

# Autophagy Portends the Level of Cardiac Hypertrophy in Experimental Hypertensive Swine Model

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## BACKGROUND

Left ventricular (LV) hypertrophy (LVH) plays an important role in hypertensive heart disease, and may be accompanied by myocardial autophagy. However, the pattern of autophagy during evolution of LVH is unclear. We hypothesized that autophagy activation indicates advancing cardiac LVH with tissue remodeling.

## METHODS

Ten domestic pigs with a 10-week unilateral renovascular hypertension (HTN) were classified as mild or moderate HTN ( $n = 5$  each group) based on the degree of renal artery stenosis (above or below 75%). Seven normal pigs served as controls. Left ventricular remodeling, function, and microvascular density were assessed using multi-detector- and micro-computed tomography and histology. Markers of myocardial autophagic and endoplasmic reticulum (ER) stress-related unfolded protein response (UPR), apoptosis, and fibrosis were examined *ex vivo*.

## RESULTS

Both HTN groups had increased myocyte cross-sectional area, but it was greater in moderate HTN, accompanied by elevated LV

muscle-mass. Moderate, but not mild HTN, also showed impaired microvascular density and impaired myocardial perfusion. Autophagy mediators were unaltered in mild HTN but UPR markers were increased, while in moderate HTN they were all upregulated, whereas UPR markers were suppressed. Myocardial apoptosis and fibrosis were also greater in moderate HTN. Autophagic proteins were correlated with LVH and fibrosis.

## CONCLUSIONS

Autophagic activity is stimulated during the exacerbation of LVH, following a transient early increase in ER stress, and may be involved in the progression of cardiac remodeling in renovascular hypertensive heart disease.

**Keywords :** autophagy; blood pressure; hypertension; left ventricular hypertrophy; renal artery stenosis; renin-angiotensin-aldosterone system; unfolded protein response.

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Almost 30% adults within the United States are affected by hypertension (HTN),<sup>1,2</sup> a leading risk factor for mortality and closely associated with chronic cardiac dysfunction.<sup>3</sup> Development of left ventricular (LV) hypertrophy (LVH), an increase in LV muscle mass in response to elevated afterload mostly caused by HTN, contributes to heart failure. The extent of LVH is directly linked to the magnitude and duration of HTN,<sup>4</sup> and its persistence associates with cardiac fibrosis and diastolic dysfunction that may advance to systolic heart failure.

The mechanisms that account for myocardial pathophysiological events in LVH are yet undetermined. Microvascular remodeling and oxidative stress are known contributors to tissue injury in HTN,<sup>5-7</sup> and recent studies have also linked it to autophagy.<sup>8</sup> During adaptive left ventricular remodeling toward LVH, a compensatory increase in protein synthesis causes accumulation of toxic misfolded molecules

and protein aggregates. To maintain cardiac integrity,<sup>9,10</sup> autophagy serves as a major cellular mechanism for clearing misfolded proteins and/or dysfunctional organelles in response to stress induced by pressure overload and resultant tissue hypoxia.<sup>11</sup> Interestingly, however, autophagy has been observed to be triggered in both LV dysfunction<sup>12,13</sup> and LVH regression,<sup>14</sup> suggesting that the activity of myocardial autophagy follows a dynamic pattern that could vary depending on the phases of LVH, serving as a protective or deleterious mechanism. In addition, in response to accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER), a coordinated adaptive program of unfolded protein response (UPR) is also activated. The UPR aims to alleviate ER stress by suppression of protein synthesis, facilitation of protein folding via induction of ER chaperones including glucose-related protein (GRP)78 and GRP94, and degradation of unfolded proteins. Indeed, ER

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stress has been implicated in ischemic heart disease and hypertrophy.<sup>15,16</sup>

Renovascular HTN, the leading cause of secondary HTN due to renal artery stenosis, may lead to hypertensive heart diseases,<sup>17–19</sup> given the marked activation of the renin-angiotensin-aldosterone system that contributes to LVH.<sup>20</sup> We have previously shown that the renovascular hypertensive heart exhibits enhanced autophagy accompanying LVH.<sup>18</sup> However, the pattern and relationship of autophagy and ER stress activation at different stages of LVH progression are unclear. We hypothesized that autophagic activity is increased during the evolution of LVH induced by renovascular HTN in a swine model, and associated with escalated tissue remodeling.

