Abstract

Background: Evidences suggest that β3-adrenoceptor (β3-AR) plays an important role in heart failure (HF), although no data is reported indicating how these effects may change with the increasing age. Pulmonary congestion and edema are the major life-threatening complications associated with HF. The purpose of this study is to explore the relationship between the anti-β3-AR autoantibody and the expression of β3-AR in the lungs and heart for both aged patients and rats with HF.

Methods: Synthetic β3-AR peptides served as the target antigens in ELISA were used to screen the anti-β3-AR autoantibody in aged patients and rats. Two aged rat models were constructed based on aortic banding and sham-operation. The expression of β3-AR mRNA and protein in the lung and heart was measured in intervention and non-intervention groups by Western blot analysis at the baseline, 5th, 7th, 9th and 11th week, respectively.

Results: The frequency and titer of anti-β3-AR autoantibody in aged patients and rats with HF were higher than those in the control group (p<0.05). The expression of β3-AR mRNA and protein in pulmonary tissues decreased continually from the 7th week (p<0.05), followed by HF observed during the 9th week. The expression of β3-AR in myocardial tissues continued to increase after the 9th week (p<0.05), and the expression of both β3-AR mRNA and protein in the BRL group (HF group with BRL37344 (4-[[2-hydroxy-(3-chlorophenyl)ethyl-amino] phenoxyacetic acid) (a β3-AR agonist) injection] was positively correlated with BRL37344 when compared with non-BRL group (HF group without BRL37344 injection) (p<0.05).

Conclusion: Anti-β3-AR autoantibody was detected in aged patients and rats with HF. The expression of β3-AR mRNA and protein in pulmonary tissues decreased continually, and began earlier than in the heart, but its expression in myocardial tissues increased continually and could be further promoted by β3-AR agonist.

Introduction

Growing evidences suggest that the autoimmune mechanism may play a major role in heart failure observed in both rats and human patients [1,2,3,4]. Previous studies have shown that autoantibodies against β3-adrenoceptors (anti-β-AR autoantibodies) display antagonist activity on the corresponding receptor and influence cardiac function [3,5,6]. It has been well established that β3-adrenoceptor (β3-AR) belongs to the family of G-protein coupled receptors, and 3 subtypes (β1, β2 and β3-AR) have been associated with different changes in heart failure. Stimulation to β1 and β2-AR produces positive chronic and inotropic effects. However, the activation of β3-AR induces a decrease in the contractility of myocardium [7,8,9,10]. Gauthier and associates recently have also reported that β3-AR is obviously coupled to a GTP/nitric oxide (NO) pathway and can reveal negative inotropic effects in ventricular endomyocardial biopsy samples of heart transplant recipients [11]. However, some disagreement exists regarding the conclusions reported by these studies [8,12], thus indicating that the relationship between β3-AR and cardiac function warrants further investigation, in aged patients or rats with heart failure.

Pulmonary congestion and edema are the major life-threatening complications associated with heart failure. The β-AR is widely distributed in lungs, where the dominant subtype is β2-AR [13]. Recently, the research has found that the administration of an inhaled β2-AR agonist can improve pulmonary function in patients with congestive heart failure, thus suggesting the involvement of β2-AR [14]. Other studies have found that β2-agonist can increase alveolar fluid clearance in rats with heart failure [15]; however, the other 2 subtypes (β1-AR and β3-AR) in pulmonary tissues have gained less attention. Recent research has
demonstrated very low expression of β3-AR mRNA in the lungs, and the authors have suggested that the expression of β3-AR mRNA could probably be accounted for by the presence of fat [16]. Additionally, new research has confirmed the presence of β3-ARs in human bronchi [13], and has further indicated that β3-adrenergic agonists may also increase alveolar fluid clearance during hypoxic lung injury in rats, and accelerate the amelioration of pulmonary edema [17].

