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Paraoxonase 2 Prevents the Development of Heart Failure

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Abstract

Background—Mitochondrial oxidation is a major source of reactive oxygen species (ROS) and mitochondrial dysfunction plays a central role in development of heart failure (HF). Paraoxonase 2 deficient (PON2-def) mitochondria are impaired in function. In this study, we tested whether PON2-def aggravates HF progression.

Methods and Results—Using qPCR, immunoblotting and lactonase activity assay, we demonstrate that PON2 activity was significantly decreased in failing hearts despite increased PON2 expression. To determine the cardiac-specific function of PON2, we performed heart transplantations in which PON2-def and wild type (WT) donor hearts were implanted into WT recipient mice. Beating scores of the donor hearts, assessed at 4 weeks post-transplantation, were significantly decreased in PON2-def hearts when compared to WT donor hearts. By using a

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Disclosures
None

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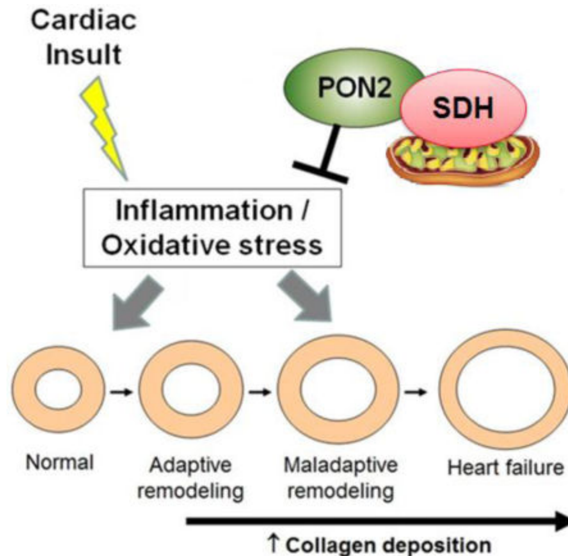
transverse aortic constriction (TAC) model, we found PON2 deficiency significantly exacerbated left ventricular remodeling and cardiac fibrosis post-TAC. We further demonstrated PON2 deficiency significantly enhanced ROS generation in heart tissues post-TAC. ROS generation was measured through dihydroethidium (DHE) using high-pressure liquid chromatography (HPLC) with a fluorescent detector. By using neonatal cardiomyocytes treated with $CoCl_2$ to mimic hypoxia, we found PON2 deficiency dramatically increased ROS generation in the cardiomyocytes upon $CoCl_2$ treatment. In response to a short $CoCl_2$ exposure, cell viability and succinate dehydrogenase (SDH) activity assessed by MTT assay were significantly diminished in PON2-def cardiomyocytes compared to those in WT cardiomyocytes. PON2-def cardiomyocytes also had lower baseline SDH activity. By using adult mouse cardiomyocytes and mitochondrial ToxGlo assay, we found impaired cellular ATP generation in PON2-def cells compared to that in WT cells, suggesting that PON2 is necessary for proper mitochondrial function.

Conclusion—Our study suggests a cardioprotective role for PON2 in both experimental and human heart failure, which may be associated with the ability of PON2 to improve mitochondrial function and diminish ROS generation.

Graphical abstract



Subject Terms

Heart Failure and Cardiac Disease; Cardiomyopathy; Heart Failure

Introduction

Despite improvements in therapeutic regimens for patients with heart failure (HF), the mortality of HF remains as high as 50% within 5 years of diagnosis[1]. Patients who develop HF following cardiac insults often experience progressive, adverse cardiac remodeling and fibrosis attributed to excessive inflammation and oxidative/nitrative stress. Although various studies suggest that phase II antioxidative enzymes may be potential therapeutic candidates

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against cardiac remodeling[2], clinical trials of antioxidative approaches to prevent cardiovascular morbidity and mortality have not yet been successful[3].

Paraoxonases (PON) constitute a family of calcium-dependent esterases with three isoforms: PON1, PON2, and PON3. While all three isoforms exhibit arylesterase and paraoxonase activities, the native enzymatic activity of PON is considered lactonase[4]. Studies have shown that both PON1 and PON3 are associated with high-density lipoprotein in the circulation, whereas PON2 is expressed in various major organs as a cell-associated enzyme[5,6]. These pleiotropic enzymes are highly conserved genetically across species, with diverse roles including protection against lipid peroxidation and oxidative stress, modulation against endoplasmic reticulum stress, and regulation of cell proliferation and apoptosis[7]. Our group, as well as others, has demonstrated the important anti-oxidative role of systemic PON activities in humans. Specifically, diminished paraoxonase/arylesterase activities in serum were directly associated with increased levels of oxidized lipoproteins[8], presence of subclinical myocardial necrosis as detected by high-sensitivity cardiac troponin I[9], and increased adverse cardiac events in stable cardiac patients[10]. In addition, diminished serum arylesterase activities were associated with patient history of HF[11] or chronic kidney disease[12], as well as poorer long-term survival. These findings have now been replicated in an independent outpatient HF cohort[13].

We observed that serum paraoxonase/arylesterase activity levels strongly track with genetic polymorphisms linked to PON1 genotype (especially Q192R), thereby confirming the contribution of circulating PON1 to these esterase activities[10]. While the majority of human studies have focused on circulating PON esterase activities and their impairment in disease states, few have specifically targeted PON isoforms. Additionally, direct quantification of tissue distributions and characteristics of PON isoforms in humans is rarely performed, and the roles of PON2 and PON3 in cardiovascular diseases are less understood.

In animal studies, PON2 is ubiquitously produced in all tissues, yet is not detected in HDL or LDL like PON1 and PON3[14]. In mice, PON2 is the most abundant PON isoform in the myocardium[5], and may contribute substantially to the lactonase activity in the myocardial tissue[15]. While subcellular localization of PON2 in the heart has not been extensively characterized, cell fractionation studies have revealed a predominant PON2 association in microsomes and lysosomes of human jejunum[16], in nuclear membrane and endoplasmic reticulum of vascular cells[17], and in the mitochondria of dopaminergic areas (e.g., striatum) and astrocytes of the brain[18]. The proximity of these intracellular locations may provide unique in situ anti-oxidative effects and cellular protection.

In this study, we demonstrate that mice with genetic deficiency of PON2 have dilated cardiac remodeling, which is aggravated upon additional cardiac insults and may be reversed via systemic overexpression of PON2. We further demonstrate that the aggravated cardiac remodeling may be associated with increased ROS generation in heart tissue upon cardiac insults. Human hearts primarily express PON2 compared to the nominal expression levels of PON1 and PON3. Although PON2 expression is higher at both mRNA and protein levels in human failing hearts compared to that in non-failing hearts, PON2 lactonase activity is lower

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in failing hearts. PON2 could be cardioprotective; however, under oxidative/nitrative stress, PON2 may be rendered “dysfunctional” and lose its cardioprotective effects.

METHODS

Mice

The PON2 deficient mouse strain (PON2-def) was backcrossed into the C57BL/6 (Jackson Laboratory, Bar Harbor, ME) background for more than 10 generations to produce PON2-def animals with a homozygous genomic background. This was extensively used in our previous studies[19,20]. PON2-def mice are fertile and do not differ from WT mice in gross appearance. Mice of both genders, aged four days to four months old, were used for the study. All animal procedures and manipulations were approved by the IACUC of Cleveland Clinic in accordance with the United States Public Health Service Policy on the Humane Care and Use of Animals, and the NIH Guide for the Care and Use of Laboratory Animals.

