subjected to Western analysis as outlined in the Western Analysis section of the Methods using anti-3-nitrotyrosine antibody. Detection was performed using a Cy3- or Cy5-labeled secondary antibody and the western blots were imaged using a Typhoon Phosphorimager.

[0058] Protein identification.

[0059] Gels were digested with sequencing grade trypsin from Promega (Madison WI) or sequencing grade chymotrypsin from Roche (Indianapolis, IN) using the Multiscreen Solvinert Filter Plates from Millipore (Bedford, MA). Briefly, bands were trimmed as close as possible to minimize background polyacrylamide material. Gel pieces were then washed in nanopure water for 5 minutes. The wash step is repeated twice before gel pieces were washed and or destained with 1:1 v/v methanol:50 mM ammonium bicarbonate for ten minntes twice. The gel pieces were dehydrated with 1.1 v/v acetonitrile: 50 mM ammonium bicarbonate. The gel bands were rehydrated and incubated with dithiothreitol (DTT) solution (25 mM in 100 mM ammonium bicarbonate) for 30 minute prior to the addition of 55 mM Iodoacetamide in 100 mM ammonium bicarbonate solution. Iodoacetamide was incubated with the gel bands in the dark for 30 min before removed. The gel bands were washed again with two cycles of water and dehydrated with 1:1 v/v acetonitrile: 50 mM ammonium bicarbonate. The protease is driven into the gel pieces by rehydrating them in 12 ng/ml trypsin in 0.01 % Protease MAX Surfactant for 5 minutes. The gel pieces were then overlaid with 40 ml of 0.01 % ProteaseMAX surfactant:50 mM ABC and gently mixed on a shaker for 1 hour. The digestion is stopped with addition of 0.5% TFA. The MS analysis was immediately performed to ensure high quality tryptic peptides with minimal nonspecific cleavage or frozen at -80^{O}C until samples were analyzed.

[0060] Mass Spectrometry.

[0061] Capillary-liquid chromatography-nanospray tandem mass spectrometry (Nano- LC/MS/MS) was performed on a Thermo Finnigan LTQ mass spectrometer equipped with a nanospray source operated in positive ion mode. The LC system was an UltiMate™ 3000 system from Dionex (Sunnyvale, CA). The solvent A was water containing 50mM acetic acid and the solvent B was acetonitrile. 5 microliters of each sample was first injected on to the µ-Precolumn Cartridge (Dionex, Sunnyvale, CA), and washed with 50 mM acetic acid. The injector port was switched to inject and the peptides were eluted off of the trap onto the column. A 5 cm 75 µm 10 ProteoPep II C18 column (New Objective, Inc. Woburn, MA) packed directly in the nanospray tip was used for chromatographic separations. Peptides were eluted directly off the column into the LTQ system using a gradient of 2-80%B over 45 minutes, with a flow rate of 300 nl/min. The total run time was 65 minutes. The *MS/MS* was acquired according to standard conditions established in the lab. Briefly, a nanospray source operated with a spray voltage of 3 KV and a capillary temperature of 200°C is used. The scan sequence of the mass spectrometer was based on the TopTen™ method; the analysis was programmed for a full scan recorded between 350-2000 Da, and a *MS/MS* scan to generate product ion spectra to determine amino acid sequence in consecutive instrument scans of the ten most abundant peak in the spectrum. The CID fragmentation energy was set to 35%. Dynamic Dynamic exclusion was enabled with a repeat count of 2 within 10 seconds, a mass list size of 200, an exclusion duration 350 seconds, the low mass width was 0.5 and the high mass width was 1.5. The RAW data files collected on the mass spectrometer were converted to mzXML and MGF files by use of MassMatrix data conversion tools (version 1.3). For low mass accuracy data, tandem MS spectra that were not derived from singly charged precursor ions were considered as both doubly and triply charged precursors. The resulting MGF files were searched using Mascot Daemon by Matrix Science version 2.2.2 (Boston, MA) and the database searched against the mouse SwissProt database version 2102_10 (16326 sequences). The mass accuracy of the precursor ions were set to 2.0 Da given that the data was acquired on an ion trap mass analyzer and the fragment mass accuracy was set to 0.8 Da.

[0062] Dihydroethidium fluorescence.

[0063] Superoxide anion generation from ischemic-reperfused myocardium was determined using dihydroethidium (DHE) fluorescence. Ex vivo hearts were rapidly embedded in OCT and solidified in liquid nitrogen. Hearts were then sectioned at 5 microns and placed on slides. Sections were covered with 10 micro M dihydroethidium (Sigma) in PBS, pH 7.4, and incubated in the dark for 30 min at 37°C. After rinsing with PBS (pH 7.4), sections were fixed with 4% paraformaldehyde (pH 7.4) for 10 minutes and fluorescence visualized at 570 nm. Superoxide generation in myocardium is expressed as relative red fluorescence at 570 nm. At least three determinations were performed in each heart.

[0064] Malondialdehyde quantitation.

[0065] Hearts were homogenized in 20 mM PBS (pH 7.4) with 0.5 M butylated hydrotoluene (Sigma) at 4°C for three 10 second cycles. Samples were centrifuged at 4 °C for 10 min at 13,000 rpm and supernatants stored at -80°C until time of Malondialdehyde (MDA) assay. Assays were performed according to the manufacturer's protocol (MDA kit, Foster City, CA). MDA concentrations (uM) were derived from linear regression of known MDA standard concentrations and expressed per mg protein.

[0066] Statistical Analysis.

