Autophagy mechanism of right ventricular remodeling in murine model of pulmonary artery constriction

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Qipshidze N, Tyagi N, Metreveli N, Lominadze D, Tyagi SC. Autophagy mechanism of right ventricular remodeling in murine model of pulmonary artery constriction. Am J Physiol Heart Circ Physiol 302: H688–H696, 2012. First published November 18, 2011; doi:10.1152/ajpheart.00777.2011.—Although right ventricular failure (RVF) is the hallmark of pulmonary arterial hypertension (PAH), the mechanism of RVF is unclear. Development of PAH-induced RVF is associated with an increased reactive oxygen species (ROS) production. Increases in oxidative stress lead to generation of nitro-tyrosine residues in tissue inhibitor of metalloproteinase (TIMPs) and liberate active matrix metalloproteinase (MMPs). To test the hypothesis that an imbalance in MMP-to-TIMP ratio leads to interstitial fibrosis and RVF and whether the treatment with folic acid (FA) alleviates ROS generation, maintains MMP/TIMP balance, and regresses interstitial fibrosis, we used a mouse model of pulmonary artery constriction (PAC). After surgery mice were given FA in their drinking water (0.03 g/l) for 4 wk. Production of ROS in the right ventricle (RV) was measured using oxidative fluorescent dye. The level of MMP-2, -9, and -13 and TIMP-4, autophagy marker (p62), mitophagy marker (LC3A/B), collagen interstitial fibrosis, and ROS in the RV wall was measured. RV function was measured by Millar catheter. Treatment with FA decreased the pressure to 35 mmHg from 50 mmHg in PAC mice. Similarly, RV volume in PAC mice was increased compared with the Sham group. A robust increase of ROS was observed in RV of PAC mice, which was decreased by treatment with FA. The protein level of MMP-2, -9, and -13 was increased in RV of PAC mice in comparison with that in the sham-operated mice, whereas supplementation with FA abolished this effect and mitigated MMPs levels. The protein level of TIMP-4 was decreased in RV of PAC mice compared with the Sham group. Treatment with FA helped PAC mice to improve the level of TIMP-4. To further support the claim of mitophagy occurrence during RVF, the levels of LC3A/B and p62 were measured by Western blot and immunohistochemistry. LC3A/B was increased in RV of PAC mice. Similarly, increased p62 protein level was observed in RV of PAC mice. Treatment with FA abolished this effect in PAC mice. These results suggest that FA treatment improves MMP/TIMP balance and ameliorates mitochondrial dysfunction that results in protection of RV failure during pulmonary hypertension.

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RIGHT VENTRICULAR (RV) failure (RVF) is the hallmark of pulmonary arterial hypertension. Pulmonary arterial hypertension patients admitted to intensive care units with RVF have a 41% acute mortality rate (46). RV function is an important determinant of long-term outcome in patients with complex congenital heart disease, chronic pulmonary obstructive diseases, or pulmonary hypertension (19, 56). In many of these patients the RV is subjected to (residual) abnormal loading conditions, including pressure overload. Some patients with pulmonary hypertension are adaptive remodelers and develop RV hypertrophy (RVH) but retain RV function; others are maladaptive remodelers and rapidly develop RVF (36). The mechanisms underlying the RVF have not been well defined. To study the mechanisms underlying the RVF in patients is very difficult, because invasive data cannot be easily obtained. For this purpose animal models are very beneficial. Small experimental animals, such as mice, are widely used in cardiovascular research since they can provide a variety of disease models including heart hypertrophy and failure. A major advantage of the use of mice disease models is that cardiac material can be easily sampled to study critically involved molecular changes over time and the possibility to study effects of transgenesis and gene ablation (2, 4, 23).