## METHODS

Seventeen domestic pigs were randomized into normal ( $n = 7$ ) and renovascular HTN ( $n = 10$ ), achieved by unilateral renal artery stenosis, and the HTN pigs were further arbitrarily classified at 10 week as mild (stenosis  $<75\%$ ,  $n = 5$ ) or moderate (stenosis  $\geq 75\%$ ,  $n = 5$ ) HTN. Left ventricular remodeling and microvascular density were studied by using multi-detector computer tomography *in vivo* and micro-computer tomography *ex vivo*, respectively. Plasma renin activity, aldosterone, and serum creatinine were assessed. Myocardial autophagy and apoptosis were measured *ex vivo* in the myocardium, and ER stress markers in isolated ER. (Detailed descriptions of all experimental methods are included in the [Supplementary Methods](#).)

## RESULTS

### Animal characteristics

Ten weeks after induction of renal artery stenosis, mean arterial pressure was elevated in both HTN groups, but was higher in moderate HTN ([Table 1](#)), while plasma renin

activity and plasma aldosterone were elevated only in moderate HTN. Serum creatinine was similarly elevated in both HTN groups ([Table 1](#)).

### LV remodeling, function, and microvasculature

HTN increased myocyte cross-sectional area in both groups, suggesting myocyte hypertrophy, but to a greater extent in moderate HTN; LV muscle mass increased only in moderate HTN ([Figure 1A,B](#)). The E/A ratios were similarly lower in both mild and moderate HTN compared to normal ([Table 1](#)), indicating diastolic dysfunction, while LV end-diastolic volume, stroke volume, and ejection fraction were unaltered ([Table 1](#)). However, moderate but not mild HTN decreased the density of microvessels (diameter  $<500 \mu\text{m}$ , [Figure 1C](#)), and upregulated hypoxia-inducible factor-1 $\alpha$  ([Figure 1D](#)). Moderate HTN also blunted the response of myocardial perfusion to adenosine ([Figure 2A](#)).

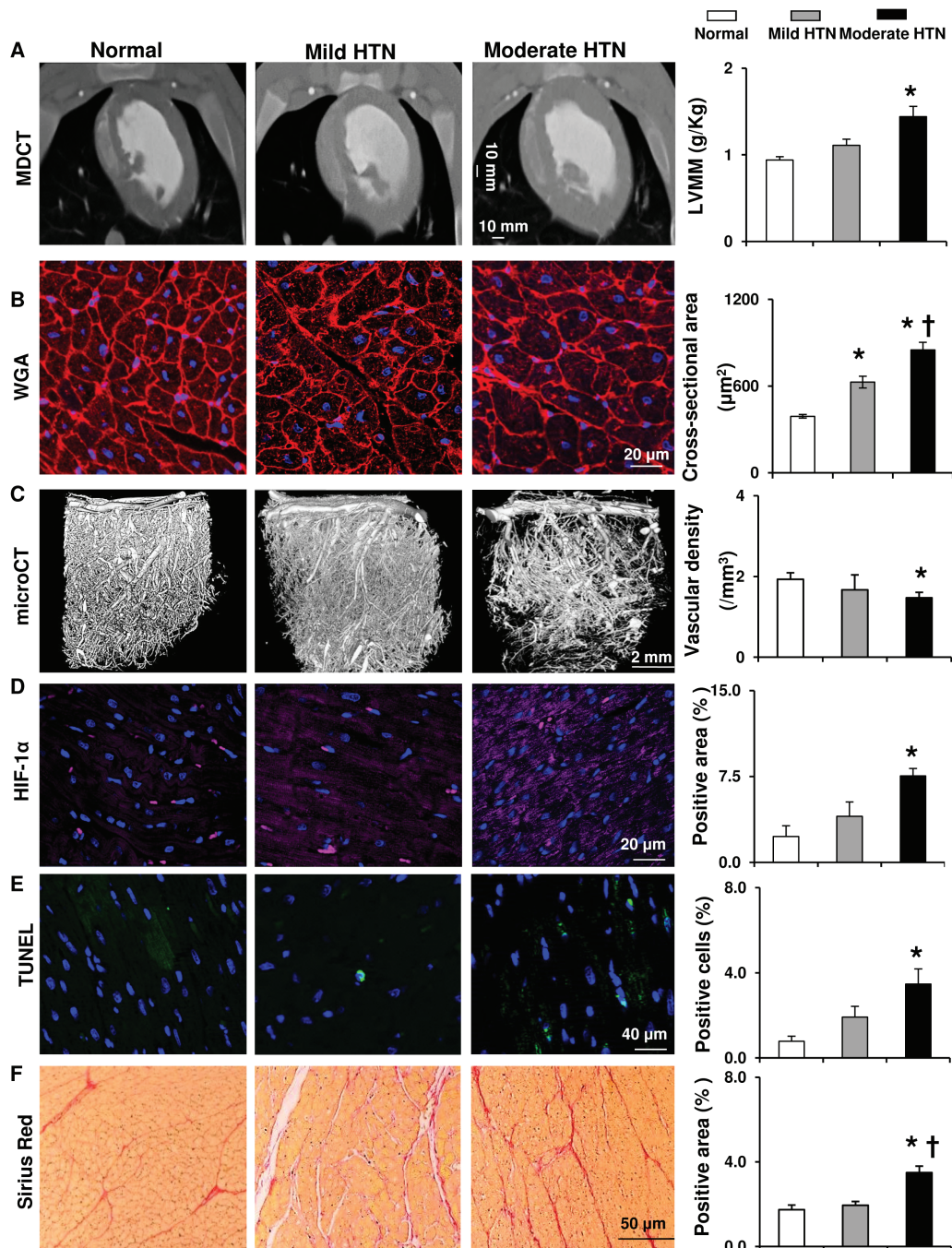
### LV autophagy, UPR, apoptosis, and fibrosis