[0067] The results of experiments were analyzed by several statistical methods (e.g. paired or unpaired t-tests, analysis of variance, chi-squared analysis, curve fitting functions, etc. using standard software (e.g. GraphPad Prism, version 4.0 and SAAS version 9.1). Results were expressed as mean ± standard error of the mean. For comparison between 2 groups, significance was determined by paired or unpaired Student t test. For comparison of multiple groups, multifactorial ANOVA with post hoc comparison of the means with Bonferroni correction was used to determine statistical significance. For all statistical evaluation, $P<0.05$ is considered significant.

[0068] Results.

[0069] Antioxidant Network Overexpressing Mice.

[0070] The generation and characterization of antioxidant network overexpressing mice (AON) has been published previously. To examine the level of expression of these antioxidant enzymes, whole heart homogenates were assayed for glutathione peroxidase and combined SOD1/SOD3 activity in untreated WT or AON animals. Analysis of the protein level revealed an increase in GSHPx, an increase in SOD1 and an increase in SOD3. Analysis of the relative enzymatic activity revealed a 20% increase in total GSHPx activity and an 80% increase in the combined SOD1 and SOD3 activity in hearts from AON mice versus WT mice.

[0071] Expression of Calcium Regulating Proteins in AON Expressing Myocardium.

[0072] To examine the effect of AON expression on calcium handling proteins, the levels of the ryanodine receptor (RyR), SERCA2a, phospholamban (PLB), and calsequestrin (CSQ) were determined by Western blot analysis. No difference in the expression of any of these proteins was observed in AON hearts compared to WT hearts.

[0073] AON Expression Protects Against Myocardial Ischemia-Reperfusion Injury.

[0074] AON Expression Protects Against ex vivo Myocardiallschemia-Reperfusion Injury.

[0075] Employing an *ex vivo* myocardial ischemia-reperfusion injury model, when compared to hearts from WT animals, AON hearts demonstrated a 68% reduction (WT:63.4 +/- 4.8% vs AON:20.3+/- 3.7%; $p<0.05$) in myocardial infarct size following 30 minutes of global ischemia and 60 minutes of reperfusion.

[0076] AON Expression Protects Against in vivo Myocardiallschemia-Reperfusion Injury.

[0077] Given the possibility for increased sensitivity of the Langendorff perfusion model to oxidative stress, an *in vivo* model of regional left coronary artery ischemia-reperfusion injury was employed. Wild-type or AON mice were subjected to *in vivo* myocardial ischemia-reperfusion injury of either 20 minutes or 60 minutes followed

by 24 hours of reperfusion. When compared to WT mice, AON mice, concomitantly overexpressing human SOD1, SOD3 and GSHPx-1, demonstrated a 92% reduction of infarct size following 20 minutes of ischemia (WT: 17.1 +/- *1.8% vs* AON:1.4 +/- 0.6%; *p<0.05;* data not shown) and a 55% reduction in infarct size following 60 minutes of ischemia and 24 hours of reperfusion (WT:40.0 +/- 3.5% *vs* AON 18.1 +/- 3.9%; p<0.05; Fig. 4; Table 2) indicating *in vivo* cardioprotective efficacy against prolonged ischemia with AON expression.

[0078] AON Expression Attenuates Formation of Reactive Oxygen Species Following Myocardial Ischemia-Reperfusion Injury.

[0079] To elucidate the mechanism of the cardioprotection conferred by AON expression, ROS generation measured, by dihyrdoethidium (DHE) staining, was examined in WT and AON hearts subjected to 30 minutes of *ex vivo* global ischemia and 60 minutes of reperfusion. AON hearts displayed significantly less DHE staining following I-R injury than WT hearts. To further assess the effect upon ROS generation, the formation of malondialdehyde (MDA), an indicator of reactive oxygen species-mediated lipid peroxidation, was measured in WT and AON hearts exposed to 60 minutes of *in vivo* ischemia and 60 minutes of reperfusion. For both the *ex vivo* and in *vivo* experiments, this 60 minutes reperfusion time was chosen so that we could observe the modifications that occur within the initial oxidative burst and the sustained oxidative production during the recovery phase of reperfusion. WT and AON hearts displayed comparable levels of MDA at baseline (Fig. 6). In contrast, following I-R injury, WT hearts demonstrated a 2-fold increase in the formation of MDA while AON hearts displayed no significant difference in MDA formation from baseline (Fig. 6). These data indicate that AON expression significantly scavenges ROS produced during myocardial ischemia-reperfusion injury, reducing its detrimental effects.

[0080] AON Expression Reduces Oxidative Posttranslational Modifications (OPTM).

[0081] Given the observed reduction of reactive oxygen species in AON mice following myocardial ischemia-reperfusion injury, the "nitroproteome" of WT and AON hearts was examined for the specific peroxynitrite-mediated oxidative

posttranslational modification of tyrosine to 3-nitrotyrosine. Again mice were subjected to 60 minutes of *in vivo* ischemia and 60 minutes of reperfusion so that we could observe the modifications that occur within the initial oxidative burst and the sustained oxidative production during the myocardial recovery phase of reperfusion. WT hearts displayed a marked increase in OPTM formation following ischemia-reperfusion compared to AON expressing animals exposed to the similar experimental protocol. Furthermore, differences in the protein banding pattern suggested that concomitant SOD1, SOD3 and GSHPx-1 expression inhibits OPTM of multiple specific proteins. To further assess 3NT OPTM of myocardial proteins, 2-D gel electrophoresis followed by 3-NT Western analysis was conducted. The number and intensity of 3-NT modified specific proteins was greater in WT hearts compared to AON hearts following myocardial ischemia-reperfusion injury, suggesting that AON expression protects specific proteins from detrimental tyrosine nitration. MS-MS analysis of two of the major proteins containing 3-NT modifications in the WT samples identified Protein A as cardiac fatty acid binding protein and Protein B as myoglobin.

