Valsartan Regulates Myocardial Autophagy and Mitochondrial Turnover in Experimental Hypertension

Xin Zhang, Zi-Lun Li, John A. Crane, Kyra L. Jordan, Aditya S. Pawar, Stephen C. Textor, Amir Lerman, Lilach O. Lerman

Abstract—Renovascular hypertension alters cardiac structure and function. Autophagy is activated during left ventricular hypertrophy and linked to adverse cardiac function. The angiotensin II receptor blocker, valsartan, lowers blood pressure and is cardioprotective, but whether it modulates autophagy in the myocardium is unclear. We hypothesized that valsartan would alleviate autophagy and improve left ventricular myocardial mitochondrial turnover in swine renovascular hypertension. Domestic pigs were randomized to control, unilateral renovascular hypertension, and renovascular hypertension treated with valsartan (320 mg/d) or conventional triple therapy (reserpine+hydralazine+hydrochlorothiazide) for 4 weeks after 6 weeks of renovascular hypertension (n=7 each group). Left ventricular remodeling, function, and myocardial oxygenation and microcirculation were assessed by multidetector computer tomography, blood oxygen level–dependent MRI, and microcomputer tomography. Myocardial autophagy, markers for mitochondrial degradation and biogenesis, and mitochondrial respiratory-chain proteins were examined ex vivo. Renovascular hypertension induced left ventricular hypertrophy and myocardial hypoxia, enhanced cellular autophagy and mitochondrial degradation, and suppressed mitochondrial biogenesis. Valsartan and triple therapy similarly decreased blood pressure, but valsartan solely alleviated left ventricular hypertrophy, ameliorated myocardial autophagy and mitophagy, and increased mitochondrial biogenesis. In contrast, triple therapy only slightly attenuated autophagy and preserved mitochondrial proteins, but elicited no improvement in mitophagy. These data suggest a novel potential role of valsartan in modulating myocardial autophagy and mitochondrial turnover in renovascular hypertension–induced hypertensive heart disease, which may possibly bolster cardiac repair via a blood pressure–independent manner. (Hypertension. 2014;64:87-93.) ● Online Data Supplement

Key Words: angiotensin receptor antagonists ● autophagy ● hypertension ● hypertrophy, left ventricular ● mitochondrial degradation ● mitochondrial turnover

Hypertension is a leading risk factor for mortality worldwide. In the United States, its prevalence in 2009 to 2010 among adults ≥18 years of age was ≥28.6% (to ≥70 million cases), and ≥74% of chronic heart failure cases are associated with hypertension. Renal artery stenosis, leading to renovascular hypertension (RVH), constitutes <5% of hypertensive cases, yet is more closely linked to hypertensive heart diseases. Indeed, left ventricular (LV) hypertrophy (LVH) is ≥3× more prevalent in patients with RVH compared with essential hypertensive cases, possibly attributed to greater activation of angiotensin II (Ang II) that contributes to LVH. 4

Recent investigations have shed light on the link between autophagy and pathophysiological LV remodeling in response to pressure overload. During adaptive remodeling toward LVH, a compensatory increase in protein synthesis causes accumulation of toxic misfolded molecules and protein aggregates. To maintain cardiac integrity, autophagy serves as a major cellular mechanism for clearing these toxic protein aggregates and dysfunctional organelles. Moreover, energy deprivation during hypertension secondary to myocardial hypoxia or ischemia might also enhance autophagy to promote cell survival by releasing energy substrates, via degradation of cellular constituents. However, excessive autophagic activity may result in elimination of essential molecules and organelles, and contribute to LV dysfunction and adverse events. Therefore, modulation of autophagy is important to functional homeostasis in the hypertensive heart. Antihypertensive drugs greatly improve cardiovascular outcomes in patients with hypertension. In particular, angiotensin-converting enzyme inhibitors and Ang II receptor blockers efficiently decrease blood pressure in RVH. Furthermore, some of their cardioprotective effects, including reversal of LVH and prevention of heart failure, exceed blood pressure control. By blocking the angiotensin II type 1 receptor (AT1R), Ang II receptor blockers allow Ang II to bind to angiotensin II type 2 receptor (AT2R), thereby ameliorating AT1R-mediated deleterious cardiac effects of Ang II such as oxidative stress, apoptosis, and inflammation.

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From the Division of Nephrology and Hypertension (X.Z., Z.-L.L., J.A.C., K.L.J., A.S.P., S.C.T., L.O.L.) and Division of Cardiovascular Diseases (A.L., L.O.L.), Mayo Clinic, Rochester, MN; and Division of Vascular Surgery, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China (Z.-L.L.).