Reactive oxygen species (ROS) such as superoxide (O2−) have been shown to be harmful to proteins, lipids, and DNA. An increase in cellular ROS levels leads to oxidative stress (47), and this has been implicated in the etiology of a wide variety of diseases, including cystic fibrosis, cancer, type 2 diabetes, Alzheimer’s disease, Parkinson’s disease, arteriosclerosis, and myocardial ischemia/reperfusion injury (14). Recently, increased ROS production was also suggested to play a role in the progression of both left ventricle and RV hypertrophy to congestive heart failure (9, 22, 45). This is supported by several animal studies showing prevention of heart failure by antioxidant treatment (6, 8, 55). ROS derived from mitochondria can promote cytotoxicity and cell death (7, 11). As a major source of ROS production, mitochondria are especially prone to ROS damage. Dysregulation of nitric oxide and increased antioxidative and nitrosative stress are implicated in the pathogenesis of heart failure. Peroxynitrite is a reactive oxidant that is produced from the reaction of nitric oxide with superoxide anion and impairs cardiovascular function through multiple mechanisms, including activation of matrix metalloproteinases (MMPs) (33). Increased oxidative stress leads to the generation of nitro-tyrosine residues in tissue inhibitor of metalloproteinases (TIMPs) and liberates active MMPs (40). Folic acid supplementation offers several clinical benefits, affecting ROS scavenging enzymes and ameliorating oxidative stress (57). Studies of RV remodeling and failure so far point to a role of diminished cellular antioxidant activity, but ROS-producing activities have not yet been determined (9, 45). In the present study, we created a novel model of RVF following the pulmonary arterial constriction (PAC) and tested the hypothesis that imbalance in MMP-to-TIMP ratio leads to interstitial fibrosis and RVF and that the treatment with folic acid alleviates ROS generation, maintains MMP/TIMP balance, and regresses interstitial fibrosis. The obtained results suggest that folic acid treatment improves MMP/TIMP balance and ameliorates mi-
METHODS

Animals. The animals were fed standard chow and water ad libitum. All animal procedures were reviewed and approved by an independent the Institutional Animal Care and Use Committee of the University of Louisville School of Medicine, according to the animal care and use guidelines of the National Institutes of Health.

Model of PAC. All mice were male C57BL/6j, 8 to 10 wk of age. Mice were anesthetized with pentobarbital sodium (65 mg/kg ip). Mice were then intubated transtracheally with an 18-gauge angiocath and ventilated using a Harvard rodent ventilator (Harvard Apparatus, Holliston, MA) at a rate of 120–140 breaths/min with a tidal volume of 10 μl/g body wt. A right lateral thoracotomy was then performed. The main pulmonary trunk was identified under the left atrial appendage and banded with a 7-0 suture, tied tight against a 27-gauge needle, which was then removed. The chest was then closed with 7-0 sutures around adjacent ribs, and the skin was closed with a 5-0 suture. Air was evacuated to avoid the need for a chest tube postoperatively. After surgery mice were given folic acid in their drinking water (0.03 g/l) for 4 wk.

Histology. Hearts were collected from experimental animals and frozen for further analyses. Frozen heart were sectioned at 5 μm thickness with a cryostat (Leica Cryocut 1800; Leica Microsystems) and mounted on slides. Each slide was stained using a Masson’s trichrome kit (Richard-Allan Scientific, Kalamazoo, MI) according to the manufacturer’s recommendations. The heart muscle and vascular smooth muscle were stained pink, and the collagen was stained blue. The level of subcellular matrix collagen was assessed by measuring the integrated optical density (IOD) of blue color.

Isolation of mouse cardiac myocytes. Mouse cardiomyocytes were isolated according to the method published earlier (31). A mouse was first injected with heparin (1,000 units/kg body wt ip) and then anesthetized with tribromoethanol (240 mg/kg of body wt ip). The chest was wiped with 70% ethanol. A skin incision was made revealing the xiphoid process. The rib cage was completely cut along starting at the xiphoid process running up the chest cavity. To avoid heart damage the diaphragm was also cut. The heart was secured with forceps and all vessels were cut. The aorta was cut so as to leave the maximal length for rapid cannulation. The dissected heart was immediately placed in a Petri dish containing ice-cold calcium free perfusion buffer (pH 7.4). To expose the aorta, all the remnant excess tissue was removed and discarded. With the aorta being held with two fine forceps, it was slid onto the vertically mounted cannula until the tip of the needle reaches the aortic valve. The heart was secured on the needle with a brass clip and was immediately perfused with perfusion buffer at a flow rate of 4.0 ml/min. The time from the heart dissection until the start of perfusion did not exceed 1 min.