[0082] AON Expression Reduces Formation of Peroxynitrite-Mediated Protein Modification of SERCA2a.

[0083] The effect of AON expression on tyrosine nitration of SERCA2a following myocardial ischemia-reperfusion injury was evaluated. Immunoprecipitation with an antibody to 3-NT and detection with an antibody to SERCA2a and immunoprecipitation with an antibody to SERCA2a and detection with an antibody to 3-NT revealed OPTM of SERCA2a on WT hearts subjected to 60 minutes of ischemia and 60 minutes of reperfusion. However, significantly less modification of SERCA2a from AON hearts was observed. Again, at baseline, there was no difference in the total level of SERCA2a between WT and AON-OE hearts. This suggests that AON expression protects SERCA2a from the detrimental oxidative posttranslational modification of tyrosine nitration.

[0084] Discussion.

[0085] The concomitant over-expression of human SOD1, SOD3 and GSHPx-1 results in a marked reduction of ischemic injury; reducing ROS-mediated-lipid

peroxidation and RNS-mediated oxidative post-translational modification (OPTM) of a multitude of proteins, including heart fatty acid binding protein, myoglobin and the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA2a). The over-expression of several antioxidant enzymes to provide a network that can efficiently scavenge and neutralize reactive oxygen species, thereby attenuating myocardial ischemia-reperfusion injury. These data further identify OPTM of cardiac fatty acid binding protein, myoglobin and SERCA2a as a potential mechanism affecting the susceptibility of the myocardium to ischemia-reperfusion injury and provide insight for the development of combined therapeutic approaches for the detection or the treatment of acute ischemic heart disease, a major cause of morbidity and mortality in the United States and worldwide.

[0086] It is widely accepted that myocardial ischemia-reperfusion induces the production of ROS. With coronary artery occlusion, myocardial ischemia quickly leads to decreased intracellular ATP levels and accumulation of intracellular calcium (Ca^{2+}) and hydrogen (H^+), resulting in bioenergetics and functional abnormalities. During ischemia, the redox status of the myocardium shifts to a more reduced state. As ischemia continues, cardiomyocyte death ensues. With reperfusion, reoxygenation of the myocardium results in exaggerated metabolic shifts and worsening myocardial damage. Generations of ROS at the onset of reperfusion shifts the redox status of the myocardium to a more oxidized state. There is a significant burst of oxygen-derived free radicals generated within the first minutes of reperfusion and peaking 4 to 7 minutes after the onset of reperfusion, followed by a persistent generation of oxygen-derived free radicals.

[0087] Generation of both ROS and RNS induce mitochondrial injury, sarcoplasmic reticulum dysfunction, and further calcium accumulation. Indeed, several studies support the concept that ROS, such as superoxide anions, hydrogen peroxide, and hydroxyl radicals contribute to myocardial tissue injury secondary to ischemia and reperfusion. Therefore, a number of studies have employed genetic modification of mice (knockout or transgenic over-expression of individual antioxidant enzymes) to explore the role of specific antioxidant enzymes in I-R injury. These results have yielded mixed interpretations of the role of individual antioxidant enzymes in myocardial ischemia-reperfusion injury. Experiments employing knockout animals have demonstrated that following 30 minutes of global ex *vivo*

ischemia and 2 hours of reperfusion, hearts genetically devoid of GSH-Px, displayed a reduced recovery of developed force, an increase in CK release and larger myocardial infarct size when compared to control hearts. Similarly, in SOD1 knockout hearts subjected to 30 minutes of global ex vivo ischemia and 2 hours of reperfusion, a decreased recovery of left ventricular developed pressure and larger myocardial infarct size were observed compared to control hearts. Complementing these reports, transgenic hearts with 4-fold over-expression of GSH-Px-1 displayed improved recovery of contractile force, reduced CK release, and reduced infarct size following 30 minutes of ex vivo global ischemia and 20 minutes of reperfusion when compared to control hearts. In an ex vivo working heart model of ischemia, 3.5-fold over-expression of SOD1 resulted in improved contractile recovery, heart rate, stroke work, and stroke volume in transgenic mouse hearts when compared to control hearts. Similarly, following 30 minutes of global ex vivo ischemia, an increase in the recovery of contractile function, a decreased in infarct size, and improved recovery of high energy phosphates was observed in a separate model employing transgenic hearts over-expressing SOD1 (10-fold) subjected to 35 minutes of global ischemia and 45 minutes of reperfusion displayed increased LVDP and a decrease in lactate dehydrogenase. Thus, in ex vivo models, significant myocardial protection has been reported with modulation of the expression of single antioxidant enzymes.

[0088] While similar protection was observed following in vivo left coronary artery ligation induced infarct size in mice overexpressing SOD2, in vivo experiments have revealed that following 30 minutes of left coronary artery ischemia and 24 hours of reperfusion, neither deficiency nor over-expression (3-fold) of SOD1 affected the extent of myocardial infarct size. Furthermore, glutathione peroxidase over-expression (8-fold) did not convey in vivo myocardial protection in the same model. The conclusion from this testing was that SOD2, but not SOD1 or GSHPx-1, modulates the susceptibility to in vivo myocardial ischemia reperfusion injury. However, given the fact that each of these enzymes acts as a critical component within an antioxidant network, one possibility for the disparate ex vivo and in vivo results is that neither enzyme alone is capable of full cardioprotective efficacy in vivo. Additionally, the modulation of individual antioxidant enzyme levels may result in detrimental changes to the overall antioxidant system. If one examines the proteomic response in models of induced oxidative stress, such as L-NAME

treatment of rats, a concomitant increase in SOD1, SOD3 and GSHPx-1 levels is observed, likely representing an adaptive upregulation of an antioxidant network mechanism to combat oxidative stress [40]. The current model attempts to mimick such a response and provide in an antioxidant network rather than a single antioxidant enzyme.