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Correspondence to Lilach O. Lerman, College of Medicine, Division of Nephrology and Hypertension, Mayo Clinic, 200 First Street SW, Rochester, MN 55905. E-mail leman.lilach@mayo.edu
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However, whether the benefit of Ang II receptor blocker in hypertensive heart disease involves modulation of autophagy is poorly characterized. We hypothesized that Ang II receptor blocker valsartan would alleviate myocardial autophagy and improve bioenergetic metabolism in RVH-induced LVH.

Methods

Domestic pigs were randomized to control, RVH, and RVH treated with valsartan (320 mg/d; RVH+valsartan) or triple therapy (reserpine+hydralazine+hydrochlorothiazide, RVH+TT) for 4 weeks after 6 weeks of RVH (n=7 each). Cardiac function and myocardial oxygenation were then studied in vivo using multidetector computer tomography and blood oxygen level–dependent MRI, respectively, and microvascular architecture ex vivo with microcomputer tomography. Myocardial protein expression and staining were measured ex vivo. (Detailed descriptions of all experimental methods are included in the online-only Data Supplement.)

Results

Animal Characteristics

There were no differences in body weight or degree of renal artery stenosis among the groups (Table). Mean arterial pressure increased in RVH groups by 6 weeks. Subsequent 4-week treatment by TT and valsartan similarly decreased mean arterial pressure compared with RVH, but neither normalized it (Table). Nocturnal mean arterial pressure was lower than daytime in normal pigs; this pattern was lost in RVH (Figure S1A and S1B in the online-only Data Supplement) and restored only by valsartan. Plasma creatinine was increased in all RVH groups, and unaltered by either treatment. Renal artery stenosis among the groups (Table). Mean arterial pressure compared with RVH, but neither normalized it (Table).

LV Remodeling, Function, and Oxygenation

RVH increased LV mass and its ratio to chamber volume (M/V ratio), suggesting LVH and concentric remodeling (Figure 1A and 1C), and induced cardiomyocyte hypertrophy (Figure 1B and 1D). Both drugs improved M/V ratio, but only valsartan alleviated LVH and cardiomyocyte hypertrophy compared with RVH. LV end-diastolic volume and early (E)/late (A) relaxations ratio were comparable among the groups, as were stroke volume and ejection fraction (Figure S1C–S1F), indicating relatively normal, but the adenosine-induced increase observed in normal pigs was abrogated in RVH (Figure S1G), suggesting impaired microvascular function, which was restored by valsartan but not TT. RVH also increased LV oxygen consumption reflected by rate-pressure product, which was restored by both drugs (Figure S1H). Congruently, blood oxygen level–dependent MRI indicated myocardial hypoxia in RVH (R*, P<0.001 versus normal) and impaired response to adenosine (P=0.26 versus baseline; Delta-R* P<0.001 versus normal; Figure 1E–1G). Although both drugs improved basal myocardial oxygenation, only valsartan bolstered its response to adenosine to normal levels (Figure 1G).

Myocardial Microcirculation

RVH suppressed the numbers of both small (20–200 μm) and large (200–500 μm) microvessels in the subendocardium, and large vessels in the subepicardium (Figure S2A and S2B), associated with decreased expression of vascular endothelial growth factor and angioptin-1, and increased hypoxia-inducible factor-1α (Figure S2C–S2E). Hence, insufficient microvascular spawning accompanied by increased oxygen consumption might promote myocardial hypoxia in RVH. TT normalized the density of both subendocardial and subepicardial large vessels, and restored the expression of vascular endothelial growth factor, angioptin-1, and hypoxia-inducible factor-1α. Moreover, valsartan additionally increased density of subepicardial small vessels and achieved greater levels of vascular endothelial growth factor and angioptin-1 than TT (Figure S2).

Myocardial Autophagy and Mitochondrial Activity

Myocardial Autophagy

RVH increased the expression of the autophagy initiator Beclin, of the autophagosome formation hallmark autophagy-related gene (Atg)12-Atg5 and microtubule-associated protein-1 light chain (LC3)-II, and the LC3-II/LC3-I ratio, indicating augmented autophagic activity (Figure S3). Valsartan normalized Beclin, Atg12-Atg5, LC3II, and the conversion of LC3 light chain (LC3)-I to LC3 II to LC3 I, whereas TT did not reverse enhanced autophagy. Interestingly, increased mammalian target of rapamycin expression in RVH was similarly restored by both drugs (Figure S3).