The heart was perfused with perfusion buffer for 2 min or until the outflow from apex was cleared from blood. The perfusion with digestion buffer consisting of 29 ml perfusion buffer and 1.0 ml 10 mg/ml Liberase Blendzyme 4 (Roche Diagnostics, Indianapolis, IN) was then continued for 7–12 min. At the end of the perfusion the tissue become soft, swollen, and light pink. After the perfusion the tissue was cut from the needle just below the atria using sterile fine scissors and was placed in a Petri dish with 10 ml of incubation buffer containing (in mM) 135 NaCl, 4 KCl, 1 MgCl2, 10 HEPES, 0.33 NaH2PO4, 10 glucose, and 10 butanedione monoxime (pH 7.4) at room temperature. The heart was cut in half, and the tissue was gently teased into small pieces with fine forceps. The obtained suspension of cardiomyocytes was gently pipetted up and down with a plastic pipette (2-mm tip) several times. The cells were then transferred to a 15-ml sterile polypropylene conical tube, and 10, 20, 30, 30, and 30 μl of a 100 mM CaCl2 solution was added at 5-min intervals to reach the final content of calcium of 1.2 mM. Isolated myocytes were maintained at room temperature in this incubation buffer.

Myocyte contractility analysis. Cardiomyocyte contractility was controlled by electrical stimulation. Contractile properties of isolated RV myocytes were assessed by a video-based edge detection method as described (31). Steady-state twitches (8–10) were analyzed for cell length changes using the Soft-Edge software and averaged for each myocyte. Cell shortening and relengthening were assessed as a percentage of peak shortening, time to 90% of peak shortening, time to 90% relengthening, and maximal velocities of shortening and relengthening (dL/dt).

Western blot analysis. Changes in protein content of MMP-2, -9, and 13 and TIMP-4, p62 (marker of autophagy), and LC3A/B (marker of mitophagy) in PAC mice were assessed by Western blot analysis (50). Briefly, frozen heart tissue was washed two times with ice-cold PBS and RV was separated and lysed with ice-cold radioimmunoprecipitation assay buffer (containing 5 mM of ethylenediaminetetraacetic acid), which was supplemented with phenylmethylsulfonyl fluoride (1 mM) and protease inhibitor cocktail (1 ml/ml of lysis buffer). Protein content of the lysate was determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Equal amounts of protein (30 mg) were resolved on 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane as described (24, 43). The blots were incubated with monoclonal anti-mouse respective antibodies for 1 h at room temperature. After incubation, the proteins on blots were detected as described (43). Membranes were stripped and reprobed for β-actin as a loading control. The blots were analyzed with Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD) as described earlier (24). The protein expression intensity was assessed by IOD of the band in the lane profile. To account for possible differences in the protein load, measurements were presented as a ratio of the IOD of each band under study (protein of interest) to the IOD of the respective β-actin band.

ROS generation. Myocardial superoxide was measured by dihydroethidium fluorescent microtopography. Fresh-frozen 5-μm RV slices were incubated for 1 h at 37°C with dihydroethidium (2 μmol/l; Invitrogen, Carlsbad, CA) and fluorescence imaged as described (21, 30). Fluorescence was visualized using a laser scanning confocal microscope (×60 objective; Olympus FluoView1000) set at the appropriate filter settings. Acquisition settings of the camera were identical for all images. Total fluorescence (red) intensity in five random fields (for each experiment) was measured with image analysis software (Image-Pro Plus; Media Cybernetics). Fluorescence intensity unit values from RV for each experimental group were averaged and presented as a measure for O2•− production.

