ONLINE SUPPLEMENT

Lack of glutathione peroxidase-1 accelerates cardiac-specific hypertrophy and dysfunction in angiotensin II hypertension.

Ardanaz, Yang et al.: Role of Gpx1 in AngII-induced cardiac hypertrophy.

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METHODS

Animals

Male Gpx1-/- mice backcrossed to the C57Bl/6J background for greater than 10 generations were kindly provided by Dr. Y. Ho (Wayne State University, Detroit, MI) and subsequently bred at our institution. Age-matched C57BL/6J mice served as wild-type controls (wildtype) and were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed in a temperature-controlled environment with a 12-h:12-h light-dark cycle, receiving standard mouse chow and tap water *ad libitum*. This study was approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital and conforms to the animal care guidelines required by the National Institutes of Health.

Radio-telemetric Measurement of Mean Arterial Blood Pressure

Eighteen- to twenty-week old mice were instrumented with telemetric transmitters (PA-C10, Data Sciences International; St. Paul, MN) as previously described [1]. Mice were anesthetized with pentobarbital (50 mg/kg, *i.p.*). Their left common carotid artery was isolated under a dissecting microscope and a transmitter catheter was introduced into the aortic arch and tied to the carotid artery and secured with tissue adhesive. The radio transmitter was placed subcutaneously along the flank between the forelimb and hind limb and the wound closed with 4-0 suture. Mice were allowed to recover for at least 1 wk. Subsequently, arterial pressure and heart rate were continuously recorded, integrated over 5 minutes and reported as 24-h means ± SEM.

Infusion of Vehicle or AngII

Mice were anesthetized with brevital (70 mg/kg, *i.p.*) to allow the subcutaneous implantation of osmotic minipumps (Alzet 1007D). Mice were implanted with minipumps infusing either vehicle (saline with 0.01 N acetic acid) or AngII (521 ng/kg*min, *s.c.*; Bachem, Torrance, CA) dissolved in vehicle for 7 days.

Echocardiographic Evaluation of Cardiac Morphology and Function

Left ventricular wall thickness, dimensions and shortening fraction (SF) were evaluated with a Doppler echocardiograph equipped with a 15-MHz linear transducer (Acuson C256) in awake mice [2]. All studies were performed on awake mice before (day 0) and after AngII treatment (day 7). Echocardiographic images were traced manually and digitized by goal-directed, diagnostically-driven software within the echocardiograph. Measurements taken at three beats of the heart were averaged. Data were expressed as values taken at days 0 and 7 as well as the per cent change from day 0. All measurements were done by leading edge to leading edge according to the American Society of Echocardiography guidelines.

Measurements of Interventricular Septum (IVST), Posterior Wall Thickness (PWT), and Left Ventricular (LV) Mass.

LV mass was calculated using the equation [2]:

$$\text{LV mass} = 1.055[(\text{IVST} + \text{LVDd} + \text{PWT})^3 - (\text{LVDd})^3]$$

where 1.055 is the specific gravity of the myocardium, IVSTd is diastolic interventricular septum thickness, LVDd is diastolic LV dimension, and PWT is diastolic posterior wall thickness. The derived LV mass was normalized for body weight and expressed as mg/10g body weight.

Measurements of Cardiac Chamber Dimensions

End-diastolic and end-systolic LV dimensions (LVDd and LVDs), interventricular septum and posterior wall thickness were measured from the M-mode tracings. During diastole, LV dimension and wall thickness were measured from the maximum chamber cavity; during systole they were measured during maximum anterior motion of the posterior wall.

LV Shortening Fraction

LV shortening fraction, a measure of LV systolic function, was calculated from the M-mode LV dimensions using the equation: SF (%) = [(LVDd - LVDs)/LVDd x 100].

Preparation of Tissue Samples

On day 7 after mini-pump implantation, mice were anesthetized and the heart was stopped during diastole by injecting a 15% potassium chloride solution into the left ventricle. The animals were transcardially perfused with phosphate-buffered saline (PBS) followed by 4 % formaldehyde in PBS under pressure (100 mm Hg) and hearts and thoracic aortas were removed. Hearts were weighed and total heart weight/body weight (THW/BW) ratio (mg/10g) served as parameter of cardiac hypertrophy. The LV was transversally cut into three pieces. The LV middle section and the descending thoracic aorta (5 mm below the subclavian artery) were processed, embedded in paraffin, and serially sectioned (6-μm sections) for a variety histological and morphometric analyses.

Myocyte cross-sectional area and interstitial collagen fraction

6-μm paraffin-embedded sections were de-paraffinized and re-hydrated with distilled water. Sections were soaked in Bouin's fluid at 56º C overnight at room temperature and washed with running tap water until the rinse fluid ran colorless (about 1 hr). Sections were incubated with 0.1% picrosirius red solution for 1.5 hr at room temperature on a rotary shaker and washed twice in 0.5% acetic acid for 3-5 seconds. Sections were dehydrated in gradient alcohol, cleared in xylene and mounted in synthetic resin. Twenty one images of each left ventricle section was captured at 400 x magnification (IX81, Olympus America, Center Valley, PA) with a digital camera (DP70, Olympus America, Center Valley, PA). Myocyte cross-sectional area (MCSA) and per cent interstitial collagen fraction (ICF) was measured using an image analysis system (Microsuite Biological imaging software, Olympus America, Melville, NY) averaging per cent values of the 21 images and expressed as per cent means ± SEM.