Apoptosis

RVH activated myocardial apoptosis, indicated by increased number of caspase-3+ and terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling–positive cells (Figure S4A), accompanied by upregulation of the

Table. Systemic Characteristics in Normal, RVH, and TT, or Valsartan-Treated RVH Pigs (n=7 each)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Normal</th>
<th>RVH</th>
<th>RVH+TT</th>
<th>RVH+Valsartan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>48.6±0.6</td>
<td>49.1±2.4</td>
<td>50.3±2.6</td>
<td>48.4±0.7</td>
</tr>
<tr>
<td>Degree of stenosis, %</td>
<td>...</td>
<td>76±8</td>
<td>78±5</td>
<td>77±6</td>
</tr>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>[108.9±4.5]</td>
<td>[111.1±1.4]</td>
<td>[109.6±1.1]</td>
<td>[107.9±2.9]</td>
</tr>
<tr>
<td>Baseline</td>
<td>[112.2±4.8]</td>
<td>[144.7±8.4*]</td>
<td>[134.5±7.6*]</td>
<td>[137.5±8.6*]</td>
</tr>
<tr>
<td>3 wk</td>
<td>[111.6±4.7]</td>
<td>[163.1±11.8*]</td>
<td>[146.9±8.8*]</td>
<td>[151.1±10.4*]</td>
</tr>
<tr>
<td>6 wk</td>
<td>[107.6±4.9]</td>
<td>[169.8±7.3*]</td>
<td>[132.2±10.7†‡]</td>
<td>[133.1±5.8†‡]</td>
</tr>
<tr>
<td>10 wk</td>
<td>[1.2±0.0]</td>
<td>[1.6±0.1*]</td>
<td>[1.7±0.1*]</td>
<td>[1.6±0.0*]</td>
</tr>
<tr>
<td>Serum creatinine, mg/dL</td>
<td>[586.9±30.0]</td>
<td>[897.2±62.3*]</td>
<td>[743.5±86.2*]</td>
<td>[697.3±61.5]</td>
</tr>
</tbody>
</table>

Data are mean±SEM. RVH indicates renovascular hypertension; and TT, triple therapy.

P<0.05 vs normal; †P<0.05 vs 6 wk; ‡P<0.05 vs RVH.
proapoptotic B-cell lymphoma-2-associated X protein and its ratio to B-cell lymphoma-extra large (Figure S4B). Valsartan normalized caspase-3 and deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling staining and restored B-cell lymphoma-2-associated X protein and B-cell lymphoma-2-associated X protein/B-cell lymphoma-extra large mitochondrial turnover.
large. TT showed antiapoptotic effects, but lesser than valsartan (Figure S4A and S4B).

**Mitochondrial Activity**

RVH enhanced myocardial mitophagy, indicated by elevated dynamin-related protein 1 (Figure 2A and 2D), and increased translocation of Parkin from the cytosol to mitochondrial outer membrane (Figure S5A–S5C). This was accompanied by elevated expression of mitochondria biogenesis regulator peroxisome proliferator–activated receptor–γ coactivator-1α and nuclear respiratory factor-1 (Figure 2B and 2E), possibly in compensation for hypoxia and mitochondrial degradation. However, the mitochondrial respiratory-chain proteins mitochondrial cytochrome c oxidase1, cytochrome c IV, and...
mitochondrial ubiquinone oxidoreductase dehydrogenase 1 were uniformly lower than normal, suggesting suppressed mitochondrial production (Figure 2C and 2F). TT did not reverse mitophagy, but restored mitochondrial proteins, possibly as a compensatory response to mitochondrial degradation. Contrarily, valsartan alleviated mitophagy (Figure 2A and 2D; Figure S5), increased mitochondrial biogenesis signals compared with normal (Figure 2B), and further stimulated the production of mitochondrial proteins (Figure 2C). Collectively, these data link valsartan to myocardial energy metabolism by regulating the balance between mitochondria degradation and biogenesis.

**Myocardial Oxidative Stress and Fibrosis**

RVH enhanced oxidative stress as demonstrated by increased myocardial dihydroethidium staining and expression of nicotinamide adenine dinucleotide phosphate oxidase gp91 (Figure S6A, S6C, and S6E), alleviated by both drugs. RVH induced myocardial fibrosis (Figure S6B and S6D) and increased the expression of tissue growth factor-β but decreased matrix metalloproteinase-2 (Figure S6F). Both drugs similarly alleviated fibrosis, although neither downregulated tissue growth factor-β, and only valsartan normalized matrix metalloproteinase-2.