Mild HTN did not alter autophagic mediators like Beclin-1, autophagosome formation hallmarks autophagy-related (Atg) 12–5, or the microtubule-associated protein-1 light-chain (LC)3-II/LC3-I ratio ([Figure 2B–D,F](#)), whereas moderate HTN increased them all. The lysosomal protease cathepsin-D was elevated in both HTN groups, but P62 decreased only in moderate HTN, suggesting enhanced lysosomal and proteasome degradation<sup>21,22</sup> ([Figure 2E,G,H](#)). Mild HTN also increased the autophagy inhibitors Akt, mammalian target of rapamycin, and its effector pS6 ([Figure 3A,C–E](#)), which were lower in moderate compared to mild HTN. These results are consistent with magnified autophagic activity in more severe LVH. Interestingly, expression of the UPR marker GRP78 was elevated, and GRP94 also tended ( $P = 0.07$ ) to increase only in mild HTN, while in moderate HTN both were not different from normal and lower than in mild HTN ([Figure 3B,F,G](#)), suggesting a transient ER stress response in the early stage of LVH.

**Table 1.** Systemic characteristics and cardiac function and structure (mean  $\pm$  SEM) of normal ( $n = 7$ ), mild ( $n = 5$ ), and moderate ( $n = 5$ ) renovascular hypertension (HTN) pigs

	Normal	Mild HTN	Moderate HTN
Body weight (kg)	47.7 $\pm$ 1.3	46.2 $\pm$ 2.7	49.8 $\pm$ 3.0
Degree of stenosis (%)	—	73 $\pm$ 6	98 $\pm$ 2 <sup>†</sup>
MAP (mm Hg)	87.1 $\pm$ 2.1	93.1 $\pm$ 2.0*	131.4 $\pm$ 6.7* <sup>†</sup>
Plasma creatinine (mg/dl)	1.2 $\pm$ 0.1	1.7 $\pm$ 0.1*	1.6 $\pm$ 0.1*
PRA (ng/ml/h)	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	10.6 $\pm$ 4.8*
Plasma aldosterone (pg/ml)	555.6 $\pm$ 33.7	519.2 $\pm$ 42.3	1,044.5 $\pm$ 44.5*
E/A ratio	1.4 $\pm$ 0.1	0.8 $\pm$ 0.1*	0.9 $\pm$ 0.1*
LVEDV (ml)	1.9 $\pm$ 0.1	1.8 $\pm$ 0.1	1.7 $\pm$ 0.1
Stroke volume (ml)	49.9 $\pm$ 1.2	54.7 $\pm$ 3.5	54.5 $\pm$ 5.0
Cardiac output (l/min)	5.3 $\pm$ 0.6	5.2 $\pm$ 0.5	4.5 $\pm$ 0.5
Ejection fraction (%)	55 $\pm$ 3.0	56 $\pm$ 4.0	63 $\pm$ 8.0

Abbreviations: LVEDV, left ventricular end-diastolic volume; MAP, mean arterial pressure; PRA, plasma renin activity. \* $P < 0.05$  vs. normal; <sup>†</sup> $P < 0.05$  vs. mild HTN.