Human heart tissue samples

Human failing hearts were from patients who underwent heart transplantation because of end-stage heart failure at Cleveland Clinic. All human studies have been approved by the Institutional Review Board (IRB) of Cleveland Clinic. Upon arrival to the lab, heart tissues were cut into small pieces, snap frozen in liquid nitrogen and then stored in −80°C for later use. Left ventricles from eight randomly selected failing hearts with ischemic cardiomyopathy were used in this study. Left ventricles of eight randomly selected unmatched donor hearts were used as non-failing controls. No clinical information was collected for this study.

Materials

Rabbit polyclonal antibodies against human PON1, PON2 and PON3 were generated by the Hybridoma Core at Cleveland Clinic Lerner Research Institute (Cleveland, OH). Antibodies to PON2 were also purchased from Abcam (Cat. #: ab183710, Cambridge, MA) and Santa Cruz (Cat.#: sc-374158, Dallas, TX). Antibody to actin was purchased from Santa Cruz (Cat.#: sc-81178). An adeno-associated virus (AAV) construct (AAV serotype 9), with a cytomegalovirus (CMV) promoter to drive the expression of murine PON2 (AAV9-PON2) was constructed by Vector Biolabs (Malvern, PA). All other chemical reagents were purchased from Sigma (St. Louis, MO), except where specifically indicated.

Heart transplantation model

To study the role of PON2 in protecting the myocardium directly, we used a heart transplantation model where the donor hearts were subjected to “hot ischemia” to enhance heart damage. Heparinized donor hearts were dissected and stored in 37°C saline for 1 hour before transplanting into WT recipient mice using the procedure described by Hasegawa et al[21]. To evaluate graft function, we scored the donor hearts at 2 minutes after implantation and 4 weeks post-transplantation based on the following criteria: score 0 = no contractions; score 1 = minimal visible ventricular motion; score 2 = weak or partial ventricular contractions; score 3 = homogeneous ventricular motion at low intensity; and score 4 = normal atrial and ventricular contraction intensity[22]. The scores were evaluated by the

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operator at 2 minutes after implantation, and by two researchers blinded to the surgery and group division at 4 weeks post-transplantation. The average scores of the two researchers were assigned to each mouse and used for statistical analysis.

Mouse transverse aortic constriction (TAC) model of heart failure

The TAC model is a previously published, reproducible model of HF predominantly due to pressure overload[23]. The sudden onset of hypertension achieved by TAC causes both an approximately 50% increase in LV mass within the first 2-4 weeks, and a well-defined HF phenotype by 12 weeks. Following intubation and mechanical ventilation of the mice, the transverse aorta was accessed through partial thoracotomy via the upper edge of the sternum. The transverse aorta was dissected free from surrounding tissues and then ligated together with a small piece of a blunted, 27-gauge needle parallel to the aorta, using a 7-0 silk thread. The 27-gauge needle was then removed to yield an approximate 0.4 mm constriction of the inner aortic diameter. Following incision closure, mice were allowed to recover and were monitored for 8 weeks with monthly echocardiograms. Mouse hearts were harvested at 8 weeks after TAC and were either snap-frozen in liquid nitrogen or fixed in 10% formalin for further studies.

Neonatal mouse cardiomyocyte culture

To directly determine the protective role of PON2 in hypoxia, we isolated cardiomyocytes from neonatal mouse hearts using the enzyme digestion method described by Yue et al[24]. In brief, 4-5 day old mouse pups were euthanized with CO_2, sterilized by dipping in 75% ethanol for one minute, and then hearts were excised under sterilized conditions. The atriums, vessels and soft tissues were separated from the ventricles. The ventricles were washed with PBS containing 100 IU/ml penicillin and 100 μg/ml streptomycin for 3 times, and then each of them was cut into 8 pieces. The heart tissues were digested in 0.07% trypsin in 10 ml PBS, with gentle agitation, for 10 minutes at 37°C. The supernatant was collected into a 50 ml centrifuge tube containing 15 ml Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum (FCS) to inactivate trypsin. Digestion of the heart tissue was repeated one time. All the cells were collected by centrifugation at 1,500 rpm for 5 min at room temperature, and then seeded into a glass plate and cultured in DMEM containing 10% FCS for 1 hour to separate the cardiomyocytes from fibroblasts, which quickly attach to the glass plate surface. The cardiomyocytes in the culture media were then collected, counted and cultured for 3-4 days before they were used for the designed experiments. All the cells used for experiments were confirmed beating actively.

MTT assay—To assess mitochondrial function and cell viability, we used MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5,diphenyltetrazolium bromide) assay with neonatal mouse cardiomyocytes as previously reported[25–27].

Measurement of ROS generation in cardiomyocytes from WT and PON2-def mice—Isolated cardiomyocytes from neonatal WT and PON2-def mice were seeded in a black 48-well plate (10^6 cells/well) and incubated at 37°C with 5% CO_2 for 4 days. Cells were then treated with 150 μM $CoCl_2$ (Cat.# AC214131000, Fisher Scientific) in complete culture media for 3 hours, washed 3 times with Hank's balanced salt solution (HBSS), and

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then incubated with 50μM dihydroethidium (DHE, Cat.#: D11347, Life Technologies, Carlsbad, CA) in HBSS for 30 minutes. After cells were washed with HBSS, fluorescence intensity was read at 510/595 nm (Excitation/Emission) on a microplate fluorometer (SpectraMax Gemini XS from Molecular Devices, Sunnyvale, CA). Cells incubated with HBSS only (without DHE) were used as negative controls.

Measurement of ROS generation in hearts from WT and PON2-def mice with TAC

Frozen heart tissues of WT and PON2-def mice harvested at 8 weeks after TAC were used for evaluating the role of PON2 on ROS generation in vivo. Since the primary ROS is superoxide, which reacts with DHE and generates a very specific product, 2-hydroxyethidium (2-OHE)[26], we measured 2-OHE levels in the TAC hearts. We adopted the methods described by Dikalov et al.[28, 29] and Laurindo et al.[30] with some modifications. Left ventricles were cut into small pieces (~ 4-6 mg) while kept frozen on dry ice. All subsequent steps were performed under dim light. The Krebs-HEPES buffer for ROS testing was composed of (in mM) 99 NaCl, 4.69 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1.03 KH_2PO_4, 20 Na-HEPES and 5.6 D-glucose, pH 7.35. Diethylenetriaminepentaacetic acid (DTPA, Cat.# D6518, Sigma, St. Louis, MO) stock solution (pH 7.35) was added into the Krebs-HEPES buffer (final DTPA concentration 100 μM) to make Krebs-HEPES/DTPA buffer. The heart tissue pieces were incubated in 1 ml argon-purged Krebs-HEPES/DTPA buffer containing freshly made 50 μM of DHE (Cat.# 37291, Sigma) at 37°C in dark for 30 min. To determine the specificity of superoxide in the TAC hearts, an aliquot of tissue pieces was pre-incubated with 100 U/ml superoxide dismutase-polyethylene glycol (PEG-SOD, Cat.# S9549, Sigma) in 1 ml argon-purged Krebs-HEPES/DTPA buffer at 37°C for 1 hour before DHE was added. The tissues were washed in 5 ml Krebs-HEPES buffer twice at room temperature and then incubated in 1 ml Krebs-HEPES buffer at 37 °C for additional 1 hour. They were then transferred to 1.5 ml homogenizing tubes (Cat.# P7811-904, Argos Technologies, Vernon Hills, IL) on ice, immediately snap-frozen and ground in liquid nitrogen with 1.5 ml pestles (Cat.# P7339-901, Argos Technologies). Cold methanol (300 μl for 5 mg tissue) was then added into each tube and tissue powder was gently mixed with the pestle. 50μL of these homogenates were used for protein concentration assay and the rest was filtered with 0.22 μm centrifugal filter units (Cat.# UFC30GV0S, Merck Millipore, Burlington, MA) at 12,000 × g, 4°C for 4 min. The filtrate was used for high-pressure liquid chromatography (HPLC) analysis.