Histological Examination of Cross-Sections of Mouse Thoracic Aorta for Measurement of Vascular Remodeling.

Sections were stained with Masson Trichrome Accustain (Sigma). Briefly, sections were de-paraffinized and hydrated, then preheated with Bouin's solution (Sigma) at 56°C for 15 min as we previously described [3]. The slides were cooled and washed in running water. Sections were stained in working Weigert iron hematoxylin solution for 5 min, rinsed in de-ionized water, stained in Biebrich scarlet-acid fuchsin (Sigma) for 5 min and rinsed in de-ionized water. Slides were placed in working phosphotungstic/ phosphomolybdic acid solution for 5 min and then in aniline blue solution (Sigma) for 5 min. Slides were washed in 1% acetic acid for 2 min, rinsed and dehydrated in alcohol, cleared in xylene, and mounted. Cross-sectional area (CSA), thickness of the media (Wm) and external perimeter (Pe) were digitally measured using

Microsuite Biological imaging software (Olympus America, Melville, NY). The diameter of the lumen (L) was calculated using the formula $L = 2 \times [(Pe/2\pi)^2-(CSA/\pi)]^{1/2}$. Remodeling was determined among groups by comparing the ratio of medial thickness to lumen diameter.

Measurements of Glutathione Peroxidase and in Aortic Tissue

Tissue homogenates were prepared by homogenizing the tissues on ice in 50 mM potassium phosphate, pH 7.0 containing 1 mM EDTA. Homogenates were centrifuged at 10,000 x g for 15 min at 4ºC. Supernatants were assayed for protein and stored at -80ºC until the day of the assay. Glutathione peroxidase (Gpx) activity was determined using assay kit # FR 17 from Oxford Biomedical Research (Oxford, MI) following the manufacturers protocol. The assay compares sample Gpx activity to a standard curve of Gpx activity generated by purified Gpx (Sigma Aldrich). Glutathione peroxidase utilizes kit substrate tert-butyl hydroperoxide to produce oxidized glutathione. Kit reagent glutathione reductase recycles the oxidized glutathione to reduced glutathione, in turn oxidizing NADPH to NADP+ which results in decreased spectrophotometric absorbance at 340 nm. The rate of decrease of A340 is directly proportional to Gpx activity.

Western Blots

Mouse hearts were homogenized in PBS-10mmole/L EDTA, 0.086 mg/ml aprotinin, with a T-50 homogenizer for 30 sec. Homogenates were centrifuged at 16000 x g, supernatant recovered and stored at -80ºC. Samples (30 μg) were run on 15 % PAGE-SDS gels, and blotted with anti-Gpx1 (Abcam), anti-AT1 antibody (Santa Cruz) and anti-β-actin. Western Blots were probed with secondary antibodies labeled with IRDye680 and 800 and analyzed by the Odyssey Imager and its software (Li-Cor Biosciences).

Data Analysis

Data are expressed as mean ± SEM. Comparisons between groups were made using analysis of variance, followed by Hochberg's method for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

References

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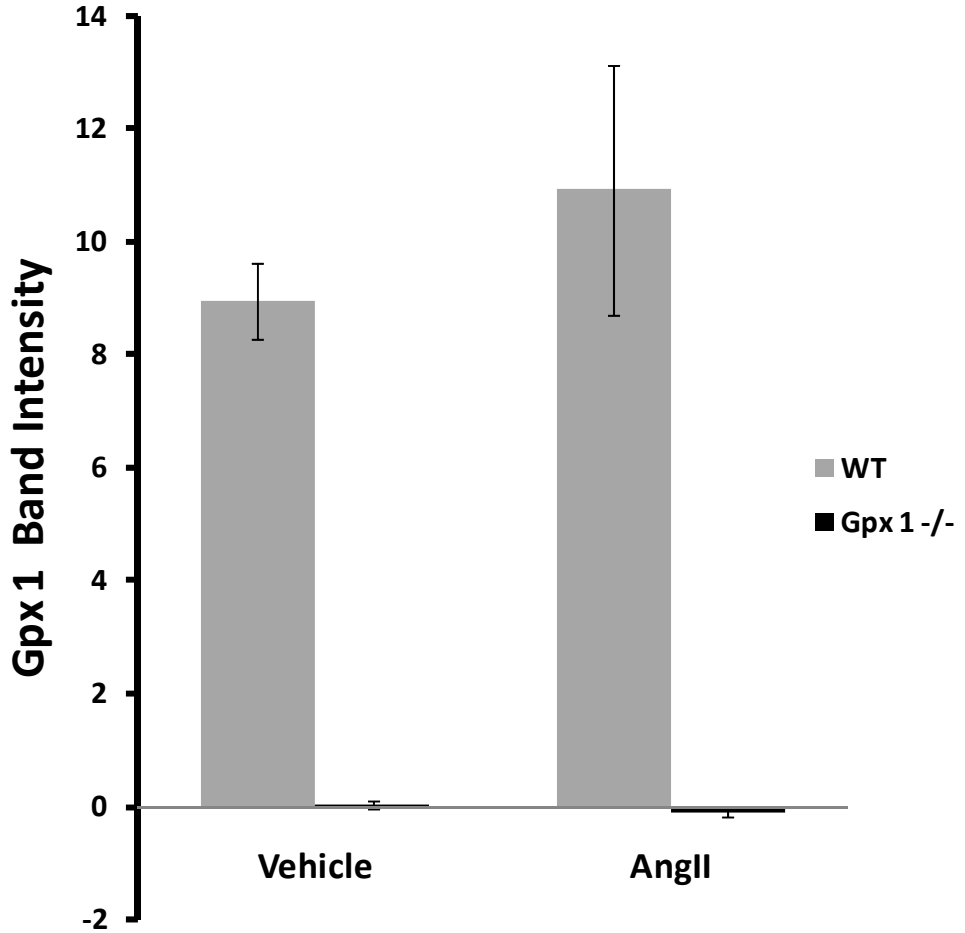