[0089] Additional differences between the ex *vivo* and *in vivo* models are due to the fact that a maintained generation of oxygen-derived free radicals during reperfusion *in vivo* may be due to inflammatory cell activity which are not present in ex *vivo* perfusion models. If the mechanism of damage is due to significant inflammatory cell contribution, protection might be observed in the ex *vivo* model but attenuated in the *in vivo* model of myocardial ischemia reperfusion injury. Finally, critical to the disparity observed between ex *vivo* and *in vivo* studies is the fact that ex *vivo* perfusion with crystalloid solutions may facilitate the production of hydroxyl radical by Fenton/Haber-Weiss reactions due to the lack of iron-binding proteins in the perfusate. Therefore, any approach aimed at reducing oxidative stress may have a more profound demonstrable effect on ex *vivo* perfused hearts but not *in vivo*. This disclosure demonstrates that AON expression not only attenuates ex *vivo* ischemia-reperfusion injury but also significantly reduces *in vivo* myocardial ischemia-reperfusion injury, reducing infarct size and ROS-mediated lipid peroxidation.

[0090] Reactive oxygen species can damage cells via peroxidation of polyunsaturated fatty acids comprising the membrane lipids. Lipid free radicals then can react with other polyunsaturated fatty acids to propagate this process. Studies suggest that ROS-mediated lipid peroxidation can occur with ischemia but predominantly occurs with reperfusion. With restoration of blood flow to and oxygenation of the ischemic tissue, increased levels of reactive oxygen and reactive nitrogen species are generated which in turn induce increased lipid peroxidation. Peroxidation of membrane lipids results in fragmentation of polyunsaturated fatty acids producing various aldehydes, alkenals, and hydroxyalkenals, including malondialdehyde and 4-hydroxy-2-nonenal (HNE) that are reactive with proteins and cause cytotoxicity. Thus, by quenching ROS and limiting lipid peroxidation, AON expression conveys cardioprotection from oxidative posttranslational modifications of proteins following myocardial ischemia-reperfusion injury.

[0091] Oxidative posttranslational modifications of proteins can range from oxidation of cysteine residues to covalent crosslinking with other proteins to adducts of protein with either lipids, carbohydrates, or nucleic acid radicals. With reperfusion of the myocardium, increased nitric oxide and superoxide results in the formation of peroxynitrite ($ONOO^-$) and suppression of myocardial tissue oxygen consumption causing a hyperoxygenation state that potentiates ROS generation. The nitration of tyrosine to 3-nitrotyrosine (3-NT) represents one of several oxidative posttranslational modifications secondary to oxidative stress from pathological conditions including hypertension, diabetes and atherosclerosis that can reduce enzymatic acitivity. Peroxynitrite mediated tyrosine nitration does not occur in all proteins but rather appears somewhat restricted as animal model of sepsis, diabetes or myocardial ischemia-reperfusion detect only about 100 proteins that are modified.

[0092] Prior work has linked ischemia/reperfusion-induced ROS production to oxidative modification of Ca^{2+} handling proteins such as sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA)2. SERCA2 also has been shown to be nitrated at two adjacent tyrosine residues (Y-294, Y-295) in both skeletal and cardiac muscle from aged animals [65, 66]. Tyrosine nitration of SERCA2a results in a marked decreased in activity. Furthermore, in myocardial samples from patients with dilated cardiomyopathy, nitrotyrosine modification of SERCA2a positive correlated with the time to half relaxation in myocytes isolated from control and OCM hearts. In a separate report, experiments with isolated SR vesicles from porcine hearts demonstrated that SERCA is inactivated by peroxynitrite exposure [68]. SERCA2a appears not only sensitive to tyrosine nitration but also to other OPTM (cysteine oxidation) that lead to decreased activity. These data demonstrate that SERCA activity is sensitive to the oxidative state of the myocardium. SERCA2a appears to be a key protein susceptible to detrimental OPTM following myocardial ischemia-reperfusion injury. AON over-expression not only results in a decrease in OPTM by tyrosine nitration of a number of myocardial proteins, but specifically attenuates tyrosine nitration of the key calcium handling protein SERCA2a.

[0093] To begin to address the differences in 3-NT modifications observed between WT and AON hearts subjected to ischemia reperfusion injury, one of the protein demonstrated by two-dimensional 3-NT Western blot analysis in WT hearts was identified using LC-MS/MS to be heart-type fatty acid binding protein (FABP3).

FABP3 is expressed in high abundance in the myocardium were it serves to translocate fatty acids and their CoA derivatives in the cytoplasm. Stimulation of cardiac myocytes with insulin leads to an increase in the amount of phosphorylated FABP compared, indicating that FABP activity may be modulated by the insulin receptor tyrosine kinase. In mice deficient in FABP the heart is unable to efficiently take up plasma long chain fatty acids and switches to glucose usage. Thus, the activity of FABP appears critical to regulating energetic homeostasis. Whether 3-NT modification affects FFA binding is under investigation. Of further interest, FABP has been examined as plasma biomarkers for the diagnosis of acute coronary syndromes. FABP3 is rapidly released into the circulation following cell damage by acute myocardial ischemia and the amount of released h-FABP correlates with infarct size in humans.

[0094] The present disclosure relates to detection of 3-NT modified FABP to provide for increased sensitivity and specificity in the detection of acute coronary syndrome.