HPLC was performed using an Agilent 1100 series HPLC system with a fluorescence detector (Agilent Technologies, Santa Clara, CA) and a C-18 reverse-phase column (Nucleosil 250 × 4.6 mm, Cat.# Z226181, Sigma). Fluorescence detection at 480 nm (excitation) and 580 nm (emission) was used to test 2-OHE and ethidium generation. The mobile phase was composed of a gradient of solution A (0.1% trifluoroacetic acid, Cat.# A116-50, Thermo Fisher Scientific, Waltham, MA) and B (pure acetonitrile, Cat.# 61001, Acros Organics, Fair Lawn, NJ) at a flow rate of 0.5 ml/min. 2-OHE and ethidium were well separated by a linear increase in acetonitrile concentration from 10% to 47% in initial 10 min, then keeping 47% for additional 10 min followed by a linear increase to 100% in the next 5 min and a linear return to 10% in the last 10 min. 2-OHE standard was purchased from Noxygen Science Transfer & Diagnostics, GmbH, Germany. The standard curve

illustrating the relationship of 2-OHE peak area and concentration was used to calculate 2-OHE concentrations of samples. 2-OHE levels were expressed as nanomole per milligram protein.

Adult mouse cardiomyocyte culture and mitochondrial ToxGlo assay

Cardiomyocytes were isolated from male WT and PON2-def mice, aged 8 weeks. Mouse was anesthetized with 200 mg/kg pentobarbital. The heart was excised under sterile conditions, gently perfused with cold PBS, minced and washed atop a 70 micron cell strainer with cold PBS. The minced heart tissue were then digested with 1U Collagenase Type II (Cat.# 17101015, Thermo Fisher) and 0.2U Porcine Elastase (Cat.# 89943132, Thermo Fisher) in sterilized PBS, with shaking at 250 rpm at 37 °C with 5% CO_2 for 5 minutes. The heart digests were then neutralized with the culture medium, 1:1 DMEM:F12 media with 15mM HEPES, 14.3mM sodium bicarbonate, 2.5mM L-glutamine, and 0.5mM sodium pyruvate (Cat.# 13-500, from the Media Core at Cleveland Clinic Lerner Research Institute) containing 10% FCS, and strained atop a 70 micro strainer, while being washed with cold PBS. Heart fragments were then digested with 1U Collagenase Type II filter-sterilized in complete media with shaking at 250 rpm, 37°C with 5% CO_2 for 1 hour. The supernatant was collected and neutralized with 1mL complete media. Cardiomyocytes were collected by centrifugation, resuspended in culture media, counted and cultured for 3 days before used for the mitochondrial ToxGlo assay.

Mitochondrial ToxGlo assay (Cat. # G8000 from Promega, Madison, WI) was performed to assess cell membrane integrity and ATP levels of WT and PON2-def mouse cardiomyocytes with the presence of sodium azide (a mitochondrial toxin, Cat. # S8032, Sigma-Aldrich) relative to no presence of sodium azide (vehicle control). Cells were assessed under non-hypoxia or $CoCl_2$-induced hypoxia conditions. Cells were seeded in a 96-well plate at 10,000 cells/well and cultured for 3 days before the assay. Cells were incubated with serial dilutions of 0.1-100 μM sodium azide in 10 mM galactose-supplemented media for 90 minutes, at 37 °C with 5% CO_2 in a cell culture incubator. Vehicle control cells were incubated with galactose media only. Galactose was used to prevent non-mitochondrial ATP generation from glycolysis. Cells were also treated with either 100 μM $CoCl_2$ in galactose media as an in vitro hypoxic agent or same volume of galactose media. As a positive toxicity control, 40 ug/mL of digitonin (Sigma) was added into culture medium. Then, the cytotoxicity reagent containing a fluorogenic substrate (bis-AAF-R110, for a distinct protease inside cells) was added and fluorescence was measured at 482 nm (Ex)/525 nm (Em) after 30-minute incubation to assess cell membrane integrity. Next, the ATP detection reagent was added and luminescence was measured after 5-minute incubation to assess ATP levels in the cells. An experimental concentration of 25 μM sodium azide was found to be acceptable for ATP detection without cell membrane instability. ATP levels and cell membrane integrity of WT and PON2-def cardiomyocytes without or with $CoCl_2$-treatment were calculated as a relative percent response of sodium azide-treated cells versus vehicle control cells.

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Western blot

Human and murine heart tissue lysates were prepared using Pierce immunoprecipitation buffer (composed of 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol) with protease inhibitors (Cat.# A32955, Thermo Scientific, Waltham, MA) added. SDS-PAGE was used to separate 30-50 μg proteins/well, which were then transferred to PVDF membranes. Membranes were probed with antibodies previously indicated, 1:1000 for primary antibodies overnight at 4°C and 1:5000 for secondary antibodies for 1 hour at room temperature. Development was facilitated using SuperSignal West Pico Stable Peroxide Solution (Cat.# 1856135) and Luminol/Enhancer Solution (Cat.# 1856136) from Thermo Scientific per the manufacturer's instructions. The membranes were stripped with Restore™ Western Blot Stripping Buffer (Cat.# 2159, Thermo Scientific) and re-blotted with actin antibody (1:1000 for 1 hour at room temperature) as a loading control.

Histological examinations

Hearts were fixed in 4% formaldehyde and embedded in paraffin. Six micrometer sections were staining by haematoxylin and eosin (H&E) staining to identify the morphology changes. Picrosirius Red staining was used to identify the fibrosis. Fibrotic areas were analyzed by an independent researcher, viewing in the whole heart longitudinal section with Image Pro Plus v7.0 software. Fibrosis was presented as the ratio of fibrotic areas to the whole myocardium areas.

Quantitative PCR (qPCR)

Total RNA was extracted from both normal, human donor hearts and ischemic cardiomyopathy (ISCM) hearts post-transplantation, using a Qiagen RNeasy® Fibrous Tissue Mini Kit as described by the manufacture (Valencia, CA). qPCR was performed using the Applied Biosystems StepOne Real-Time PCR system and StepOne Software v2.3 (Carlsbad, CA), to analyze 10 ng of RNA per sample. Predesigned primers (Cat#: 4331182; assay IDs: Hs00166557_m1 for human PON1, Hs00165563_m1 for human PON2, Hs01023629_m1 for human PON3, and Mm00447159_m1 for mouse PON2) and TaqMan Gene Expression Assays were purchased from Life Science Technology. Expression of each individual gene was adjusted with the housekeeping gene, β-actin.

PON2 activity assay

Human heart tissues were ground in liquid nitrogen and homogenized in lysis buffer containing 25 mM Tris–HCL (pH 7.4), 1 mM $CaCl_2$, 0.05% n-dodecyl-b-maltoside (Dojindo Molecular Technologies, Gaithersburg, MD) and protease inhibitors (Cat.# A32955 from Thermo Scientific). The lactonase activity assay was performed on the Roche Cobas C311 (Roche Diagnostics, Indianapolis, IN) using y-thiobutyrolactone (Sigma Aldrich) as a substrate, by the Preventive Research Lab (PRL) at Cleveland Clinic Lerner Research Institute. The final reaction mixtures were composed of 10 μL of heart tissue lysate, 5 mM y-thiobutyrolactone, 10mM Tris-HCl, pH 8, 1M NaCl, and 2mM $CaCl_2$. The reaction was performed at 37°C and the rate of generation of free thiol was determined at 412 nm using 5,5′-Dithiobis (2-nitro-benzoic acid) as an indicator. An extinction coefficient (at 412 nm) of 150,000/M/cm was used for calculating units of thiolactonase activity, which

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is expressed as the amount of free thiol produced in micromoles per minute per milliliter of reaction. The value was adjusted to protein concentration, resulting in the amount of free thiol produced in micromoles per minute per gram protein (μmol/min/gram protein).