Cryosectioning. After mice were euthanized, hearts were harvested, thoroughly washed in PBS, preserved in Peel-A-Way disposable plastic tissue embedding molds (Polysciences, Warrington, PA) filled with tissue freezing medium (Triangle Biomedical Sciences, Durham, NC), and stored at 70°C until analysis. Tissue sections 5 μm in thickness were made with a Leica CM 1850 Cryocut (Bannockburn, IL). Sections were placed on Superfrost Plus glass slides, air-dried, and processed for histological and immunohistochemistry (IHC) staining.

IHC. IHC was performed on 5-μm-thick frozen tissue sections according to a standard IHC protocol (Abcam). Primary antibodies applied overnight included anti-p62 and anti-LC3A/B (Abcam) antibodies. A secondary antibody labeled with Alexa Fluor 488 (Invitrogen) was applied for immunodetection of these proteins. Stained slides were analyzed for fluorescence intensity in samples with a laser scanning confocal microscope Olympus FluoView1000 with (60× objective) set at the appropriate filter settings. Total fluorescence (green or red) intensity in five random fields (for each experiment) was measured with Image-Pro Plus. Fluorescence intensity values from RV for each experimental group were averaged and presented as measure for autophagy or mitophagy, respectively.
**Pressure-volume loop measurements.** Using a standard Millar (Millar Instrument, Houston, TX) protocol, after a two-point calibration was performed, steady-state pressure-volume loops were recorded followed by saline bolus and cuvette calibration for conversion of relative volume units to microliters. RV blood pressure and hemodynamic parameters were measured by a Millar catheter and analyzed by PVAN software (32).

**Statistical analysis.** Values are reported as mean ± SE. Differences between groups were tested by two-way ANOVA. If ANOVA indicated a significant difference ($P < 0.05$), Tukey’s multiple comparison test was used to compare group means and were considered significant if $P < 0.05$.

**RESULTS**

**Level of fibrosis.** Histological analysis of collagen was performed in the slices of RV. The intensity of trichrome blue stain demonstrated development of significant collagen accumulation in the RV samples from PAC mice hearts compared with those from the Sham-operated mice (Fig. 1). Treatment with folic acid mitigated the formation of fibrosis in the RVs from PAC + folic acid group (Fig. 1). RV wall thickness in PAC mice was thinner compared with that in PAC mice treated with folic acid (Fig. 1). RV was dilated in PAC mice compared with that in sham-operated mice (Fig. 1). Treatment with folic acid decreased RV dilatation in PAC mice (Fig. 1).

**RV function.** RV pressure and volume were increased in mice with PAC compared with the Sham group (Table 1). Supplementation with folic acid decreased RV pressure and volume in PAC + folic acid group compared with PAC group, although they were still lower than in the Sham group (Table 1). Treatment with folic acid for 4 wk did not change RV pressure in Sham + folic acid compared with the Sham group (Table 1).

**Myocyte function.** PAC significantly increased length and decreased thickness of myocytes (Fig. 2A and Table 1). Treat-

**Table 1. Comparison of RV pressure and RV volume in Sham, Sham + FA, PAC, and PAC + FA mice treated with or without FA**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Sham + FA</th>
<th>PAC</th>
<th>PAC + FA</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV pressure, mmHg</td>
<td>18 ± 2</td>
<td>17 ± 1.5</td>
<td>50 ± 3*</td>
<td>35 ± 1.8*#</td>
</tr>
<tr>
<td>RV volume, µl</td>
<td>17 ± 2.1</td>
<td>16 ± 1.1</td>
<td>30 ± 2.2*</td>
<td>22 ± 1.3*#</td>
</tr>
<tr>
<td>Length, µm</td>
<td>260 ± 28</td>
<td>280 ± 15</td>
<td>496 ± 20*</td>
<td>450 ± 30*</td>
</tr>
<tr>
<td>Thickness, µm</td>
<td>53 ± 6</td>
<td>60 ± 8</td>
<td>30 ± 4*</td>
<td>110 ± 3*#</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 4$ for all groups. Sham, sham-operated mice; RV, right ventricular; FA, folic acid; PAC, pulmonary artery constriction. *$P < 0.05$ vs. Sham group; #P < 0.05 vs. PAC.
ment with folic acid improved myocyte properties in PAC mice (Fig. 2A and Table 1). PAC significantly impaired contractility of isolated cardiomyocytes (Fig. 2, B and C). Folic acid improved myocyte contractility in PAC mice (Fig. 2, B and C).