The major objectives of this study are summarized by three hypotheses: 1) The anti-β3-AR autoantibodies can be detected in the plasma of aged patients and rats with heart failure. 2) The abnormal expressions of β3-AR mRNA and protein, and changes in the levels of anti-β3-AR autoantibodies in pulmonary congestion or edema emerge prior to the appearance of heart failure in aged rats. 3) These latter changes may involve continuing deterioration during heart failure, and may further be altered by the introduction of the β3-AR agonist.

**Results**

**Mortality Rate of Aged Rats**

Mortality rate with Banded group was significantly higher than that in the Sham group (p<0.01, 50.0% vs. 8.6%) during the postoperative period (0–11 weeks). The major causes of early death were over anesthesia or heart failure, especially in the Banded group. From the 5th week, the rats from the Banded group (n = 90) were divided randomly into two groups as the BRL group [heart failure group with a β3-AR agonist called BRL37344 (4-[2-hydroxy-(3-chlorophenyl)ethyl-amino] phenoxyacetic acid) injection] (n = 50) and the non-BRL group [heart failure group without BRL37344 injection] (n = 40). Mortality rates were different at various stages in the non-Banded and BRL groups from the 5th to 11th week. The accumulative mortality rate in the BRL group was significantly higher than that in the non-BRL group (p<0.05; 32% vs. 12%), and these results suggested that β3-AR agonist (BRL37344) might be associated with an increased risk of death in aged rats with heart failure.

**Hemodynamic Parameters and Left Ventricle/Body Weight Ratio**

The hemodynamic parameters are summarized in Table 1. In both the BRL group and the non-BRL group, the HR, LVESP and dP/dtmax decreased, and LVEDP and dP/dtmin increased significantly, when compared with the Sham group (p<0.05). However, at the 9th week, left ventricular function was significantly impaired in the BRL group compared to the non-BRL group (p<0.01). Thus, left ventricular function was progressively damaged.

The left ventricle weight and left ventricle weight/body weight ratio in both BRL group and non-BRL group were significantly higher than those in the Sham group, but overall body weight was lower. However, the average ratio in the BRL group was significantly higher than in the non-BRL group at the 7th week (Table 2). Body weight did not reveal a significant difference between non-BRL and BRL groups. Our results indicate that left ventricular function was significantly reduced in the BRL group.

**Anti-β3-AR Autoantibody in Aged Patients and Rats**

The frequencies of anti-β3-AR autoantibody in aged patients with heart failure were 39.6% (mean antibody titer: 142.3±3.1), which was obviously higher than 12.7% (mean antibody titer: 64.3±2.1) in aged control group.

Similar results were observed in aged rats with heart failure. The frequencies and mean titer of anti-β3-AR autoantibody in the Banded group increased significantly from the 5th week, with a high peak (from 20.4% and 28.3±1.4 to peak 41.4% and 82.3±3.2) at the 10th week, followed by a decrease after the 10th week, although these results were not observed in the Sham group (p<0.05). It was worth noting that the frequencies and mean titers of anti-β3-AR autoantibodies in the BRL group increased markedly at the 5th week, and reached a maximum at the 10th week (from 37.5% and 68.4±2.3 to peak 66.5% and 142.6±1.1) when compared with the Sham group (p<0.05) (Figure 1).

**Lung Histopathology in Aged Rats With Heart Failure**

To our knowledge, this is the first reported observation of the changes occurring in the lungs (the 7th week after operation) prior to the appearance of heart failure in aged rats (the 8th week after operation). During the 2–7th week after operation, no significant difference was observed in the alveolar space, lung interstice or peri-alveolar blood vessels in the BRL group, non-BRL group or Sham group. However, from the 5th to the 7th week after operation, the peri-alveolar blood vessels in the BRL group reveal slight dilation and blood stasis. Subsequently, during the 7–11th weeks after operation, lung lesions in the non-BRL group were gradually characterized by interstitial edema, acinar cell vacuolization, and infiltration of inflammatory and congestion edema (Figure 2).