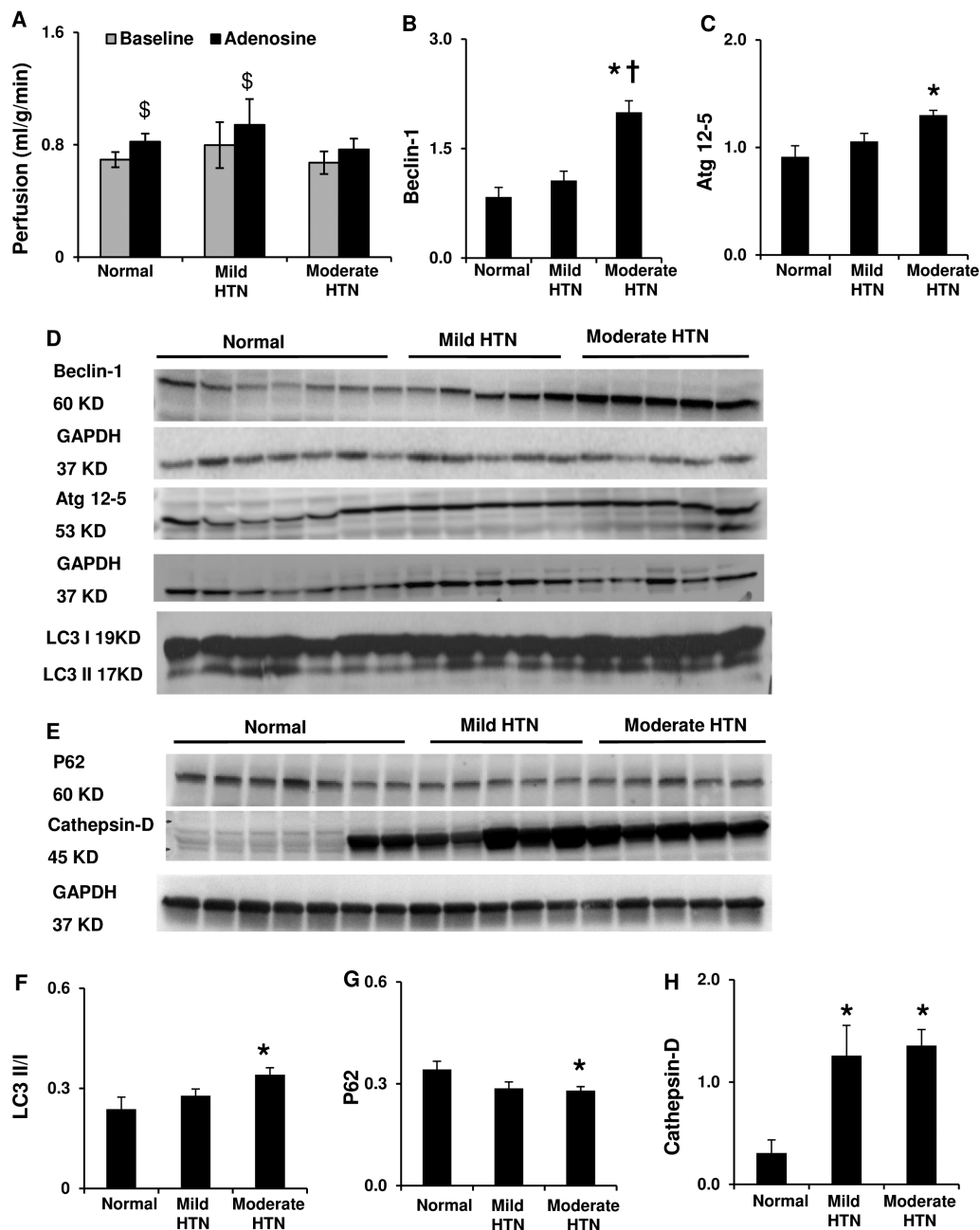
**Figure 1.** Alterations in left ventricular structure in hypertension. (A,B) Representative multi-detector computed tomography (CT) and wheat germ agglutinin (WGA) staining images establishing left ventricular and myocyte hypertrophy in pigs without or with mild or moderate hypertension (HTN), and their quantification. (C) Representative micro-CT images of the myocardial microvasculature and quantification of vascular density ( $<500\ \mu\text{m}$  in diameter). (D–F) Representative staining and quantification of hypoxia-inducible factor (HIF)-1 $\alpha$ , dUTP nick-end labeling (TUNEL), and Sirius Red. \* $P < 0.05$  vs. normal, † $P < 0.05$  vs. mild HTN.

HTN increased the number of apoptotic cells only in moderate HTN (Figure 1E). Both HTN groups decreased expression of anti-apoptotic B-cell lymphoma extra-large (Bcl-xl), while moderate HTN additionally decreased Bcl-2, while Bax remained unchanged (Figure 4A,C–E), suggesting that the enhanced apoptosis in moderate HTN was possibly due to attenuated anti-apoptotic activities.

Sirius Red staining revealed an increase of collagen deposition only in the moderate HTN myocardium (Figure 1F),

as did tissue inhibitor of metalloproteinase-1 expression (Figure 4B,F).