Statistics

Data were expressed as mean ± SEM. Results were analyzed by 2-tailed Student's *t* test or 1-way ANOVA with Bonferroni post-hoc test for multiple comparisons using SigmaStat 3.5. A *p* value less than 0.05 was considered statistically significant.

RESULTS

PON2 deficiency impairs cardiac graft function in a heart transplantation model and increases reactive oxygen species generation in cardiomyocytes

In PON2-def mice, it is reported that 5-10% of the PON2 gene remains in some organs[20]. qPCR analysis specifically indicates that 2% PON2 expression remains in the hearts of PON2-def mice, as compared to WT (Supplemental Figure I). Serum PON1 activity has been concurrently reported to decrease by 26% in PON2-def mice, which may play an additive role in the oxidative environment of atherogenesis[20]. Thus, the overall phenotype found in PON2-def mice cannot completely exclude the role of PON1. Therefore, to specifically elucidate the function of cardiac PON2 independently of serum PON1 differences, a heart transplantation model was adopted in which both WT and PON2-def donor hearts were implanted into the WT recipients, which had similar circulating PON1. To enhance the injury, the donor hearts were subjected to ischemia for 1 hour in 37 °C saline before transplanting into recipient mice. Graft function was assessed at 2 minutes and 4 weeks post-transplantation, based on the criteria previously reported[22]. Although donor hearts completely stopped beating after 1 hour of hot ischemia, and no donor heart-beating was identified at 2 minutes post-transplantation, graft function assessed 4 weeks after transplantation was significantly higher in the WT donor hearts than in the PON2-def donor hearts (Figure 1A). These data suggest that cardiac PON2 is protective in post-ischemic transplantation.

Since a large amount of reactive oxygen species (ROS) are generated after reperfusion and these ROS subsequently induce injury to the hearts, we sought to determine whether the protective role of PON2 is related to its antioxidative effect. By using cardiomyocytes isolated from neonatal WT and PON2-def mice and DHE as a ROS indicator[25], we examined ROS generation in response to $CoCl_2$ treatment as an in vitro hypoxia model[31]. As shown in Figure 1B, PON2 deficiency dramatically increased production of ROS upon $CoCl_2$ treatment, as compared to WT. These data further demonstrate that cardiac PON2 is protective and may play an important role in reducing oxidative stress associated with cardiac dysfunction after cardiac insult.

PON2 deficiency in murine hearts impairs cardiac function and aggravates cardiac remodeling in a TAC model

Having shown that cardiac PON2 is protective via graft function and reduces oxidative stress in a hypoxia model, we next examined whether PON2 deficiency impairs cardiac function

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and if PON2 may prevent progression of heart failure, using a TAC model. The heart mass was significantly increased in PON2-def mice at 8 weeks after TAC surgery as compared to that of WT mice (Figure 2A). Echocardiography was conducted on both PON2-def and age- and sex-matched WT mice before surgery and at 4 and 8 weeks post-TAC. It was determined that PON2 deficiency did not affect cardiac function by observing ejection fraction (EF) and fractional shortening (%FS) in 8-week-old mice without any cardiac insult (Supplemental Figure IIA and B, Before TAC). However, left ventricular internal diameter end diastole (LVIDd) was significantly larger in PON2-def mice than in WT mice before surgery (Figure 2B), suggesting that PON2 deficiency leads to dilated cardiac remodeling under physiological conditions. Both LVIDd (Figure 2B) and left ventricular internal diameter end systole (LVIDs) (Figure 2C) were greater in PON2-def mice when compared with those of WT mice at 4 weeks post-TAC, suggesting a rapid progression of cardiac remodeling in PON2-def mice. TAC treatment also did not lead to significant differences in cardiac function (measured by EF% and %FS) between WT and PON2-def mice at 4 and 8 weeks post-surgery (Supplemental figure IIA and B, Post-TAC). However, when comparing cardiac function pre-TAC to that at 8 weeks post-TAC, cardiac function was significantly decreased in PON2-def mice but not in WT mice (Supplemental Figure IIC and IID). These data further suggest that PON2-def mice are more vulnerable to cardiac insult compared to WT mice. Additionally, Picrosirius Red staining demonstrated more severe fibrosis in PON2-def hearts than in WT hearts at 4 (Supplemental figure IIE) and 8 weeks post-TAC (Figure 2D). In an attempt to rescue PON2 expression with delivery of AAV9-PON2 to the PON2-def mice, we found a dose-dependent induction of PON2 in the heart by Western blot and qPCR (Figure 2E) 4 weeks after venous injection. Although overexpression of PON2 did not induce a statistically significant change in basal cardiac function when assessed by EF, it did appear to elevate the EF of PON2-def hearts to the level seen in WT hearts (Figure 2F). These data suggest that cardiac PON2 may play a protective role in development of HF in response to cardiac insults.

PON2 deficiency is associated with more ROS generation in heart tissue in the TAC model

Based on the above findings in PON2-def and WT hearts after TAC, we further explored whether the cardioprotective role of PON2 in the TAC model is related to its antioxidative effect, namely reducing ROS generation in the heart. Recent studies have shown ROS (superoxide) stay in frozen heart tissues and its level can be successfully measured[32]. Meanwhile, DHE combined with HPLC-fluorescence detection has been demonstrated to be an accurate approach to measure intracellular superoxide production from cells and tissues[26,33,34]. We thus used this method to compare superoxide levels in PON2-def and WT hearts harvested at 8 weeks post-TAC. We found a significantly higher level of 2-OHE in the PON2-def hearts than in the WT hearts (Figure 3A), suggesting that PON2 deficiency resulted in higher superoxide accumulation upon cardiac insult. Meanwhile, pre-incubation of WT tissue samples with PEG-SOD significantly diminished 2-OHE production (Figure 3A), confirming that 2-OHE was a product from DHE's reaction with superoxide instead of other oxidants.

In line with other groups[28,29,30,34], we also observed generation of ethidium (E) after incubation of the tissues with DHE. Ethidium is a product of nonspecific redox reactions of

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DHE, not a product from the reaction of DHE and superoxide[28]. 2-OHE and ethidium were well separated in fluorescence HPLC (Figure 3C) and this allowed us to quantify 2-OHE accurately. We found significantly higher 2-OHE/E peak area ratio in PON2-def hearts than in WT hearts (Figure 3B), which was consistent with the higher level of 2-OHE in PON2-def hearts. Ethidium levels (peak area/ug protein) were lower in PON2-def heart tissues compared to WT heart tissues and this further contributed to the higher 2-OHE/E peak area ratio in PON2-def TAC heart tissues. SOD-treated tissues showed the lowest 2-OHE/E peak area ratio (0.58 ± 0.14, $p=0.007$ when compared with WT group), supporting strong inhibition of 2-OHE production by PEG-SOD even though their ethidium levels were significantly lower than those of WT TAC hearts and at the same levels as those of PON2-def TAC hearts.