**Cardiac Histopathology in Aged Rats with Heart Failure**

No nuclear degeneration was observed in the intercellular spaces in any of the 3 groups during the 2–7th weeks after operation, however, during the 9–11th week after operation, cardiac lesions in the BRL and non-BRL groups were gradually characterized by interstitial fibrosis, edema, degeneration, necrosis, and myocyte hypertrophy. It is notable that the cardiac lesions in the BRL group were more pronounced than those observed in the non-BRL group (Figure 3).

**The Expression of β3-AR mRNA and Protein in Lung and Heart**

In the present study, we first demonstrated the polarity of the expression of β3-AR mRNA and protein in the lung and cardiac tissues from rats with heart failure. The expression levels of β3-AR mRNA and protein in lungs from the non-BRL group exhibited a negative correlation with the severity of heart failure (p<0.001) (Figure 4).

Conversely, the expression levels of cardiac β3-AR mRNA and protein in both BRL and non-BRL groups exhibited a positive correlation with the severity of heart failure (p<0.001). More importantly, compared with the non-BRL group, the BRL group revealed a more obviously positive correlation with the severity of heart failure (p<0.01). These results suggested that β3-AR agonist might further activate the expression of β3-AR mRNA and protein in heart of the aged rats with heart failure (Figure 5).

**Discussion**

Three subtypes of β-receptors are expressed in normal hearts at various levels. The expression levels of β1-AR and β2-AR subtypes are approximately 70–80% and 20–30%, respectively, but β3-AR subtype can not be detected under normal conditions [18]. The β3-AR subtype is first characterized in human endomyocardial biopsies from transplanted hearts [7]. Moreover, different subtypes of β-receptors have different impacts on myocardial contractility. The myocardial contractility is increased by activated β1-AR and β3-AR subtypes but decreased by activated β2-AR subtype [19]. In senescent heart, both β1-AR and β3-AR subtypes are down-
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regulated [20,21], but the up-regulation of b3-AR is reported by Birenbaum et al. [21], and it is never reported previously. In the present study, we have first demonstrated that the ascending aorta banding-induced heart failure in aged rats is an accurate and reliable model, but with a mortality up to 50%. In addition, our results suggest that the b3-AR agonist may be associated with the increased risk of death in aged rats with heart failure. Third, the frequencies and mean titer of anti-b3-AR autoantibody in aged patients and rats with heart failure are significantly higher than those within the control groups, and reveal an increase with

Table 1. Hemodynamic effects following injection.

| Group   | Time (Weeks) | N  | HR (bpm) | LVESP (mm Hg) | LVEDP (mm Hg) | dp/dt max (mmHg/ms) | dp/dt min (mmHg/ms) |
|---------|--------------|----|----------|----------------|----------------|---------------------|---------------------|
| Sham    | 5            | 8  | 353±27   | 154.9±12.5     | 0.96±0.60      | 9.40±0.97           | -6.28±0.52          |
|         | 7            | 8  | 379±15   | 154.1±3.0      | 1.01±0.53      | 9.38±0.50           | -6.08±0.35          |
|         | 9            | 8  | 395±15   | 152.6±4.0      | 0.90±0.40      | 9.08±0.52           | -5.80±0.62          |
|         | 11           | 8  | 394±16   | 153.8±5.2      | 0.89±0.49      | 9.38±0.26           | -6.08±0.54          |
| Non-BRL | 5            | 8  | 364±29   | 178.3±6.1*     | 7.25±0.71*     | 8.88±1.87           | -6.29±0.76          |
|         | 7            | 8  | 381±32   | 173.2±9.3*     | 7.47±0.76*     | 8.74±1.56           | -5.72±0.95          |
|         | 9            | 8  | 269±20*  | 88.3±7.5*      | 10.31±1.62*    | 5.85±0.76*          | -2.68±0.55*         |
|         | 11           | 8  | 254±14*  | 61.5±2.4*      | 10.74±0.48*    | 1.40±0.21*          | -1.18±0.41*         |
| BRL     | 5            | 8  | 356±11   | 178.3±1.8*     | 7.30±0.98*     | 9.94±1.66           | -6.50±0.78          |
|         | 7            | 8  | 370±13   | 170.9±7.6*     | 7.69±0.83*     | 8.35±0.51           | -5.81±0.17          |
|         | 9            | 8  | 222±23*  | 73.2±7.8*      | 11.13±0.61*    | 2.36±1.19*          | -1.84±0.75*         |
|         | 11           | 8  | 216±20*  | 42.5±3.8*      | 12.17±0.50*    | 1.75±0.52*          | -1.08±0.33*         |