**RVF-induced increased ROS production.** Production of ROS in the RV was measured using oxidative fluorescent dye, DHE. The extent of DHE fluorescence indicated ROS production (Fig. 3). A robust increase of DHE fluorescence was observed...
in PAC mice (Fig. 3). Treatment with folic acid ameliorated DHE fluorescence intensity in PAC + folic acid mice, it was lower compared with that in PAC mice but it was still higher than in age-matched sham-operated mice (Fig. 3).

Role of MMPs during RVF. Representative Immunoblots for MMPs are shown in Fig. 4A. The protein levels of MMP-2, -9, and -13 were robustly increased in RVs of PAC mice compared with those in RV of the sham-operated mice (Fig. 4A). Supplementation with folic acid reversed this effect in RVs of PAC mice (Fig. 4A).

Role of TIMP-1 and -4 during RVF. The protein level of TIMP-4 was decreased in RV of 4-wk PAC mice compared with that in sham-operated mice (Fig. 4B). Supplementation with folic acid reversed PAC-induced decrease in TIMP-4 level in RV of mice with PAC (Fig. 4B). At the same time protein level of TIMP-1 was increased in PAC mice (Fig. 4B), treatment with FA reduced expression of TIMP-1 in PAC mice (Fig. 4B).

Expression of LC3A/B and p62. To support the claim that mitophagy occurs during RVF, the levels of mitophagy (LC3A/B) and autophagy (p62) markers were assessed by Western blot analyses (Figs. 5 and 6, respectively). Protein level of p62 was increased in RV of PAC mice (Fig. 5). Similarly, increased LC3A/B protein level was observed in RV of PAC mice (Fig. 6). Expression of LC3A/B and p62 was also defined by immunohistochemical staining of cryosectioned RV samples with anti-LC3A/B (Fig. 5) and anti-p62 (Fig. 6) antibodies. The results replicated the data obtained by a Western blot analyses (Fig. 7).

DISCUSSION

The principal findings of this study were that folic acid mitigates mitophagy due to amelioration of oxidative stress and leads to improve RVF during PAC. Mitochondria are the sites of oxidative phosphorylation, respiration, and ROS formation (25, 54). As a major source of ROS production, mitochondria are especially prone to oxidative damage (7, 11). Development of PAC-induced RVF is associated with an increased capacity of ROS production (39). Our data suggest that treatment with folic acid decreases ROS production and improves RV function via decreasing mitophagy in RV.

Increases in oxidative stress lead to the generation of nitrotyrosine residues in TIMP and liberate active MMP (15, 51). MMPs are membrane-bound, zinc-dependent endoproteinases that can be divided into two structurally distinct groups: the secreted MMPs and the membrane-type MMPs (28). We showed that most of the MMPs in the myocardium are inactive (53). Our present data suggest that during chronic heart failure, increase in load and oxidative stress lead to MMP activation, causing a generation of nitro-tyrosine residues in TIMP and the release of active MMP (49). TIMPs are a family of proteins that regulate activity of MMPs. Four types of TIMPs have been
identified: TIMP-1, -2, -3, and -4 (5). Balance of the MMP/TIMP axis is very important to maintain normal physiological matrix constriction (42), imbalance of which results in cardiac fibrosis (52). It is known that TIMP-1 induces formation of fibrosis (48, 52); TIMP-2 causes cell proliferation (26). TIMP-3 results in apoptosis (1). TIMP-4 is more expressed on cardiac tissue than other TIMPs and induces apoptosis in transformed cells without affecting normal cells (48). We found a decrease in TIMP-4 in RV of PAC mice. Interestingly, the level of TIMP-4 was restored in PAC animals given folic acid supplementation, suggesting an effect of folic acid on expression TIMPs that confirms our earlier findings (38). The differential role of MMP-2 versus MMP-9 has been suggested (10, 13). Targeted detection of MMP-2 amplifies the cardiac failure (17, 27). The present data suggest that this effect on RV remodeling, it was not surprising to find increased MMP-2,-9, and -13 expressions in RV of mice after 4 wk of PAC compared with those found in sham-operated mice and PAC + folic acid mice. Thus, an increase in MMP-9 may result in increased RV size. The data indicate that the level of MMPs was returned to its normal level in PAC mice given folic acid supplementation.