Spearman rank correlation analysis showed a significant association of the expression of autophagy proteins with the extent of LVH as well as tissue fibrosis, whereas UPR markers was not associated with either (Figure 5A–E), revealing a close link of autophagic activity to cardiac remodeling in hypertensive heart. dUTP nick-end labeling (TUNEL) was also correlated to fibrosis. In addition, while an association between



**Figure 2.** Left ventricular myocardial perfusion and autophagic activity. (A) Myocardial perfusion at baseline and in response to adenosine. (B–D,F) Myocardial protein expression and quantification for Beclin-1, autophagy-related (Atg) 12-5, and microtubule-associated light-chain (LC) 3-II and LC3-I ratio. (E,G,H) Myocardial protein expression and quantification of P62 and cathepsin-D. \* $P < 0.05$  vs. normal, † $P < 0.05$  vs. mild HTN. ‡ $P < 0.05$  vs. baseline.

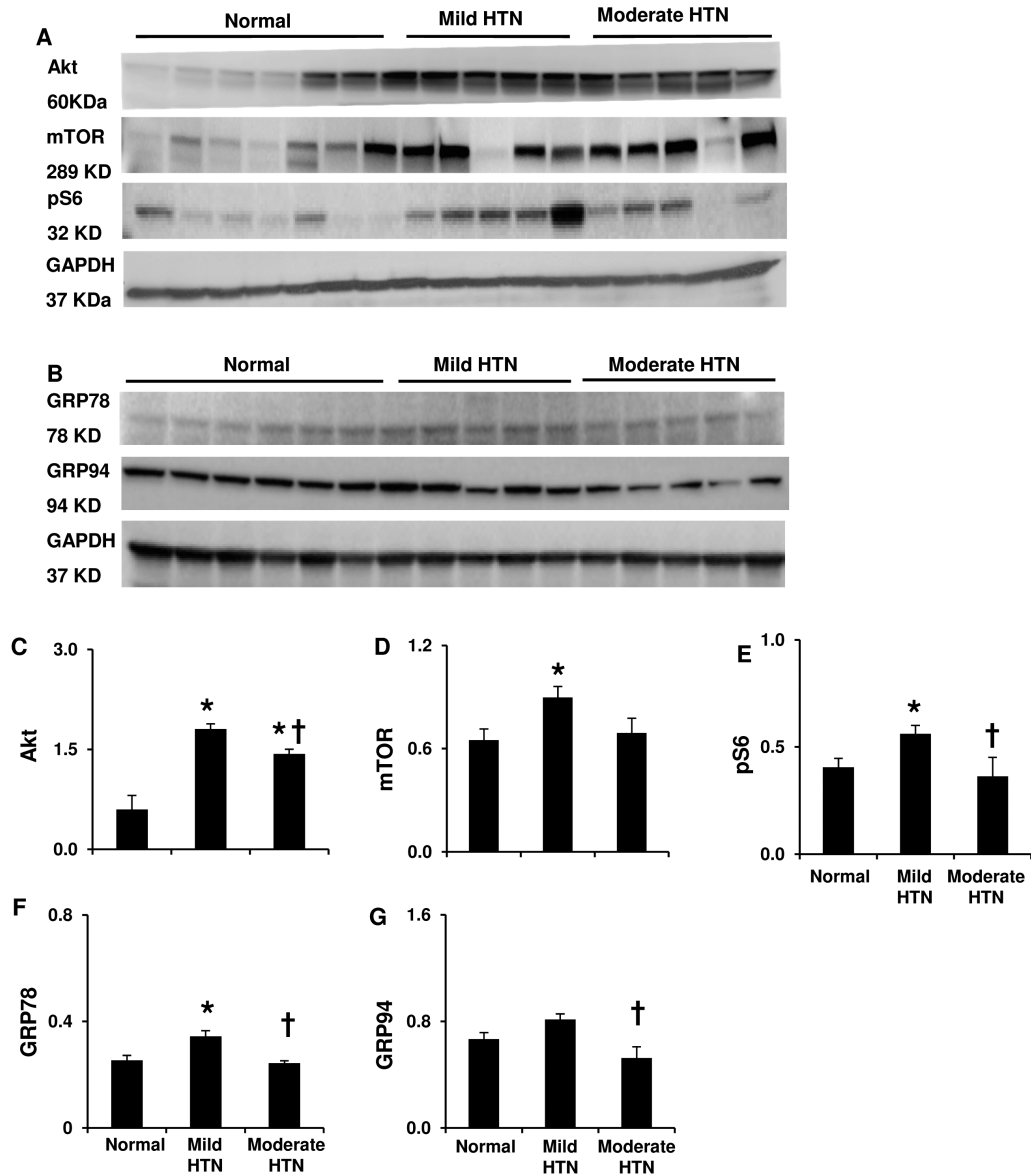
TUNEL and Beclin-1 did not reach a statistical significance level ( $P = 0.07$ ), the anti-apoptotic Bcl-2 was significantly related to autophagic proteins, suggesting a possible link between autophagic and apoptotic activities (Figure 5G,H).