**Discussion**

The present study shows that in addition to inducing LV hypertrophy, RVH reduced microvascular growth and function, decreased oxygen supply, and increased fibrosis. Although overall cardiac function was relatively preserved at this early stage of RVH, its biological effects were highlighted by altered bioenergetic metabolism, including enhanced cellular autophagy, apoptosis, and signals for mitochondrial degradation, but impeded production of mitochondrial respiratory-chain subunit proteins. Valsartan showed effects superior to conventional therapy in alleviating LVH, improving myocardial microcirculation and oxygenation, associated with restored cellular survival and instigation of mitochondrial biogenesis. Accordingly, this study suggests novel, blood pressure–independent cardioprotective effects of valsartan in RVH-induced heart disease.

The role of myocardial autophagy in advanced hypertensive cardiomyopathy has been described,15 whereas its involvement in early RVH is unclear. Interestingly, despite interactive crosstalk between autophagy and apoptosis, 2 cell death processes that regulate and balance each other,16–18 we found enhanced activities of both, suggesting maladaptive cell survival in RVH-induced cardiomyopathy. Although the underlying mechanism is unclear, excessive Ang II, via its AT1R, may be partly responsible.19,20 Furthermore, prominent mitophagic activity was observed in RVH, dynamin-related protein-1, richly expressed in heart,21 might have been triggered by upregulated hypoxia-inducible factor-1α, secondary to compromised microcirculation and oxygenation in RVH. Activated dynamin-related protein-1 promotes mitochondrial fragmentation, an early step in mitophagy22 and its interaction with E3 ubiquitin ligase Parkin.23 Parkin translocates to damaged mitochondrial outer-membrane,24 ubiquitinates mitochondrial proteins, and signals mitochondrial degradation.25 Despite increased expression of mitochondrial biogenesis signals (proliferator-activated receptor γ coactivator-1α, nuclear respiratory factor-1), which may reflect an attempt to replenish energy production during exposure to Ang II,26 the expression of mitochondrial proteins in RVH was suppressed. As a result, inadequate mitochondrial production coexisting with increased oxygen demand in RVH may deplete bioenergetic reserve in remaining mitochondria, thereby accounting for overactivated cell death processes. Whether the imbalanced cell death processes and mitochondrial turnover underpin development of myocardial hypertrophy in RVH and progression of cardiac dysfunction needs further investigation.

Although LVH is a major factor triggering myocardial autophagy, its partial reversal by valsartan may not suffice to achieve normalization of autophagic activity. The present study hence discloses a novel role of valsartan in regulating myocardial autophagy in RVH, which involves several potential mechanisms. AT1R and AT2R in essence play opposite roles in autophagy, where the stimulatory effect of AT1R is antagonized by AT2R.18 AT1R-mediated aldosterone production also elevates LC3II expression in cardiomyocytes.27 Valsartan has moderate binding affinity but is highly specific and selective for AT1R, with low (10%) nonspecific binding and virtually no affinity for AT2R.26,29 Hence, by selectively blocking the binding of Ang II to AT1R in myocardium, valsartan impedes the proautophagic effect of AT1R. Conceivably, valsartan may also ameliorate Ang II–driven mitochondrial degradation in response to hypoxia25 and oxidative stress30 via conferring Ang II binding to AT2R. Despite minor regulation of autophagy, TT also restored the antiapoptotic mammalian target of rapamycin similarly to valsartan. Given its important role in regulating cardiac hypertrophy in pressure overload,31 the activity of mammalian target of rapamycin is likely regulated by blood pressure level. Moreover, valsartan distinctly upregulated mitochondrial biogenesis signals and subsequent synthesis of core proteins that perform mitochondrial function, possibly via release of nitric oxide, which, through activation of AT2R,22,33 modulates mitochondrial electron-transport-chain subunits, biogenesis, and degradation.34 Collectively, our findings imply potential biological benefits of AT2R in regulation of mitochondrial turnover. Further studies need to identify the causal link of Ang II receptors to mitochondrial dynamics and its therapeutic effect by Ang II receptor blocker in myocardial repair.