[0095] WT (wild-type littermate control animals) or AON (antioxidant network expressing animals) were subjected to 60 minutes of left coronary artery ligation induced ischemia and 60 minutes of reperfusion as described in the Material and Methods section. Myocardial lysates were processed, subjected to one dimensional (FIG. 1A) or 2-dimensional (FIGS. 1B and 1C) gel electorphoresis and samples examined for the 3-NT modification.

[0096] WT (wild-type littermate control animals) or AON (antioxidant network expressing animals) were subjected to 60 minutes of left coronary artery ligation-induced ischemia and 60 minutes of reperfusion as described in the Material and Methods section for in vivo testing. Myocardial lysates were processed, subjected to immune precipitation with either (FIG. 2A) anti-SERCA2a polyclonal antibody or (FIG. 2B) anti-3NT antibody. Following electrophoresis and transfer of the samples detection was achieved with either (FIG. 2A) anti-3NT antibody or (FIG. 2B) anti-SERCA2a antibody. (FIG. 2C) Quantitation of blot (FIG. 2B) revealed significant differences in the level of 3-NT modified SERCA2a in WT versus AON hearts.

[0097] The exemplary embodiment has been described with reference to the preferred embodiments. Obviously, modifications and alterations will occur to others upon reading and understanding the preceding detailed description. It is intended

that the exemplary embodiment be construed as including all such modifications and alterations insofar as they come within the scope of the appended claims or the equivalents thereof.

CLAIMS:

1. A method for detecting myocardial injury, comprising:

determining the presence of nitrotyrosinylated fatty acid binding protein and/or myoglobin.

2. The method of claim 1, further comprising measuring the amount of nitrotyrosinylated fatty acid binding protein and/or myoglobin.

3. The method of claim 1, wherein said presence is determined using an anti-3-nitrotyrosine antibody and a secondary antibody.

4. The method of claim 1, wherein the presence of nitrotyrosinylated fatty acid binding protein and/or myoglobin is determined by western blot analysis.

5. The method of claim 4, wherein the western blot analysis includes use of a primary anti-3-nitrotyrosine antibody and a secondary antibody.

6. The method of claim 5, wherein the secondary antibody is either a horseradish peroxidase conjugated, Cy3- or Cy5-labeled antibody.

7. A method for diagnosing ischemic injury in an animal, comprising:

determining the presence of nitrotyrosinylated fatty acid binding protein and/or myoglobin in a protein sample from the animal.

8. The method of claim 7, wherein said presence is determined using a primary anti-3-nitrotyrosine antibody and a secondary antibody.

9. The method of claim 8, wherein the secondary antibody is a either a horseradish peroxidase conjugated, Cy3- or Cy5- labeled antibody .

10. The method of claim 7, wherein the animal is a mammal.

11. The method of claim 10, wherein the mammal is a human.

12. The method of claim 10, wherein the mammal is a mouse.

13. The method of claim 7, wherein the protein sample comprises a myocardial lysate.

14. The method of claim 7, wherein the ischemic injury is acute coronary syndrome.

15. A method for diagnosing acute coronary syndrome in an animal, comprising:

rehydrating an isoelectric focusing strip using a protein sample from the animal;

electrophoresing the strip on a gel; and

performing a western analysis on the gel to determine the presence of nitrotyrosinylated fatty acid and/or myoglobin in the protein sample from the animal.