## DISCUSSION

The present study shows that progression of LVH in HTN involves a dynamic pattern of cellular turnover modulation. As LVH evolved, ER stress was upregulated as an early response, while autophagic mediators rose at a later stage

(Beclin-1, Atg 12-5, LC3) as did lysosomal and proteasome degradation (increased cathepsin-D and decreased P62, respectively). The change in autophagy-related proteins paralleled magnification of apoptosis and fibrosis. Therefore, autophagic and apoptotic activities may be implicated in the pathways promoting LVH, and possibly reflect the severity of hypertensive heart disease.

Myocardial autophagy has been largely considered a defensive response to pressure overload.<sup>18,23</sup> Due to a compensatory increase in protein synthesis during adaptive cardiac remodeling toward LVH, autophagy is triggered to clear

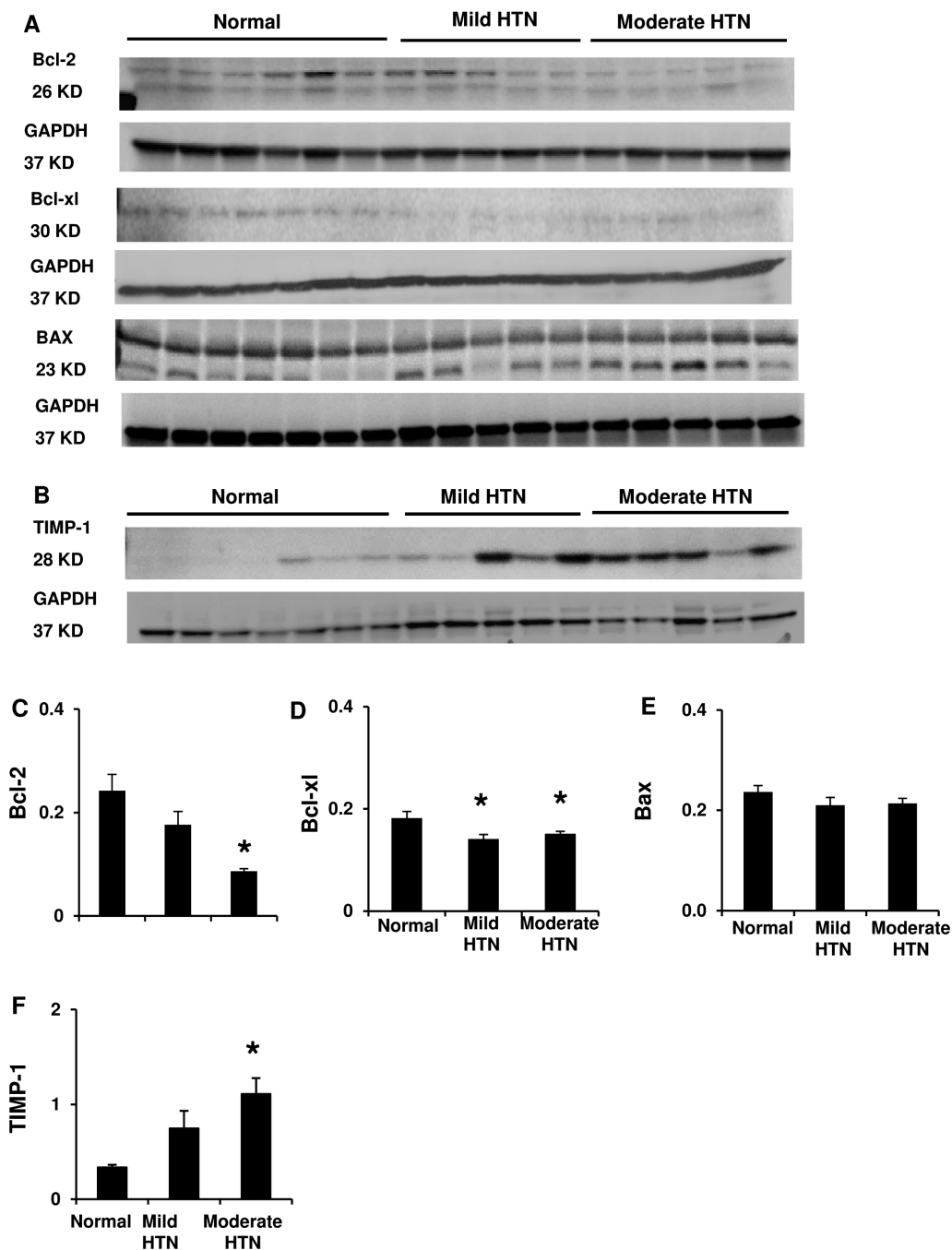


**Figure 3.** Left ventricular autophagic activity, and endoplasmic reticulum stress. (A,C–E) Myocardial protein expression and quantification for phospho-/total Akt, mammalian target of rapamycin (mTOR) and phospho-s6. (B,F–G) Protein expression and quantification for unfolded protein response markers glucose-regulated protein (GRP)-78 and GRP94 in isolated endoplasmic reticulum. \* $P < 0.05$  vs. normal, † $P < 0.05$  vs. mild HTN.