Microvascular remodeling characterizes tissue adaptation in hypertension.8,35 Early RVH is associated with myocardial neovascularization to match blood supply to the evolving LVH, but subsequent vascular loss ensues,3,8,36 which is tightly linked to oxidative stress.9 In the current study, basal myocardial perfusion was relatively preserved in RVH, yet reduced vascular endothelial growth factor expression suggested a transition from functional to structural rarefaction that characterizes disease progression.9 Importantly, autophagy, which was magnified by LVH and hypoxia in RVH, inhibits angiogenesis37 and mediates the effect of angiogenesis inhibitors,38,39 thereby constituting a vicious circle interfering with maintenance of the microcirculation. Indeed, despite similar alleviation of oxidative stress to TT, valsartan alone preserved microvascular density and function (myocardial perfusion and oxygenation), which paralleled amelioration of autophagy. In
addition, enhanced mitochondrial biogenesis by valsartan may also boost energy provision for restoration and maintenance of microvascular function. The role of autophagy in modulating myocardial microcirculation merits further examination.

Our study is limited by short duration of the disease, yet cardiac structure and function in our swine model are similar to human. We were also unable to explore autophagosome formation or mitophagic activity using electron microscopy or isolated mitochondria, and relied on changes in protein expression in tissue. The effects of valsartan on myocardial autophagy and mitochondrial turnover under more aggressive blood pressure control warrant further studies. Further studies also need to clarify the causal link between myocardial bioenergetic metabolism and LVH reversal, and explore strategies for alleviating tissue fibrosis and slowing the progression of cardiac dysfunction.

Perspectives

Our study demonstrated that improvement of LVH and myocardial microcirculation by valsartan is associated with restoration of autophagy activity and regulation of mitochondria-related bioenergetic metabolism. Conventional therapy also confers some cardioprotective benefits, possibly linked to blood pressure control, vasodilation, and direct antioxidant effects. However, the distinct effects of valsartan in regulating myocardial autophagy and mitochondrial turnover may contribute to its superior efficacy in relieving cardiovascular complication and, therefore, they constitute a unique and important therapeutic component in the management of cardiovascular disease in RVH.

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Disclosures

None.

References


Novelty and Significance

What Is New?

• Our study implicates restoration of autophagic activity and regulation of mitochondrial turnover as novel protective targets in the management of hypertensive heart secondary to renovascular hypertension.

What Is Relevant?

• Renovascular hypertension is linked to adverse cardiac outcomes, and the mechanism by which Angiotensin II receptor blockers protect the hypertensive heart is incompletely understood. Our study proposes that unique modulation of autophagy and mitochondria turnover by valsartan may mediate its therapeutic benefit in hypertensive heart disease.

Summary

Angiotensin receptor blocker, valsartan, may convey blood pressure-independent cardiac repair in renovascular hypertension by directly improving cell survival and mitochondrial turnover.
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Valsartan Regulates Myocardial Autophagy And Mitochondrial Turnover In Experimental Hypertension

Xin Zhang, M.D.¹, Zi-Lun Li, M.D., Ph.D.¹,³, John A. Crane¹, Kyra L. Jordan¹, Aditya S. Pawar, M.D.¹, Stephen C. Textor, M.D.¹, Amir Lerman, M.D.², Lilach O. Lerman, M.D., Ph.D.¹,²

¹Division of Nephrology and Hypertension and ²Division of Cardiovascular Diseases, Mayo Clinic, Rochester, MN, United States; ³Division of Vascular Surgery, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China

Correspondence:

Lilach O. Lerman, MD, PhD, Division of Nephrology and Hypertension, Mayo Clinic, 200 First Street SW, Rochester, MN 55905. lerman.lilach@mayo.edu

Phone: (507)-266-9376; Fax: (507)-266-9316
METHODS

1. Experimental protocol

This study was approved by the Institutional Animal Care and Use Committee. Four experimental groups of domestic female pigs (50-60 Kg, n=7 each) were studied over 10 weeks of observation. Pigs were randomized to untreated shams (Normal), RVH (n=7), and RVH treated with either Valsartan (RVH+Valsartan, n=7) or conventional triple therapy including Hydralazine, Reserpine, and Hydrochlorothiazide (RVH+TT, n=7). All pigs were fed the same amount of isocaloric diets of standard chow containing 0.15-0.65% sodium chloride (NaCl), and had free access to water.

Unilateral RAS was induced by placing a local irritant coil in the right main renal artery, leading to gradual obstruction of its lumen within the following days, as previously described. A telemetry transducer (TA-D70, Data Sciences International, MN) was implanted in the left femoral artery in each animal to continuously monitor blood pressure during the observation. The day-night patterns of the blood pressure were also evaluated as previously described. The degree of RAS was determined by renal angiography at 6 weeks, before initiation of antihypertensive regimens.