Data are presented as means ± standard error. Sham: control group; Non-BRL: HF group without BRL37344 injection; BRL: HF group with BRL37344 injection; HR: heart rate; LVESP: left ventricular end-systolic pressure; LVEDP: left ventricular end-diastolic pressure; dp/dt max: maximum rate of rise of left ventricular pressure; dp/dt min: maximum rate of fall of left ventricular pressure.

*P<0.01 vs. Sham group; †P<0.05 vs. Non-BRL group; ‡P<0.01 vs. Non-BRL group.
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Table 2. The ratio of left ventricle weight to body weight following injection.

| Group   | Time (Weeks) | N  | BW (g) | LVW (g) | LV/BW (mg/g) |
|---------|--------------|----|--------|---------|--------------|
| Sham    | 5            | 8  | 529.13 | 1.00±0.04 | 1.89±0.06 |
|         | 7            | 8  | 585±29 | 1.11±0.02 | 1.89±0.03 |
|         | 9            | 8  | 601.19 | 1.09±0.07 | 1.81±0.10 |
|         | 11           | 8  | 622±23 | 1.15±0.07 | 1.85±0.06 |
| Non-BRL | 5            | 8  | 485±29 | 1.07±0.08 | 2.22±0.18 |
|         | 7            | 8  | 574.36 | 1.28±0.09 | 2.24±0.17 |
|         | 9            | 8  | 584±27 | 1.34±0.06 | 2.30±0.11 |
|         | 11           | 8  | 581±16 | 1.35±0.05 | 2.32±0.09 |
| BRL     | 5            | 8  | 484±33 | 1.06±0.13 | 2.19±0.20 |
|         | 7            | 8  | 595±35 | 1.45±0.21 | 2.42±0.22 |
|         | 9            | 8  | 591±57 | 1.48±0.19 | 2.50±0.21 |
|         | 11           | 8  | 596±26 | 1.51±0.17 | 2.52±0.23 |

Data are presented as means ± standard errors. Sham: control group; Non-BRL: HF group without BRL37344 injection; BRL: HF group with BRL37344 injection; BW: body weight; LVW: left ventricular weight; LV/BW: left ventricular weight/ body weight.

*P<0.05 vs. Sham group; †P<0.01 vs. Sham group; ‡P<0.05 vs. Non-BRL group; ‡P<0.01 vs. Non-BRL group.
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Figure 1. The frequencies of anti-b3-autoantibodies in non-BRL and BRL groups of aged rats with heart failure. 2 mL of blood samples were collected before treatment, and at the 5th, 6th, 7th, 8th, 9th, 10th and 11th week after treatment. The sera were separated by centrifugation (3000 rpm, Beckman CS-15R Centrifuge) for 10 min and stored at –80°C until needed for assay. Anti-b3-AR autoantibody was determined by enzyme-linked immunosorbent assays (ELISA). The frequencies of anti-b3-autoantibodies in the BRL group increased markedly at the 5th week, and reached a maximum at the 10th week in both BRL group and non-BRL group. The frequencies of anti-b3-autoantibodies were significantly higher in BRL group than non-BRL group and Sham group (P<0.05). BRL group: HF group with b3-AR agonist of BRL37344 injection; non-BRL group: HF group without BRL37344 injection.
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Figure 2. Lung histopathology at different times and in different groups. The lung tissues were observed with a Brightfield microscope under 40× magnification (Olympus BX 51, Japan). During the 2–5th week after operation, no significant difference in alveolar space, lung interstice and peri-alveolar blood vessels from BRL group, non-BRL group or Sham group was observed. However, during the 5–7th week after operation, the peri-alveolar blood vessels in the BRL group revealed slight dilation and blood stasis, and then during the 7–11th week after operation, the lung lesions was gradually characterized by interstitial edema, acinar cell vacuolization, and infiltration of inflammatory and congestion edema in the BRL group. BRL group: HF group with β3-AR agonist BRL37344 injection; non-BRL: HF group without BRL37344 injection.