accumulating toxic or misfolded/dysfunctional organelles and maintain cardiac integrity.<sup>9</sup> In addition, HTN-induced LVH impairs coronary flow reserve<sup>24</sup> possibly due to the regression of microvessels that is particularly discernible in moderate HTN. Although basal myocardial perfusion was relatively preserved by compensatory mechanisms, amplified oxidative stress by persistent HTN and activated renin-angiotensin system<sup>18</sup> might have interfered with microvascular function to diminish the response to adenosine. Insufficient microcirculation subsequently limits oxygen (reflected in increased hypoxia-inducible factor-1 $\alpha$ ) and nutrient supply,<sup>5</sup> both of which may stimulate autophagic signals for recycling energy substrates.<sup>25</sup> Activation of the renin-angiotensin system (elevated plasma renin activity and aldosterone) in moderate HTN might not only exacerbate autophagy via

aggravation of hypertrophy, but also directly modulate its activity.<sup>26,27</sup> Interestingly, autophagy inhibitors were also upregulated in mild HTN. Given their contributions to mediating hypertrophy in various tissues, activated Akt/mammalian target of rapamycin/s6 may reflect elevated protein synthesis that initiates LVH.<sup>28–30</sup> However, production of toxic proteins requires activation of autophagy to limit their accumulation. As LVH continues to progress in moderate LVH even though the mammalian target of rapamycin pathway is lower than mild HTN, other mechanisms leading to progression of LVH in RVH warrant further investigation.

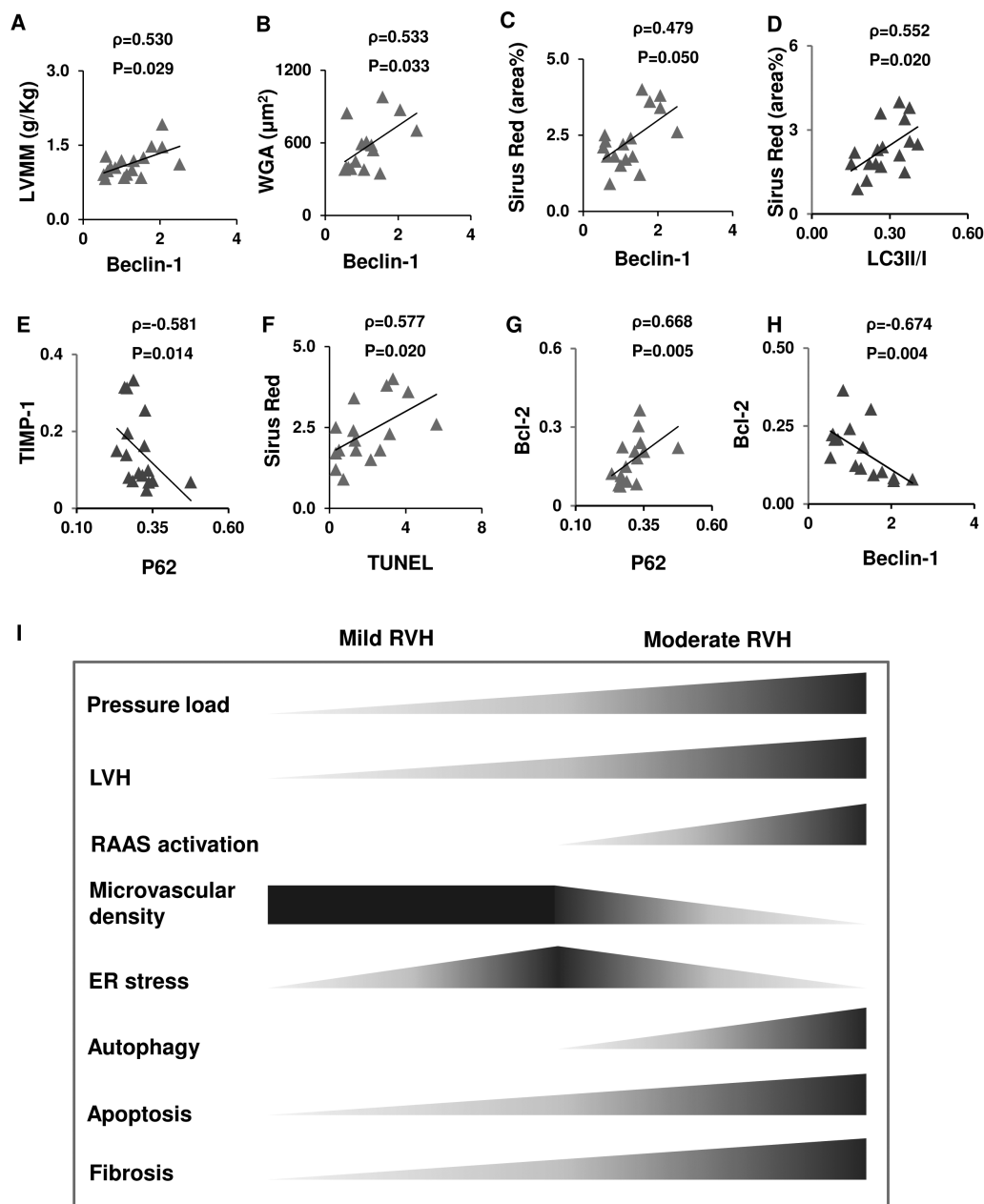
The levels of misfolded proteins and damaged organelles during cardiac hypertrophy are also sensed by the ER, which subsequently elicits the UPR to reduce the stress.<sup>31,32</sup> In the present study, ER stress appears to be involved transiently