Medications were started and fed with food 6 weeks later for 4 additional weeks. Magnetic resonance imaging (MRI) and multi-detector computed-tomography (MDCT) studies were then performed to assess left ventricle (LV) myocardial oxygenation, remodeling, and function. Blood samples were collected at the time of imaging studies.

For each in vivo study animals were weighed, induced with Telazol and xylazine (5 mg/kg and 2 mg/kg, respectively, intramuscular injection) and intubated. For MDCT, animals were anesthetized during study by continuous intravenous infusion of Ketamine and ventilated with room air. During MRI, pigs were maintained anesthetized by ventilation of 2% isoflurane-contained oxygen.

Three days following the completion of in vivo studies, pigs were euthanized, by intravenous sodium pentobarbital (100mg/kg, Fatal Plus, Vortech Pharmaceuticals, Fort Washington, PA). Hearts were removed, preserved, and prepared for ex-vivo tissue studies.

2. Antihypertensive treatment and dosage determination

Valsartan (Molecular Weight 435.5) was delivered at 320mg daily. The dosage of TT was initiated at Reserpine 0.1 mg/day, hydralazine 25 mg/day, and hydrochlorothiazide 12.5 mg/day, then titrated based on the telemetry records to achieve comparable blood pressure control to the RVH+Valsartan groups.

3. Cardiac hemodynamic, oxygenation, and function

Cardiac function and structure were assessed in vivo using 64-slice multi-detector computer tomography (MDCT, Somatom Definition-64, Siemens Medical Solution, Forchheim, Germany). Two parallel 6-mm-thick mid-LV levels were selected for evaluation of myocardial perfusion and LV function. A bolus injection of nonionic, low
osmolar contrast medium (Isovue-370, 0.33 ml/Kg over 2 seconds) into the right atrium was followed by a 50-s flow study during respiratory suspension. Subsequently, the entire LV was scanned 20 times throughout the cardiac cycle to obtain parameters of cardiac function, including LV end diastolic volume, E/A ratio, stroke volume, and ejection fraction. LV muscle mass was acquired at the end-diastole by tracing the LV endocardial and epicardial borders, and LV mass/chamber volume (M/V) calculated to assess remodeling.\textsuperscript{9, 10} LV myocardial perfusion was measured at both baseline and after adenosine infusion to assess microvascular function. The rate pressure product (RPP; systolic blood pressure x HR) served as an index of oxygen demand. After a 15-minutes interval, the same process was repeated during a 5-minute intravenous infusion of adenosine (400\textmu g/kg/min).\textsuperscript{3, 7, 11} The images were analyzed with the Analyze\textsuperscript{TM} software package (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN).\textsuperscript{7}

To assess LV myocardial oxygenation, pigs were positioned in the MRI scanner (Signa EXCITE 3T system, GE, Waukesha, WI) and blood oxygen level dependent (BOLD) images (4-5 axial-oblique) were acquired along the cardiac short axis during suspended respiration.\textsuperscript{7, 12} Gated Fast Gradient Echo sequence was used with TR/TE/number of echoes/Matrix size/FOV/Slice thickness/Flip angle=6.8 ms/1.6-4.8 ms/8/128x128/35/0.5 cm/30°. Data was acquired before and after intravenous injection of adenosine (400 mg/kg/min) through the ear vein catheter, to evaluate the oxygenation level under basal conditions and its response to a vasodilator. In each slice on T2*-weighted images obtained, the BOLD index, R2*, was estimated in each voxel by fitting the MR signal intensity vs. echo times to a single exponential function and calculating the MR intensity decay rate. Images were subsequently analyzed using MATLAB 7.10 (MathWorks, Natick, MA).

Blood samples were collected from inferior vena cava during in-vivo studies to measure plasma creatinine and aldosterone.

4. Ex vivo studies

1) Myocyte hypertrophy:

Wheat germ agglutinin (WGA, Invitrogen) staining was performed on tissue sections of left ventricular myocardium to assess cardiomyocyte hypertrophy. Immunofluoresecnt images were taken at 40x magnification at areas of transversely cut muscle fibers and examined using a computer-aided image-analysis program (AxioVision\textsuperscript{®} v4.7.2.0, and ZEN 2012, Carl Zeiss MicroImaging, Thornwood, NY). Cardiomyocytes with round nuclei were included for measurement. Lines were drawn to delineate the border of each cell according to WGA staining that highlights the cell membrane, then the area within the border were automatically calculated. Around 50-100 cardiomyocytes were measured and averaged for each animal.