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Figure 3. Cardiac histopathology at different times and in different groups. Representative images of myocardial cells at the 5th, 7th, 9th, and 11th week observed with a Brightfield microscope under 40× magnification (Olympus BX 51, Japan) were presented. During the 5–7th week after operation, the myocardial cells revealed an ordered arrangement and normal intercellular space without nuclear degeneration. However, at the 9th week, the time-dependent increase of interstitial fibrosis, edema, degeneration, necrosis, and myocyte hypertrophy was observed in the BRL group when compared to the Banded group. BRL group: HF group with β3-AR agonist BRL37344 injection; non-BRL: HF group without BRL37344 injection. doi:10.1371/journal.pone.0068747.g003
Finally, the expression of lung β3-AR mRNA and protein could exhibit a negative correlation with the severity of heart failure, and is observed earlier than expression in the heart in the experiment. Conversely, the expression of β3-AR in cardiac tissues exhibits a positive correlation, which is further increased by the presence of β3-AR agonist. It is also notable that the serum levels of anti-β3-AR autoantibody are negatively correlated with the expression of lung β3-AR, but positively correlated with the expression of heart β3-AR. These results suggest that the plasma levels of anti-β3-AR autoantibody may act as a clinical reference index of prognosis in patients with heart failure, and may indirectly reflect the expression of β3-AR mRNA and protein in both lungs and heart. These findings suggest that the β3-AR and anti-β3-AR autoantibody play an important role in the senescent heart failure. But we think it is the first step to reveal the link between the activation of β3-AR and the aggravated heart failure in the senescent heart. Further studies are needed to reveal the underlying mechanism.

Mortality Rate and Heart Failure in Aged Rats

The model of ascending aorta banding-induced chronic heart failure has been widely used to produce a time-dependent impairment of cardiac function [19,22]. However, the model has not been previously applied using aged rats. The results of the current study indicate that ascending aorta banding-induced heart failure in aged rats is an accurate and reliable model, but with a mortality rate exceeding 50%. These early deaths appear to be due to anesthesia intolerance or acute heart failure. The mortality rate in BRL group is significantly higher than that in non-BRL group, suggesting that β3-AR agonist (BRL37344) may further increase mortality rate in aged rats with heart failure.

On the other hand, we have used male rats in the experiment because estrogen plays a cardioprotective role in female rat hearts. The effects of estrogen during ischemia-reperfusion injury are associated with the decreased cardiomyocyte contraction and expression of β1-AR, and increased expression of β2-AR [23].

Anti-β3-AR Autoantibody and Heart Failure

In recent years, several studies have reported that the positive rates of anti-β3-AR autoantibody in patients with heart failure are remarkably higher than those in the control group [20,24]. The

Figure 4. Expression of β3-AR mRNA by RT-PCR and β3-AR protein by Western blot in lung. (A) Representative analysis of β3-AR mRNA level by RT-PCR. Lane M: marker; bp: base pair; lane n5: the 5th week, non-BRL group; lane n7: the 7th week, non-BRL group; lane n9: the 9th week, non-BRL group; lane n11: the 11th week, non-BRL group. (B) Bar plot showed the data from all animals in the non-BRL group. Data were analyzed by ANOVA. β3-AR revealed a decrease with the deterioration of heart failure. (*p<0.05, versus non-BRL group 2 weeks prior to treatment). (C) Immunoblot of β3-AR protein in representative animals. Lane n5: the 5th week, non-BRL group; lane n7: the 7th week, non-BRL group; lane n9: the 9th week, non-BRL group; lane n11: the 11th week, non-BRL group. GAPDH: glyceraldehyde phosphate dehydrogenase. (D) Bar plot showed the data from all animals in each group. The data are analyzed by ANOVA. β3-AR protein revealed a decrease with the deterioration of heart failure. (*p<0.05, versus non-BRL group 2 weeks prior to treatment). BRL group: HF group with β3-AR agonist BRL37344 injection; non-BRL: HF group without BRL37344 injection.