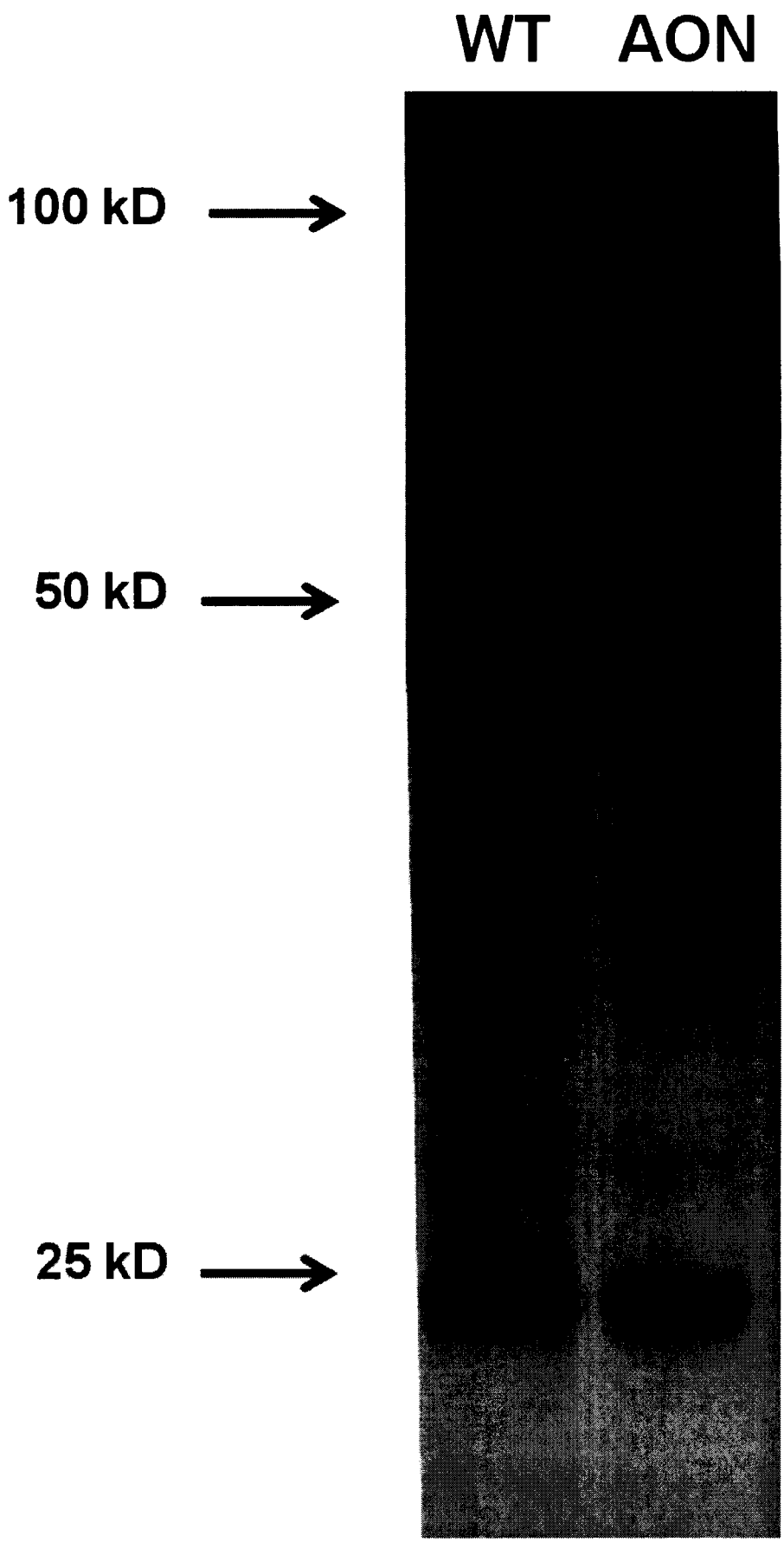


FIGURE 1A

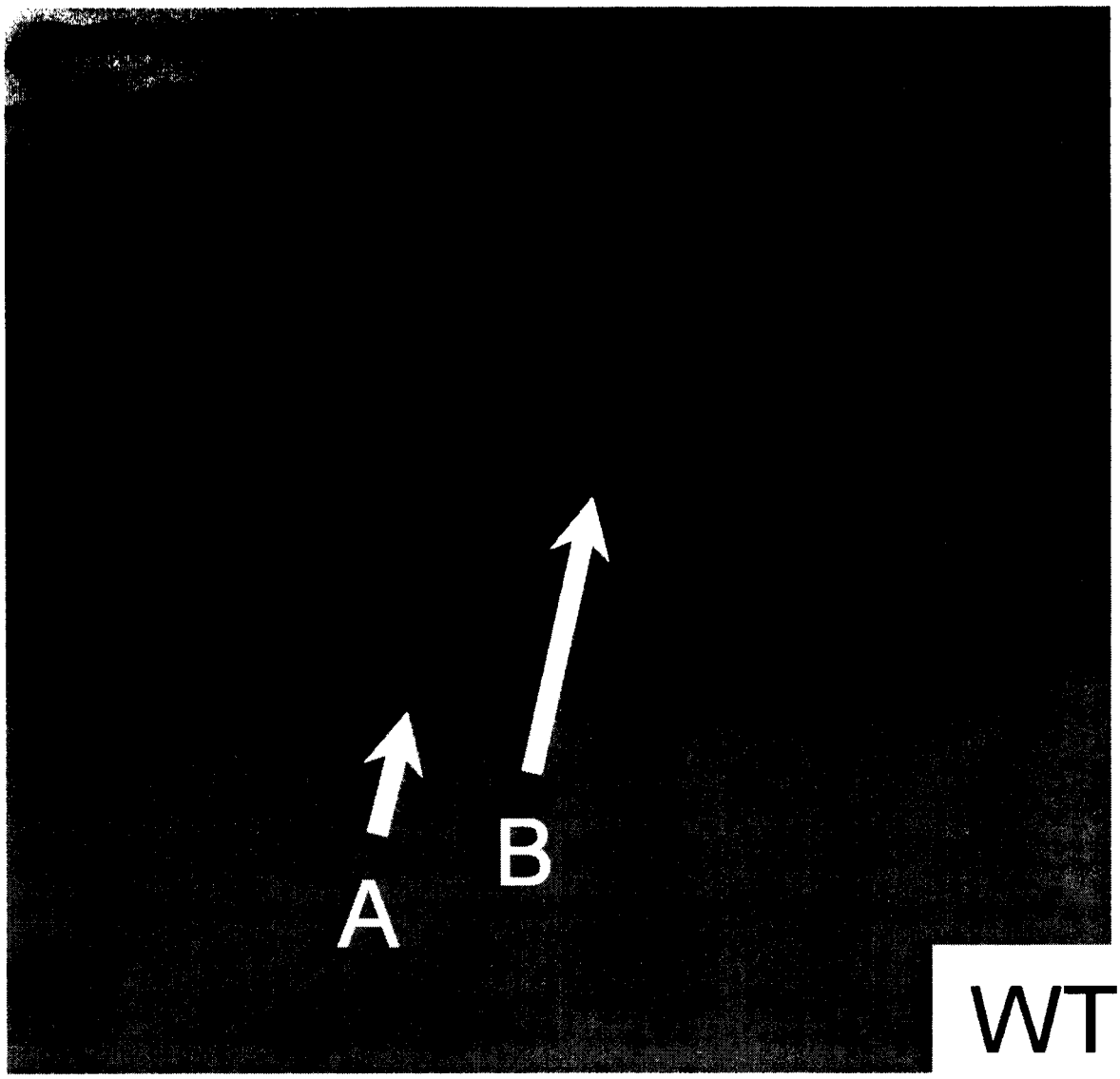


FIGURE 1B