Taken together, these data demonstrate that PON2 deficiency is associated with enhanced superoxide generation in heart tissue after TAC and PON2 has an important role in reducing superoxide production in the failing hearts. Combined with the data showed in figure 2, these data suggest increased superoxide generation in PON2-def mice after TAC may be related to more rapid progression of cardiac remodeling and more severe fibrosis in these mice, and the ability of PON2 to reduce superoxide generation may contribute to its cardioprotective role following cardiac insults.

Expression of PON2 is increased but lactonase activity is decreased in human failing hearts

Having shown that the cardio-protective role of PON2 may be related to its antioxidative effect, we then tested potential increased expression of PON proteins in human ischemic cardiomyopathy (ISCM) that may counterbalance increased oxidative stress. Firstly, we examined the expression profile of PON genes in human hearts by qPCR. As shown in Figure 4A, expression of PON1 and PON3 in human hearts was nominal in contrast to abundant expression of PON2. Histological examination also confirmed the predominant PON2 expression in the heart, compared to PON1 and PON3 (Figure 4B). Western blot further demonstrated PON2 as the predominant PON protein in ISCM hearts (Figure 4C). Minimal levels of PON1 were detected with SuperSignal West Femto Maximum kit, which may be from plasma residue in heart tissues, and no PON3 expression was detected. In addition, both PON2 mRNA and protein were significantly increased in ISCM hearts compared to those in non-failing hearts (Figure 4A and 4C). However, lactonase activity was significantly decreased in ISCM hearts (Figure 4D). Although these data suggests that PON2 may be compensatorily increased to counterbalance increased oxidative/nitrative stress in failing hearts, excessive oxidative/nitrative stress may also disrupt PON2 functioning and its protective effect.

PON2 is indispensable for proper mitochondrial function

Upon hypoxia, cells promptly respond by regulating their metabolic pathways to overcome exhausted energy resources. PON2 is present in mitochondria and assists Co-Q10 function[35], but the detailed mechanism is still unclear. The MTT assay detects mitochondrial SDH activity and has been used as a determinant of mitochondrial function and cell viability[25]. Through MTT assay, the SDH activity response to a short treatment with $CoCl_2$

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(150 uM), mimicking hypoxia, was significantly increased in the WT cardiomyocytes, but no changes were observed in PON2 deficient cells (Figure 5A). Further analysis revealed that PON2-def cardiomyocytes (vs. WT) had lower baseline SDH activity (Figure 5A), suggesting that PON2 may function in mitochondrial complex II and is necessary for SDH to function properly.

To further confirm that PON2 is necessary for proper mitochondrial function, we performed mitochondrial ToxGlo assay and measured cellular ATP production in WT and PON2-def cardiomyocytes with and without $CoCl_2$-induced hypoxia. ATP levels reflect important mitochondrial function – oxidative phosphorylation. We found significantly lower ATP levels in PON2-def cardiomyocytes compared to those in WT cells (Figure 5B). Likewise, we appreciated the similar results under $CoCl_2$-induced hypoxic condition with a 17% difference (compared to 36% difference without hypoxia) between the two cell groups (Figure 5B). There were no significant differences in cell membrane integrity between the two cell types with or without $CoCl_2$ treatment (Figure 5C), suggesting that the differences of ATP levels between the two cell types were not due to defects of cell membrane integrity or cell injury/death. These results suggest that PON2 deficiency in cardiomyocytes increases mitochondrial susceptibility to mitochondrial toxin and impairs cellular ATP generation. PON2 is indispensable for maintaining normal mitochondrial function for cardiomyocytes with or without stress.

DISCUSSION

Although technologies and treatment regimens have significantly developed over the past decades, the morbidity and mortality of heart failure remain high in the US[36, 37]. Developing new heart failure treatment strategies remains a high priority. It is widely accepted that persistent inflammation and generation of reactive oxygen and nitrogen species plays a pivotal role in HF. Despite the physiological and pathological roles of ROS, current anti-oxidative therapies remain unsuccessful in treating HF. Our study demonstrates that cardiac PON2 is protective following cardiac insults and this protection may be attributed to the anti-oxidative effect of PON2, namely inhibition of ROS generation. Our study also evidenced that PON2 is necessary for maintaining myocardial mitochondrial function, probably through regulating the function of mitochondrial complex II. While PON2 is increased in ischemic cardiomyopathy, the oxidative/nitrative environment possibly renders PON2 dysfunctional, thereby minimizing its protective effect. Strategies to restore PON2 function may lead to a novel therapy for HF.

PON2 exhibits lactonase activity[38], and is the most abundant PON isoform in murine myocardium[5]. As represented in our mRNA and protein expression data, human myocardium also has elevated levels of PON2, compared to nominal levels of PON1 and PON3 (Figure 4). By comparing PON2 haploinsufficient, PON2-def, and WT mice, we found that PON2 deficiency dramatically decreased lactonase activity in the myocardium (Supplemental Figure III), suggesting that a y-thiobutyrolactone substrate is sensitive enough to measure cardiac PON2 activity. Thus, cardiac lactonase activity was measured with this method to evaluate the function of increased PON2 in human failing hearts. Although PON2 expression was significantly increased, cardiac lactonase activity was

significantly decreased in ischemic cardiomyopathy hearts compared to non-failing hearts. Since little PON1 and no PON3 was found in human hearts via Western blot analysis, this difference in lactonase activity can reasonably be attributed to PON2. These data suggest that increased PON2 in the failing heart may be not fully functional.

Unlike PON1 and PON3, which bind to circulating lipoprotein and prevent their oxidation[39], the function of locally expressed PON2 remains unclear. PON2 has noted functions of mitochondrial expression, coenzyme Q10 functional assistance[35], and reduction of ER-stress-mediated apoptosis[40]. Several key amino acid residues were determined to be essential for PON2 lactonase activity, although Altenhöfer et al. demonstrated that disruption of lactonase activity by mutation of these residues does not affect the anti-oxidative or anti-apoptotic functions of PON2[6]. This suggests that additional mechanistic studies are necessary to clarify the cellular function of PON2.

Our study is the first to demonstrate that PON2 is involved in the inhibition of ROS (superoxide) generation in both cardiomyocytes (after hypoxia) and mouse heart tissues (after TAC), which might contribute to the cardioprotective effects we observed in the study. In the meantime, our study is the first to demonstrate that PON2 may be necessary for maintaining the function of mitochondrial complex II. The presence of PON2 in complex II may promote rapid cellular response to external insults such as hypoxia. As increasing evidence suggests that mitochondrial complex II can generate ROS[41], PON2 may contribute to the reduction of ROS generation by regulating the function of the complex II during hypoxia (Figure 1B), ischemia/reperfusion injury (Figure 1A) and heart failure (Figure 3A). Additional studies are necessary to clarify whether PON2 binds to mitochondrial complex II components and how it affects the complex function and ROS generation. HF and other conditions that predispose patients to HF are associated with oxidative/nitrative stress[1,2,11,13,33,42], which can lead to protein post-translational modification, such as nitration of tyrosine residues. Tyrosine nitration leads to protein degradation and loss of function, which was identified in various human disorders, including HF[43]. Structural studies suggested that some of the 14 tyrosine-residues in human and murine PON2 are available for exposure to solvent. Therefore, further study is necessary to clarify if PON2 could be nitrated and whether nitration of these residues alters PON2 function.

Overall, our study demonstrates that human myocardium primarily expresses PON2, similar to murine hearts. PON2 is essential for maintaining normal cardiac structure, and deficiency of PON2 aggravates cardiac remodeling upon cardiac insults. The cardioprotective role of PON2 upon cardiac insults may be related to its antioxidative effect (reduction of superoxide generation), which may be achieved by regulating the function of complex II. PON2 is necessary for mitochondrial complex II function in mitochondrial response to insults. Strategies to augment PON2 expression or function may lead to a novel therapy for heart failure.