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results of our study demonstrate for the first time that, in aged patients with heart failure, the positive rate is associated with a 3- to 4-fold increase in anti-β3-AR autoantibody when compared with the normal control. Similarly, the positive rate in aged rats is positively correlated with the severity of heart failure. These results strongly suggest that the plasma level of anti-β3-AR autoantibody can act as a clinical reference index of the prognosis in patients with heart failure, especially aged patients.

The Expression of Pulmonary β3-AR in Heart Failure

One of the most important findings from the present study is that the expressions of lung β3-AR mRNA and protein reveal gradual decrease in aged rats with heart failure, exhibiting a negative correlation with the severity of heart failure. Additionally, β3-AR agonist (BRL 37344) can further inhibit the expression of β3-AR mRNA and protein in pulmonary tissues. Moreover, it has been reported that β3-AR agonist can increase alveolar fluid clearance during lung injury and accelerate the amelioration of pulmonary edema. We therefore speculate that, with the anti-β3-AR autoantibody, similar to a β1-AR agonist, these protective effects may greatly decrease or even disappear with gradually decreasing β3-AR expression in pulmonary tissues. Furthermore, β3-AR reveals a significant decrease in density and activity, thus resulting in a significant pulmonary congestion or edema.

Cardiac β3-AR Expression in Heart Failure

Multiple studies have established that in normal heart tissues, the negative inotropic effect induced by β3-AR agonist may play a safety role during intense adrenergic stimulation such as stress and strong physical effort. Thus, over-expression of β3-AR may protect against cardiac damage induced by the high catecholamine level present in heart failure, particularly during the early stages. However, during the worst stages of heart failure, the down-regulation of cardiac β1-AR and β2-AR, and concomitant decrease in adenylyl cyclase activity result in a significant enhancement of cAMP-mediated negative inotropic effects, thereby enhancing myocardial depression [3,10,25]. Interestingly, β3-AR expression is up-regulated 2–3 times faster in the failing heart when compared with the non-failing heart [26]. The negative inotropic effect of the β3-AR agonist may therefore exacerbate cardiac dysfunction and profoundly aggravate systolic and diastolic dysfunction.

The Relationship between β3-AR and Anti-β3-AR Autoantibodies

In our aged rat model, increasing impairment of heart function is associated with a 3- to 4-fold increase in β3-AR expression in the heart. At the same time, the serum level of anti-β3-AR autoantibodies is associated with a 3- to 4-fold increase. Contrary to the results observed in the heart, a gradual decrease in the expression of β3-AR in the lung is observed. These data suggest
that in the case of lung, the anti-β3-AR autoantibody, as β3-AR agonists, can increase continuous negative inotropic effects that can further inhibit the heart function, and at the same time, due to the significant increase in the expression of pulmonary β3-AR, the action of alveolar fluid clearance may be inhibited, resulting in increasingly impaired heart function. We therefore conclude that the serum level of anti-β3-AR autoantibody can act as a reference index of the prognosis in aged patients with heart failure, and indirectly reflect the expression of β3-AR mRNA and protein in the lungs and heart at different stages.

Conclusion
The ascending aorta banding-induced heart failure model in aged rats is an accurate and reliable model, but is associated with high mortality rate. During the development of heart failure, pathological changes of lung injury preceded myocardial injury. The expression of β3-AR mRNA and protein in pulmonary tissues reveals a gradual decrease, but the expression in myocardial tissues reveals an increase and can further be promoted by β3-AR agonist. The anti-β3-AR autoantibodies can increase gradually as the progression of heart failure. The serum levels of anti-β3-AR autoantibody may then act as a reference index, indirectly reflecting the expression of β3-AR mRNA and protein in lungs and heart at different stages.