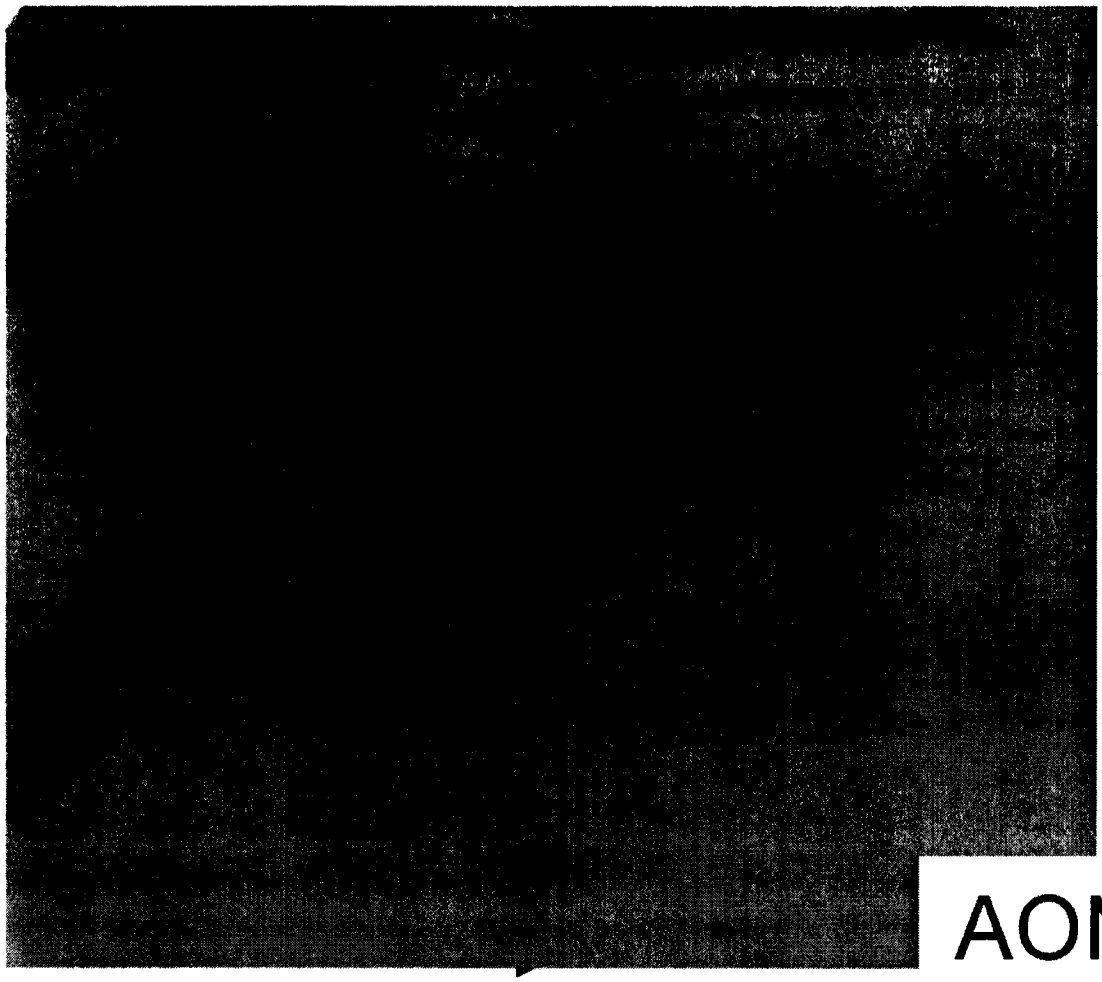
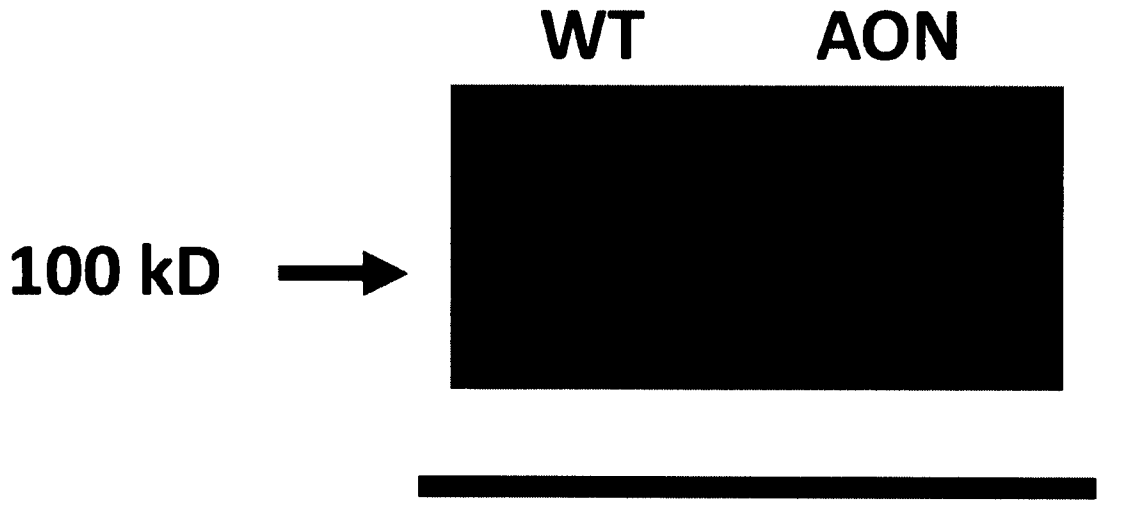
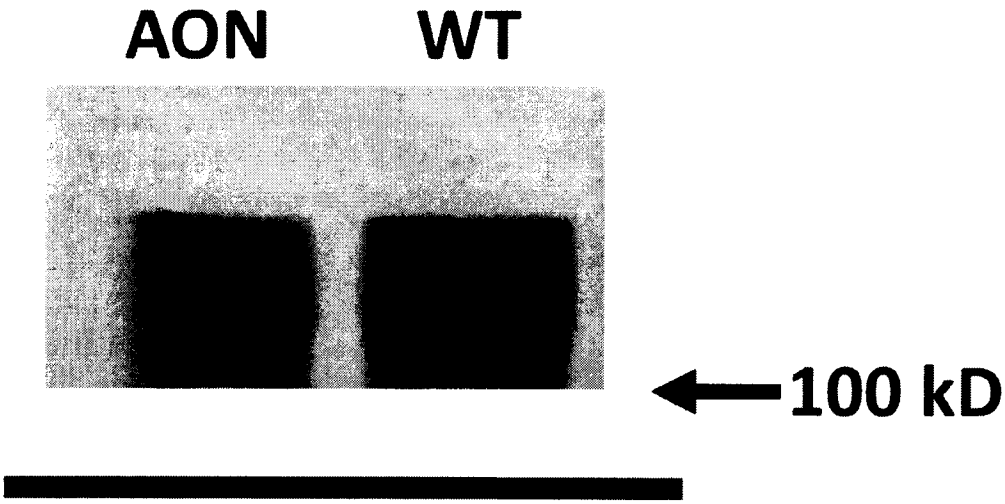