P62 forms complexes with a number of proteins and acts as a central player in regulating NF-κB activation (41). Another function of p62 is to facilitate the packing and delivery of misfolded or aggregated proteins for disposal through the autophagolysosomal degradation pathway (3). Indeed, a well-known binding partner of p62 is LC3 (18, 35, 44). LC3 is enriched on intracellular membranes during autophagy (16), which can be induced by proteasome inhibition (34). In the present study we have shown that LC3A/B and p62 are upregulated in PAC mice. Treatment with FA lowers their expression in RV.

We have shown that folic acid decreases collagen accumulation in hypertensive hearts (38). Others have indicated that folic acid supplementation lowers collagen and scar formation (12). In this study we showed that supplementation with folic acid lowered RV dilatation size and increased RV wall thickness, in addition to the lowered collagen formation in PAC mice. It is known that folic acid affects endothelial function and rapidly modulates arterial stiffness (37); this may explain the effect of folic acid causing an improvement in RV systolic blood pressure. Positive net effect of folic acid in improving the RV volume in our study can be explained by its effect of preventing changes in arteriolar stiffness (20, 37) leading to an improved arterial compliance and diminishing collagen formation in the heart (12, 24, 43). Interestingly, the positive net effect of folic acid in improving the RV volume in our study can also be explained by its effect of preventing changes in arteriolar stiffness. Hence, the improvement in the RV remodeling could simply be because they are subjected to lower pressure and in addition to the beneficial effects of folic acid in high energy phosphates in cardiomyocytes (29). As also reported in Table 1, the RV pressure is significantly lower in the folic acid-treated group. This can only be clarified by in vitro studies.
where the pressure (and the influence of arterial compliance) will not mask the direct effects of folic acid on cardiomyocyte hypertrophy and remodeling.

In addition, the left ventricle is also grossly enlarged (hypertrophied) in the PAC group. This may be secondary to RV dilation. This may suggest that the impact of PAC is not restricted to the RV for the duration of this study.

In conclusion folic acid treatment improves MMP/TIMP balance and ameliorates mitochondrial dysfunction that results in protection of RVF during PAC (Fig. 7).

![Image of Fig. 6: Effect of FA on LC3A/B in mice. A: RV immunohistochemical staining and colocalization of LC3A/B. Cryocut frozen sections of (8-10 μm) were stained and secondarily conjugated with FITC. B: bar diagrams depicted the intensity quantification of LC3A/B in Sham, Sham + FA, PAC, and PAC + FA mice. C: Western blot analysis for LC3A/B protein levels in RV of the above mentioned mice groups. β-Actin was used as a loading control. D: relative protein expression is reported as a ratio of IOD of each band to the IOD of the respective β-actin band. *P < 0.05 vs. Sham, Sham + FA; #P < 0.05 vs. PAC; n = 5 for all groups.]

![Image of Fig. 7: Schematic presentation of possible mechanism involved in FA induced cardiac function during PAC. A: PAC caused reactive oxygen species (ROS) production in RV, leading to mitophagy and MMP/TIMP imbalance. That caused RV failure (RVF). B: treatment with FA, which decreased ROS production, ameliorates mitophagy and MMP/TIMP imbalance and protects the heart from RVF.]