Supplementary Material

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Highlights

- Human heart primarily expresses PON2, and only expresses nominal level of PON1 and PON3.
- Mice with genetic deficiency of Pon2 had dilated cardiac remodeling, which was aggravated upon additional cardiac insults.
- PON2 deficiency dramatically decreased function of transplanted graft hearts and increased cardiac fibrosis in a murine HF model.
- PON2 deficiency lowered baseline SDH activity, and increased ROS production upon hypoxia.
- While expression of PON2 increased in human HF hearts, cardiac PON lactonase activity was decreased.
- Taken together, our findings demonstrate that cardiac PON2 is protective and strategies to upregulate PON2 function might lead to a novel therapy for HF.

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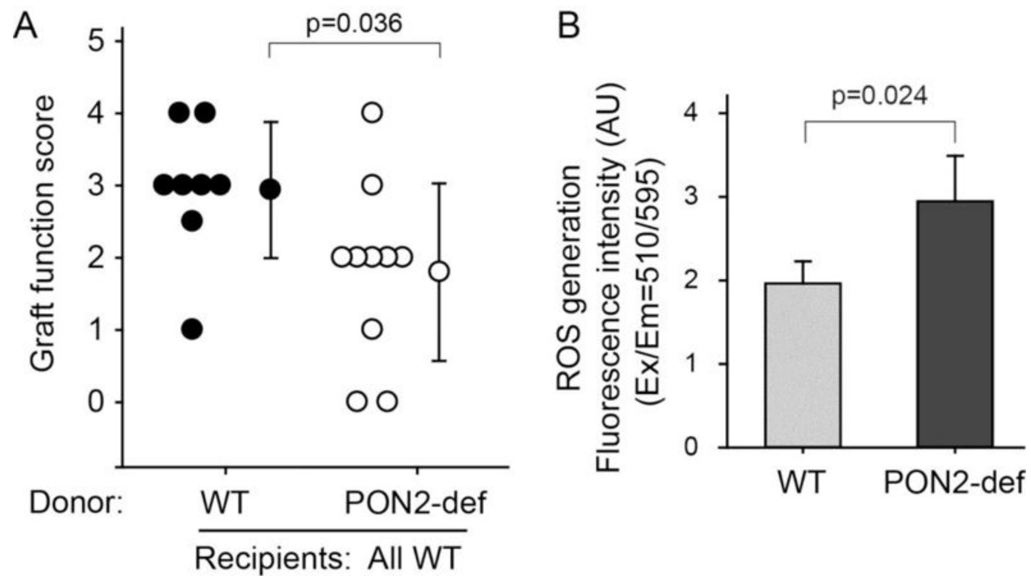

Figure 1. PON2 deficiency impairs cardiac graft function in a heart transplantation model and increases ROS generation in cardiomyocytes

A Comparison of cardiac graft function scores (assessed at 4 weeks post-transplantation) between WT donor hearts (n=8) and PON2-def donor hearts (n=10). Significant lower function scores were found in PON2-def donor hearts (p=0.036). **B.** Comparison of fluorescence intensity (reflecting ROS generation) between cardiomyocytes isolated from WT and PON2-def mice after $CoCl_2$ treatment for 3 hours (mimic hypoxia) and incubation with DHE for 30 min. Significant more ROS generation were detected in PON2-def cardiomyocytes than in WT cells (p=0.024).

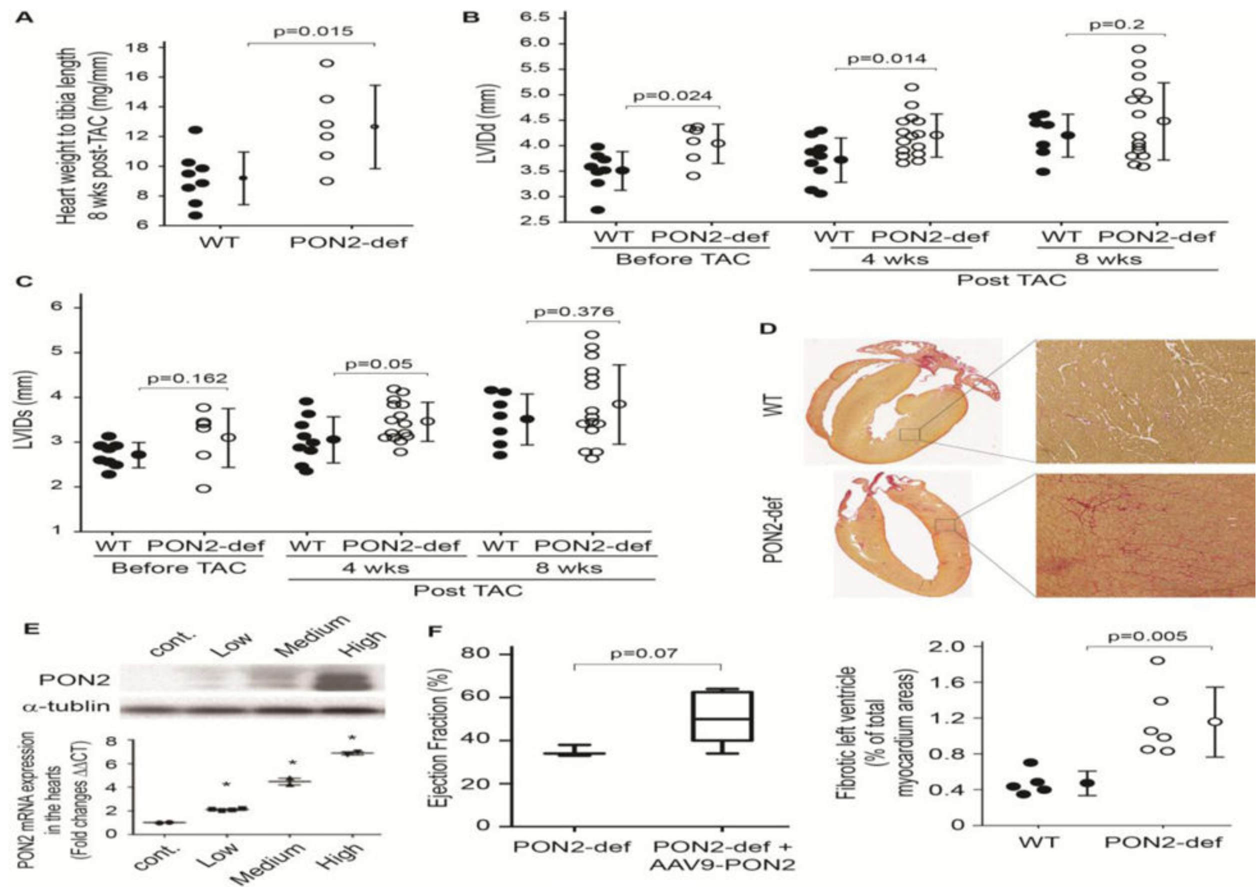

Figure 2. Systemic deficiency of PON2 aggravates cardiac remodeling and fibrosis in response to cardiac insults

A Ratio of heart weight to tibia length was assessed at 8 weeks post-TAC. PON2-def mice have significant higher ratio than WT mice after TAC (p=0.015). **B** (LVIDd) and **C** (LVIDs). Echocardiography was performed on PON2-def mice and age- and sex-matched WT mice before TAC and at 4 and 8 weeks after TAC. **D.** Picrosirius Red staining was performed on longitudinal sections of the PON2-def and WT hearts at 8 weeks after TAC. Fibrosis areas were analyzed with Image Pro Plus v7.0 software. **E.** Upper panel shows Western blot analysis of PON2 expression in mouse heart tissues at 4 weeks after intravenous injection of AAV9-PON2. “Control” = AAV9 empty vector injection, “Low” = Low dose (3.1×10^{11} viral particles) of AAV9-PON2 injection, “Medium” = Medium dose (6.7×10^{11} viral particles), “High”= High dose (1.3×10^{12} viral particles). Low panel shows PON2 mRNA expression from the same mice. **F.** PON2-def mice (n=3) were treated with AAV9-PON2 (3.1×10^{11} viral particles/mouse) and cardiac function was assessed 4 weeks later.