2) Microcirculation:

The left-anterior-descending coronary artery was perfused with a radio-opaque polymer under physiological pressure, and a transmural portion of the LV was then prepared and scanned at 0.5° angular increments at 20-\textmu m resolution, as previously
The spatial density of microvessels (defined as diameters <500 µm) in the subepicardium and subendocardium was calculated and classified according to diameter as small (20-200 µm) and large (200-500 µm) microvessels. Expression of vascular endothelial growth factor (VEGF), angiopoietin-1 and hypoxia-inducible factor (HIF)-1α were examined by western blotting and immunofluorescence for angiogenesis.

3) Myocardial autophagy and mitochondrial turnover

**Autophagy** was examined by the expression of the autophagy initiator Beclin, autophagosome formation hallmarks autophagy-related gene (Atg)12-Atg5, and microtubule-associated protein1 light chain (LC) 3-II, as well as the LC3-II/LC3-I ratio. Expression of mammalian target of rapamycin (mTOR) was also assessed. **Apoptosis** was evaluated by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and caspase-3 staining, western blotting for Bax and Bcl-xL, and their ratio (Bax/Bcl-xL).

**Mitochondrial degradation**: Myocardial expression of dynamin-related protein (DRP) which participates selective degradation of mitochondria was examined by Western blotting. Cardiomyocyte mitophagy was also examined by immunofluorescence staining for Parkin (green, Santa Cruz) and the mitochondrial outer membrane marker Tom20 (red, Santa Cruz) for Parkin translocation (yellow). Images were taken at 40x with a zoom factor of 1.5 using the LSM780 microscope (Carl Zeiss MicroImaging), and with a zoom factor of 2.5 for inserts. Parkin-translocation+ cells were counted to represent mitophagic cardiomyocytes.

**Mitochondrial biogenesis** Signals for mitochondrial biogenesis were detected by the expression of chief modulator peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α, and its effectors nuclear respiratory factor (NRF)-1, and uncoupling protein (UCP)-2. Mitochondrial respiratory chain subunits proteins were examined by cytochrome c oxidase (COX) I, COXIV and mitochondrial NADH dehydrogenase (MTND)-1.

4) Oxidative stress and fibrosis

Oxidative stress was assessed by dihydroethidium (DHE) staining and western blotting for the expression of NAD(P)H oxidase gp91. Myocardial fibrosis was evaluated by Masson's trichrome staining, and expression of tissue growth factor (TGF)-β and matrix metalloproteinase (MMP)-2.

5) Western blotting

Standard blotting protocols were followed, using specific polyclonal antibodies against target proteins VEGF (Santa Cruz 1:200), angiopoietin-1(Santa Cruz, 1:200), and HIF-1α(abcam, 1:1000) for angiogenesis; Beclin (abcam, 1:500), Atg12-Atg5 (Cell Signaling, 1:1000), LC3 (abcam, 1:500), and mTOR (abcam, 1:2000) for autophagy; Bax and Bcl-xL (both Santa Cruz, 1:200) for apoptosis; DRP-1 (Cell Signaling, 1:1000) for mitophagy; PGC-1α, NRF-1, and UCP-2 for mitochondrial biogenesis signals (all Abcam, 1:1000); MTCO1 (abcam, 1:2000), COXIV (Cell Signaling, 1:1000), and MTND-1 (abcam, 1:1000) for mitochondria production; NAD(P)H oxidase gp91 (abcam, 1:1000) for oxidative stress, and TGF-β and MMP-2 (both Santa Cruz, 1:200) for fibrosis.
Horseradish peroxidase secondary antibodies (GE Healthcare UK Limited) were used and chemiluminescence determined using the SuperSignal West Pico Chemiluminescent Substrate or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, IL) according to vendor's instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:5000 Covance, Emeryville, CA) served as loading control.