Materials and Methods

Ethics Statement
The present human study complied with the Declaration of Helsinki and was approved by the Ethics Committee and the Prescription and Therapeutic Committee (10-S-6) of Capital Medical University Beijing Chao-yang Hospital. All subjects provided written informed consented prior to admission into the study.

All animal experiments were approved by the Institutional Animal Care and Use Committee of Capital Medical University (10-A-33). All surgeries were performed under isoflurane/oxygen anesthesia and all efforts were made to minimize suffering including the use of buprenorphine for pain management after surgery.

Study Cases
Outpatients from Beijing Chao-yang Hospital of China were the source of the samples collected for the determination the anti-β3-AR autoantibody. Sixty-nine heart failure patients were selected for this study. Criteria for patient selection included: aged 65 years or older defined according to New York Heart Association (NYHA) functional classes II-IV; less than 45% left ventricular ejection fractions on echocardiograms; no apparent severe hepatic and renal dysfunction; no previous medication of ACEI, diuretic and/or digoxin. The normal control groups (NC) were selected for this study. Animals were classified randomly into 2 experimental groups and a control group. Experimental animals (n = 140) were subjected to aorta banding, in which the ascending aorta was banded (steel wire area/ascending aorta area ≥ 75%) according to the methods of Brown, et al [22]. In the sham-operated control group (n = 35), the animals were treated with the same surgical procedures, but without the aortic banding. After 5 weeks, aged Wistar rats from the aorta-banding group were divided into a BRL group (n = 50) and a non-BRL group (n = 40). The BRL group received β3-AR-selective agonist (BRL37344 -2-[2-(3-chlorophenyl)-2-hydroxyethyl] amino) propyl] phenoxyacetic acid) (Sigma-Aldrich Co. USA) at a dose of 0.4 nmol·kg⁻¹·min⁻¹ via a vena caudal injection sustained for 10 minutes, twice weekly for 6 weeks, while an equivalent dose of normal saline was administrated in the non-BRL group.

Hemodynamic Measurement and LVW/BW
Eight animals were anesthetized with urethane (20%, 1 g/kg). A cannula connected to a pressure transducer was inserted along the carotid artery into the left ventricle to measure the following primary and derived variables: heart rate, left ventricle end-systolic pressure, left ventricle end-diastolic pressure and the maximal rate of rise and decline in left ventricle pressure (dP/dtmax and dP/ dtmin) by opened thorax. All pressures were measured with a pressure transducer connected to a polygraph (BL-420F, Chengdu Taimeng Technology Co., LTD, China), stored, and displayed on a personal computer. The lung and heart tissues were rapidly excised and frozen in liquid nitrogen, and then stored at −80°C until use. The bodies and the left ventricles of each rat were weighed, and the ratio of left ventricular weight to body weight (LVW/BW) was calculated.

Microscopic Structure Inspection of Lung and Heart
The lung and heart were filled with 10% formalin for fixation, and coated in gauze for histopathological examination. All tissue specimens were cut into 5-mm-thick cross-sections, fixed in 10% formalin, dehydrated, and embedded in paraffin. Slices with 5 μm in thickness were obtained from the sections and stained with hematoxylin-eosin. The tissues were observed with a Brightfield microscope under 40× magnifications (Olympus BX 51, Japan).

Serum Sampling and Anti-β3-AR Determination
Two milliliters of blood were withdrawn from the antecubital vein in patients with heart failure and in the control cases. Throughout the experiment of aged rats, 2 mL of blood samples were taken from the carotid arteries of subjects from all groups. Samples were collected before treatment, and at the 5th, 6th, 7th, 8th, 9th, 10th and 11th week after treatment. The sera were separated by centrifugation (3000 rpm, Beckman CS-5R Centrifuge) for 10 min and stored at −80°C until needed for assay.