Fig. S1: Gpx1 protein levels detected by Western blot. Rat heart homogenates were run on SDS-PAGE and blotted with a rabbit antibody against Gpx 1. Western blots were analyzed using secondary antibodies labeled with IRDye 800CW, the Odyssey Imager and software from Li-COR Biosciences. Data are expressed average band intensities of Gpx 1 ± SEM (n = 2-5).

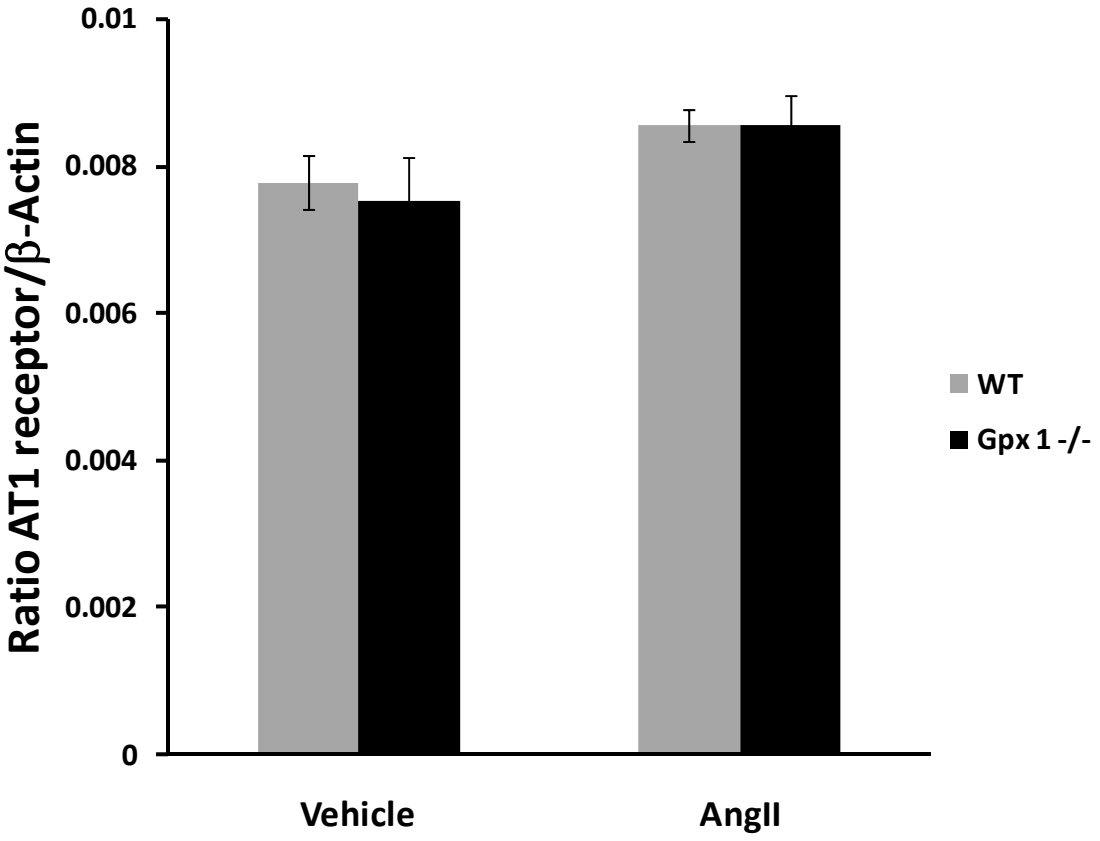

Fig. S2: AT-1 receptor levels detected by Western blot. Rat heart homogenates were run on SDS-PAGE and blotted with a rabbit antibody against AT-1 receptor and a goat antibody against β-actin. Western blots were analyzed using secondary antibodies labeled with IRDye680 and 800CW, the Odyssey Imager and software from Li-COR Biosciences. Data are expressed as the ratio of band intensities of AT-1 receptor/β-Actin ± SEM ($n = 3$).