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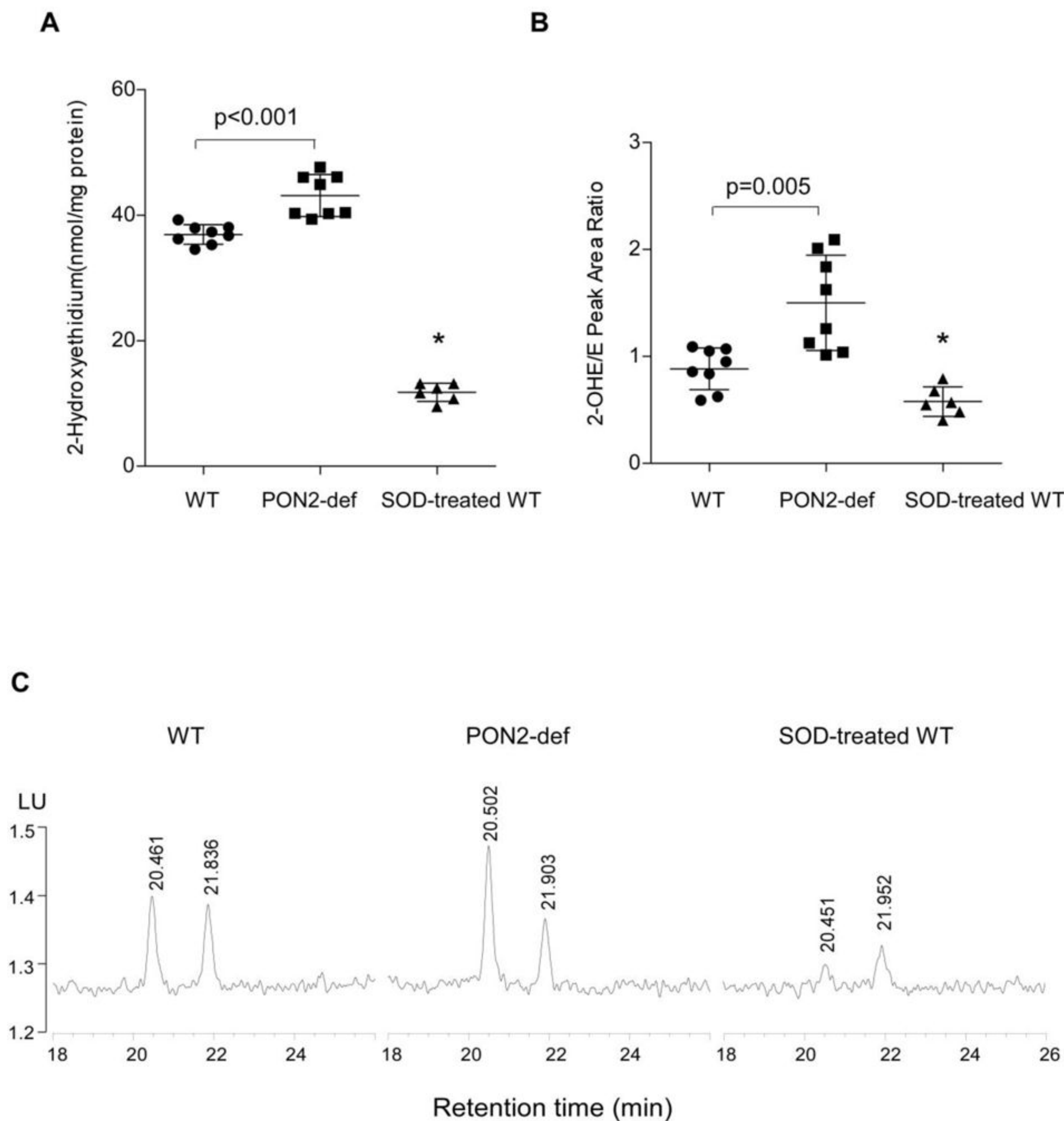

Figure 3. Systemic PON2 deficiency enhances superoxide ($O_2^{\cdot-}$) generation in heart tissue following cardiac insult (TAC)

Heart tissues were harvested at 8 weeks post-TAC from WT and PON2-def mice and accumulation of superoxide in the tissue was analyzed with DHE and fluorescence HPLC. **A.** Compared to WT heart tissues, PON2-def heart tissues had higher levels of 2-OHE (43.14 ± 3.34 vs. 36.95 ± 1.55 nmol/mg protein, p<0.001, n=8), reflecting more $O_2^{\cdot-}$ generation in PON2-def hearts at 8 weeks post-TAC. 2-OHE levels were significantly reduced when TAC heart tissues were pre-treated with PEG-SOD. * means p < 0.0001 when compared to those of WT TAC heart tissues. n=6. **B**. PON2-def TAC heart tissues demonstrated significantly higher 2-OHE/E peak area ratio compared to WT TAC heart

samples (1.50 ± 0.45 vs. 0.88 ± 0.19, $p=0.005$, $n=8$). PEG-SOD-treated samples had the lowest peak area ratio among all the sample types. * means $p=0.007$ when their peak area ratio was compared to that of WT TAC heart tissues. $n=6$. **C.** Representative HPLC tracing of 2-OHE and ethidium peaks in WT TAC, PON2-def TAC and PEG-SOD-treated WT TAC heart tissue samples, showing well-separated 2-OHE and ethidium peaks through HPLC as well as different 2-OHE/E ratios among different sample types.