The peptides with terminal cysteines corresponding to the sequence of the second extracellular loop of the human β3-AR (residues 176–202: Q-W-W-R-V-G-A-D-A-E-A-Q-R-C-H-S-N-P-R-C-G-A-F-A-S-N-M-C) were synthesized by the Biological Institute of CAMS &PUMC, Beijing, using the solid phase method of Merrifield. The purity of these peptides was evaluated by HPLC on a Vydac C18 column and by amino acid analysis on an automated amino acid analyzer (Beckman instruments, Inc, Palo Alto, CA, USA). The peptides in rats were nearly identical, except for residues shaded in the following sequence of the second extracellular loop of the human β3-AR (residues 173–199: Q-W-W-R-V-G-A-D-A-F-A-Q-E-C-H-S-N-P-R-C-G-S-F-A-S-N-M-C).

Anti-β3-AR autoantibody was determined by enzyme-linked immunosorbent assays (ELISA). Both the study population and rats were defined as being positive or negative based on the presence or absence of anti-β3-AR autoantibody. The ELISA protocol as described by Fu, et al. was used to screen for the presence of autoantibodies [1,2,3,4].
RT-PCR and the Expression of β3-AR

Amplification of β3-AR by PCR was completed in a 50-μL reaction mixture containing 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM MgCl₂, 200 μM dNTPs, 20 pmol each of the primers, 1 unit of Taq polymerase, and 2 μL of DNA template. Amplification was performed with a thermal cycler (PTC-200 Peltier, Bio-RAD) using the following parameters: initial denaturation at 95°C for 3 min; denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 40 s; and a final extension at 72°C for 10 min. For the β3-actin reaction, a 50-μL reaction mixture containing 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 200 μM dNTPs, 20 pmol each of the primers, 15 mM MgCl₂ and 1 unit of Taq polymerase, was used. The conditions for the reaction were as follows: initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 30 s, and extension at 72°C for 90 s; and a final extension at 72°C for 10 min. PCR products were separated using 1% agarose gel and visualized on a gel documentation system (Vilber Lourmat Co., France) after staining with ethidium bromide.

Western Blot and Expression Level of β3-AR Protein

Lung and cardiac tissues were homogenized in ice-cold homogenization buffer. Total protein was isolated from tissues and the concentration was determined using the Bradford method. Crude protein extracts (80 μg) were separated on a 10% SDS-polyacrylamide gel and then transferred to nitrocellulose membranes. The membranes were blocked for 1 h at room temperature in TBS-T containing 5% non-fat milk, and probed with primary antibodies against GAPDH and β3-AR (Santa Cruz Biotechnology, USA). The primary antibodies were diluted in TBS-T (1:1,000). Either alkaline phosphatase conjugated goat anti-mouse (GAPDH) or goat anti-rabbit (β3-AR) antibody was diluted to 1:3,000 in TBS-T and used as a secondary antibody with 1 h incubation. The bands on the blots were visualized using western blue (Promega, USA) and were semi-quantified using a computer image analysis system. The expression level of β3-AR protein was normalized to GAPDH.

Data Analysis

All data, with the exception of antibody titers, are expressed as mean value ± SEM. Antibody titers reported as geometric means and differences between data sets were evaluated by unpaired Student’s t-test. All other data were evaluated by analysis of variance. In all cases, a statistically significant difference was considered at p<0.05. An anti-β3-AR positive score were defined as a ratio [(sample OD - blank OD)/(negative contrast OD - blank OD)] of ≥2.1. Analyses were performed with the GraphPad Prism 4.0 software package (San Diego, CA).

Author Contributions

Conceived and designed the experiments: LZ. Performed the experiments: GBM ZC YXF MBL GH. Analyzed the data: HLA ZYZ JZ. Contributed reagents/materials/analysis tools: LQL JZ. Wrote the paper: GBM ZC LZ.

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