**Figure 4.** Left ventricular apoptotic and pro-fibrotic activities. (A,C–E) Protein expression and quantification of the anti-apoptotic B-cell lymphoma (Bcl)-2, Bcl-xl, and pro-apoptotic Bax. (B,F) Tissue inhibitor of metalloproteinase (TIMP)-1 expression. \* $P < 0.05$  vs. normal.

in the relatively early stage of HTN, while in the later stage autophagy became predominant. This phenomenon supports the notion that ER stress mediates autophagy,<sup>33,34</sup> and suggests its potential role as the first-step reaction to the adaptive process in maintenance of intracellular homeostasis. Besides, as a survival mechanism, the UPR may extensively upregulate the autophagy machinery using the ER itself to provide the membrane needed for autophagosome formation.<sup>34,35</sup> Particularly, GRP78 has been shown to be obligatory for autophagy in mammalian cells,<sup>36</sup> possibly in an Atg protein-dependent

manner.<sup>34</sup> Indeed, the present study found no association between ER stress and LVH or fibrosis, whereas autophagic and apoptotic proteins were correlated to LV myocardial remodeling. Previous studies have shown that autophagic and apoptotic proteins may interact. Bcl-2 inhibits autophagy by binding to Beclin-1,<sup>37</sup> and P62 modulates the level of autophagy by interfering with the Bcl-2-Beclin-1 interaction.<sup>38</sup> Therefore, these results implicate a sequential activation of ER stress followed by autophagy and apoptosis, which may play a role in the progression of LVH. While ER stress represents



**Figure 5.** Proposed links between left ventricular remodeling and the associated pathological events. (A–F) Spearman rank correlation revealed associations of autophagy and apoptosis with LV myocardial remodeling. (G,H) Anti-apoptotic B-cell lymphoma (Bcl)-2 was correlated with autophagy proteins. (I) Schematic illustration of the proposed temporal pattern of pathophysiological events during the evolution of LVH. Abbreviations: RVH, renovascular hypertension; RAAS, renin-angiotensin-aldosterone system; ER, endoplasmic reticulum.

an early response to facilitate control of toxic or misfolded cellular components in LVH, autophagy might be stimulated later to dispose damaged whole cells. As angiotensin receptor blockers attenuate ER stress in cardiac injury,<sup>39</sup> the interaction of the renin-angiotensin-aldosterone system with ER stress and autophagy in RVH also warrants further investigation.

#### Limitations

This study includes relatively small group sizes without comorbidities, which might affect the severity and time-course

of the evolution of LVH and myocardial tissue injury in HTN. Nevertheless, cardiovascular physiology and pathophysiology in our model resemble those in humans and thus our study has translational power. Longitudinal studies with extended and successive observation and the use of specific inhibitors of ER stress and autophagy may assist in identification of the dynamic interaction and their impact in LVH.

#### CONCLUSIONS

As summarized schematically in Figure 5I, our findings suggest that autophagic activity may participate in the

evolution of LVH along with enhanced apoptosis, and is associated with progression of tissue remodeling including microvascular rarefaction and fibrosis. Contrarily, ER stress seems to be a transient early process during LVH. Autophagy activation may be implicated in hypertrophic cardiac injury under substantial pressure overload.

## SUPPLEMENTARY MATERIAL

Supplementary materials are available at *American Journal of Hypertension* (<http://ajh.oxfordjournals.org>).

## ACKNOWLEDGMENTS

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## DISCLOSURE

The authors declared no conflict of interest.

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