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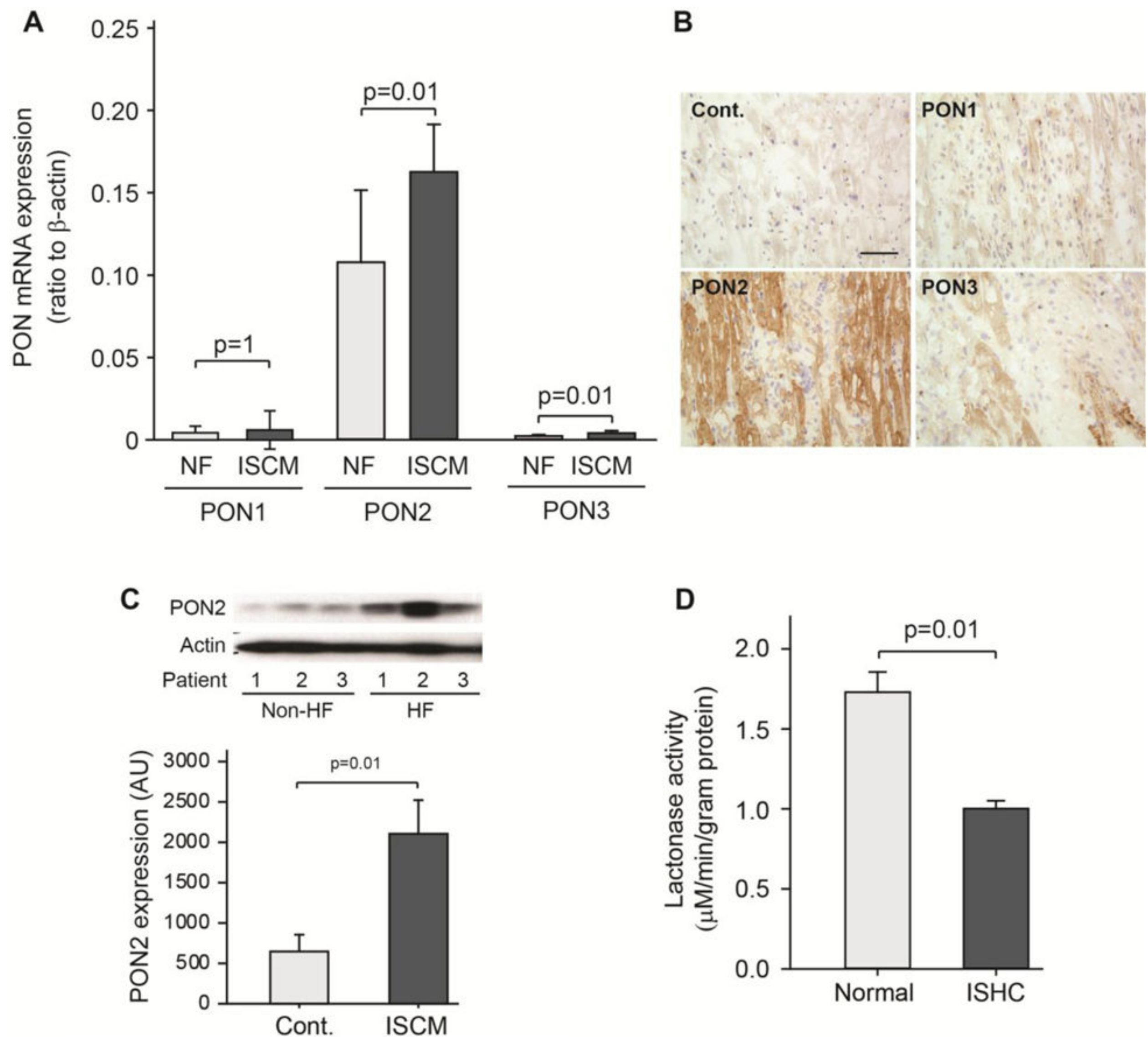

Figure 4. PON2 expression is increased in human failing hearts that possesses lower lactonase activity

A Total RNA was extracted from human non-failing and ischemic cardiomyopathy (ISCM) hearts and qPCR was performed to determine expression of PON genes. Beta-actin was used as internal control and data were presented as a ratio of expression of PON to beta-actin. n=8 in each group. **B.** Immunohistochemical staining of PON2 using the new antibodies generated in our lab. Brown represents positive staining and nuclei were counter-stained with hematoxylin. **C.** Western blot assay of PON2 expression in non-failing and ISCM hearts. Upper panel shows representative blots, and lower panel shows densitometry analysis of the western blots. n=6. **D.** Cardiac lactonase was determined using y-thiobutyrolactone as a reaction substrate and determined at 412 nm using 5,5′-Dithiobis (2-nitro-benzoic acid) as an indicator, n=8.

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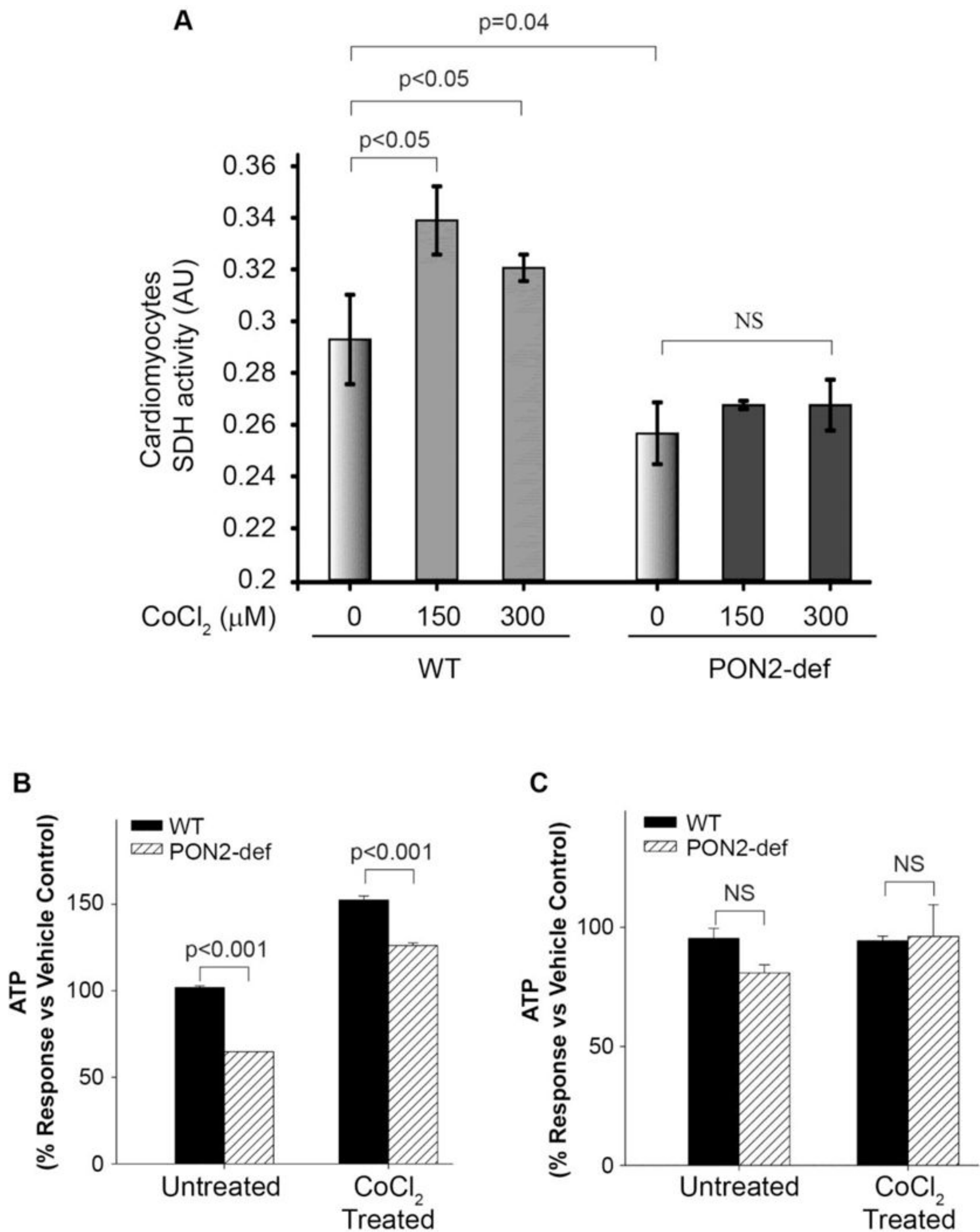

Figure 5. PON2 preserves cell viability and mitochondrial ATP generation with or without hypoxic insults

A Neonatal mouse cardiomyocytes cultured from WT and PON2-def mice were treated with $CoCl_2$ at indicated concentrations for 2 hours and then the cell viability was measured by MTT assay. **B & C.** Mitochondrial ToxGlo assay was performed to measure relative ATP levels (**B**) and cell membrane integrity (**C**) (vs. vehicle controls) in WT and PON2-def adult mouse cardiomyocytes treated with 25 μM sodium azide under non-hypoxic or $CoCl_2$-

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induced hypoxic condition. NS= No significant difference. N=3 for WT cells, n=5 for PON2-def cells.