FIGURE 1C



IP: 3NT
Detection: anti-SERCA2a

FIGURE 2A



IP: anti-SERCA2a
Detection: 3NT

FIGURE 2B

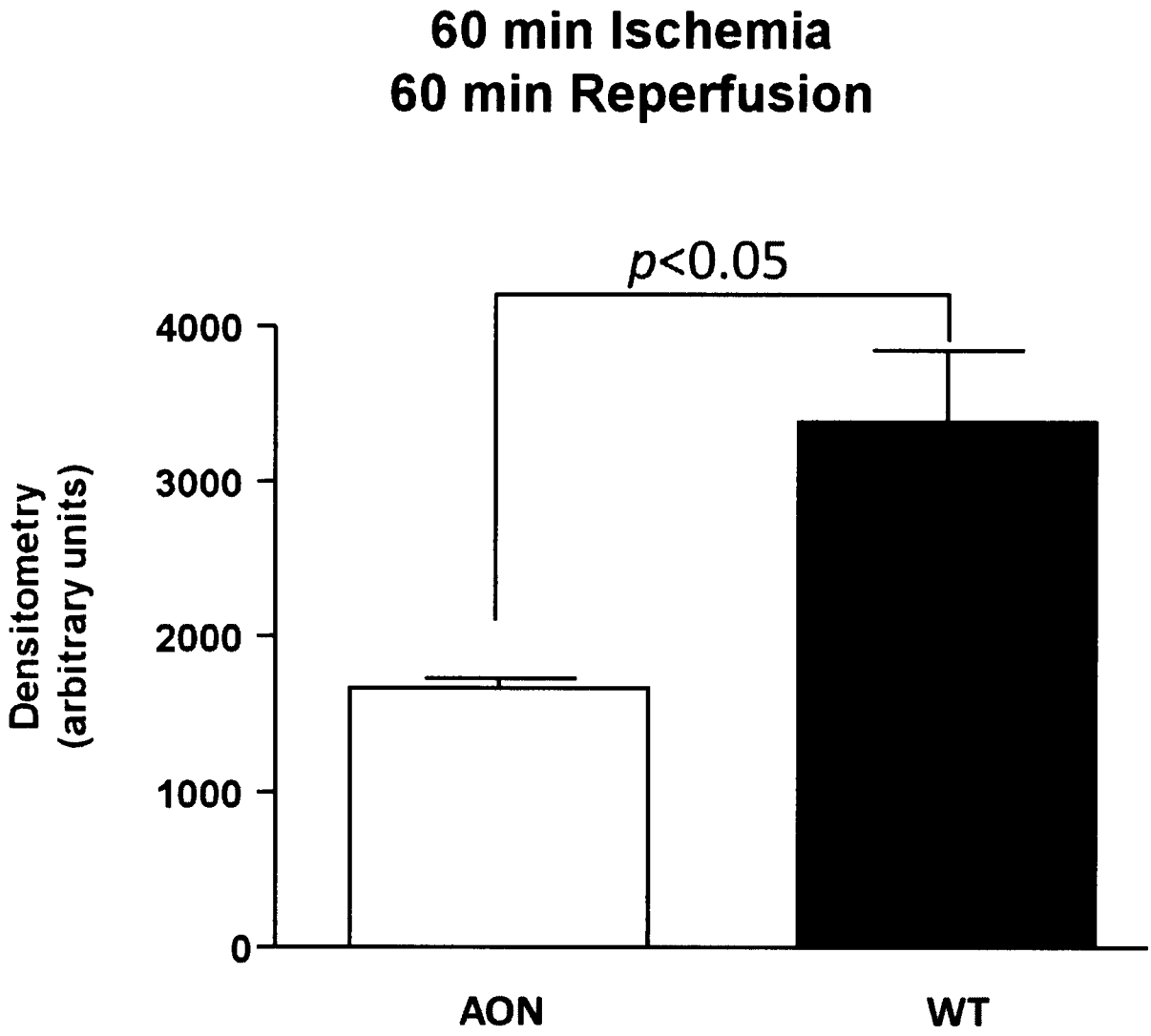

60 min Ischemia
60 min Reperfusion
p<0.05
Densitometry (arbitrary units)
4000
3000
2000
1000
0
AON
WT

FIGURE 2C