5. Statistics

Data are expressed as mean±SEM. Comparisons within groups were performed using the paired Student’s *t*-test and among groups ANOVA followed by unpaired *t*-test. Statistical significance was accepted at *P*< 0.05.
REFERENCES FOR ONLINE SUPPLEMENT


Figure S1

A and B: Diurnal patterns of mean arterial pressure in Normal, renovascular hypertension (RVH), RVH+triple therapy (TT) and RVH+Valsartan pigs. C-F: Diastolic and systolic function was relatively preserved in early RVH. G: Myocardial perfusion measured by multidetector CT showed diminished response to adenosine in RVH that was normalized only by Valsartan. H: Rate-pressure product indicated elevated oxygen demand in RVH but restored by both regimens. *p<0.05 vs. Normal; $p<0.05 vs. RVH; #p<0.05 vs. its control.
Figure s2

**A**

![Images of normal, RVH, RVH+TT, and RVH+Valsartan subepicardial sections.](image)

**B**

![Bar graphs showing microvascular density (vessels/mm²) in subepicardium and subendocardium.](image)

**C**

![Western blot images for VEGF, GAPDH, Angiopoietin-1, and HIF-1α.](image)
Figure S2. A: Representative 3-D micro CT images of the LV myocardium in the 4 groups. White arrow indicates the left anterior descending artery. B: Quantification of microvascular density. C: Expression of the angiogenic factors vascular endothelial growth factors (VEGF), angiopoietin-1 and hypoxia-inducible factor (HIF)-1α. D and E: Representative immunofluorescence images (40x) and quantification for VEGF (red) and HIF-1α (green) expression in the myocardium. Valsartan restored microvascular density, and upregulated angiogenesis to a greater extent than TT. *p<0.05 vs. Normal; &p<0.05 vs. RVH+TT.
Figure S3. Expression of autophagic proteins in Normal, RVH, RVH+TT and RVH+Valsartan pig hearts. Atg: autophagy-related gene; LC3: microtubule-associated protein1 light-chain-3; mTOR: mammalian target of rapamycin. Valsartan alone reversed enhanced autophagy in RVH myocardium. *p<0.05 vs. Normal; $p<0.05 vs. RVH.
Figure S4. A: Immunofluorescent staining for Caspase-3 (red) and TUNEL (green, blue nuclei), and B: Expression of apoptotic proteins for Bax and Bcl-xL. RVH activated apoptosis. Both regimens alleviated apoptosis, but Valsartan more than TT. *p<0.05 vs. Normal; $p<0.05 vs. RVH; &p<0.05 vs. RVH+TT.
Figure S5. A: Immunofluorescence staining of Parkin (green), the mitochondrial outer membrane marker Tom20 (red), nuclei (blue), and their merged images (40x). Mitophagy induction was indicated by co-localization of Parkin and Tom20. In the top row images, in normal pig cardiomyocytes both Parkin and Tom20 were evenly distributed across the cytosol. Second row images, obtained from an RVH pig, show markedly increased peri-nuclear aggregates (yellow) positively stained for both Parkin and Tom20, indicating translocation of Parkin to damaged mitochondria membrane, and hence mitophagy induction (Inserts shown in panel B). In the third row images, TT did not alter Parkin translocation in RVH. Bottom row images show that Valsartan alleviated the number of mitophagic cells. C: Quantification of mitophagic/Parkin translocation+ cells. *p<0.05 vs. Normal; $p<0.05 vs. RVH.
Figure S6. A, C and E: Representative immunofluorescence images (40x) and quantification for dihydroethidium (DHE), and expression of NAD(P)H oxidase gp91. RVH increased oxidative stress, which was alleviated by both regimens. B, D and F: Representative images of myocardial trichrome staining (20x), its quantification, and expression of tissue growth factor (TGF)-β and matrix metalloproteinase (MMP)-2 and activated MMP-2. Myocardial fibrosis in RVH was alleviated by both regimens, but to a slightly greater extent in Valsartan. *p<0.05 vs. Normal; $p<0.05 vs. RVH.
Figure S7

<table>
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<tr>
<th>Protein</th>
<th>Normal (n=6)</th>
<th>RVH (n=6)</th>
<th>RVH+TT (n=6)</th>
<th>RVH+Valsartan (n=7)</th>
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<td>VEGF 42 KDa</td>
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Figure S8

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- LC3 I 19KDa
- LC3 II 17KDa
- mTOR 289KDa
- Bax 23KDa
- Bcl-xL ~30KDa
- DRP1 ~78KDa
Figure S9

Normal (n=6)  RVH (n=6)  RVH+TT (n=6)  RVH+Valsartan (n=7)

- PGC-1α ~110 KDa
- NRF-1 54KDa
- UCP-2 ~30KDa
- MTCO1 ~ 40KDa
- COX IV 17 KDa
Figure S10

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Figure S7-S10. Whole gel images for all proteins examined by western blotting. Images are shown in the order in which the proteins expression is reported in the Results section of the manuscript.