Nrf2-Mediated Cardiac Maladaptive Remodeling and Dysfunction in a Setting of Autophagy Insufficiency

Qingyun Qin, Chen Qu, Ting Niu, Huimei Zang, Lei Qi, Linmao Lyu, Xuejun Wang, Mitzi Nagarkatti, Prakash Nagarkatti, Joseph S. Janicki, Xing Li Wang, Taixing Cui

Abstract—Nuclear factor erythroid-2–related factor 2 (Nrf2) appears to exert either a protective or detrimental effect on the heart; however, the underlying mechanism remains poorly understood. Herein, we uncovered a novel mechanism for turning off the Nrf2-mediated cardioprotection and switching on Nrf2-mediated cardiac dysfunction. In a murine model of pressure overload–induced cardiac remodeling and dysfunction via transverse aortic arch constriction, knockout of Nrf2 enhanced myocardial necrosis and death rate during an initial stage of cardiac adaptation when myocardial autophagy function is intact. However, knockout of Nrf2 turned out to be cardioprotective throughout the later stage of cardiac maladaptive remodeling when myocardial autophagy function became insufficient. Transverse aortic arch constriction–induced activation of Nrf2 was dramatically enhanced in the heart with impaired autophagy, which is induced by cardiomyocyte-specific knockout of autophagy-related gene (Atg)5. Notably, Nrf2 activation coincided with the upregulation of angiotensinogen (Agt) only in the autophagy-impaired heart after transverse aortic arch constriction. Agt5 and Nrf2 gene loss-of-function approaches in combination with Jak2 and Fyn kinase inhibitors revealed that suppression of autophagy inactivated Jak2 and Fyn kinase inhibitors and nuclear translocation of Fyn, while enhancing nuclear translocation of Nrf2 and Nrf2-driven Agt expression in cardiomyocytes. Taken together, these results indicate that the pathophysiological consequences of Nrf2 activation are closely linked with the functional integrity of myocardial autophagy during cardiac remodeling. When autophagy is intact, Nrf2 is required for cardiac adaptive responses; however, autophagy impairment most likely turns off Fyn-operated Nrf2 nuclear export thus activating Nrf2-driven Agt transcription, which exacerbates cardiac maladaptation leading to dysfunction. (Hypertension. 2016;67:00-00.)

DOI: 10.1161/HYPERTENSIONAHA.115.06062.) • Online Data Supplement

Key Words: angiotensinogen • autophagy • myocardial infarction • NF-E2-related factor 2 • ventricular remodeling

Nuclear factor erythroid-2–related factor 2 (Nrf2) is a member of the Cap ‘n’ Collar family of basic leucine zipper transcription factor.1–5 Nrf2 binds to a cis-acting enhancer sequence known as the antioxidant response element with a core nucleotide sequence of 5′-RTGACNNNGC-3′ to control the basal and inducible expression of >200 genes that can be grouped into several categories, including antioxidant genes, phase II detoxifying enzymes, transcriptional factors, transporters, scavenger receptors, and chaperone proteins.1–3 Thus, the function of Nrf2 ranges broadly from the classical antioxidant defense to cell cycle regulation and protein quality control.

A cardioprotective role of Nrf2 has been demonstrated in several animal models, including the transverse aortic arch constriction (TAC) model of pressure overload–induced cardiac maladaptive remodeling and dysfunction.4,5 At the molecular level, Nrf2 drives antioxidant and detoxifying defense to suppress oxidative stress–mediated cardiac injury and dysfunction.4,5 Also, Nrf2 facilitates autophagic clearance of toxic ubiquitinated protein aggregates thereby protecting against proteocytotoxicity in the heart.6 In contrast, a recent study has shown that knockout of Nrf2 prevents myocardial reductive stress, attenuates cardiac accumulation of ubiquitinated proteins and pathological hypertrophy, and ameliorates heart failure in the aged human missense (R120G) mutant of alpha B-crystallin (hCryABR120G) transgenic mouse, a mouse model of protein aggregate–induced cardiomyopathy.9 Therefore, sustained activation of Nrf2 is proposed to cause reductive stress thereby contributing to hCryABR120G-induced cardiomyopathy.9 However, Nrf2 cardiomyocyte-specific transgenic mice are healthy and resistant to cardiac maladaptive remodeling and dysfunction after 4 weeks pressure overload,4 arguing against the theory of Nrf2-mediated reductive...
stress. However, previous studies have demonstrated that Nrf2 activation leads to liver damage in a setting of autophagy impairment.10,11 In addition, autophagy insufficiency plays an essential role in mouse CryABR120G (mCryABR120G)-induced cardiomyopathy.12 Given that the mCryABR120G and hCryABR120G have virtually identical amino sequences13,14 and aging is associated with autophagy insufficiency or impairment,15 it is likely that the Nrf2-mediated adverse effects may be related to impaired autophagy in the aged hCryABR120G heart. Collectively, these findings indicate that Nrf2 may exert either a protective or detrimental effect on the heart depending on the nature of pathological settings. However, the precise mechanism for the dual effects of Nrf2 remains unknown.

Nrf2 is a protein whose half-life is estimated to be <20 minutes in the cell.2 Although precise molecular mechanisms of Nrf2 activation are not fully understood, it is generally accepted that Kelch-like ECH-associated protein 1 (Keap1) plays a central role in the regulation of Nrf2 protein stability and Nrf2-driven transcriptional activity.2 Keap1 serves as an adaptor for the interaction of the Cul3-based E3-ubiquitin ligase complex with Nrf2 leading to Nrf2 ubiquitination and consequent proteosomal degradation. Several alternative mechanisms of Nrf2 regulation have been proposed, such as phosphorylation of Nrf2 by various protein kinases, including mitogen-activated kinases, protein kinase C, phosphatidylinositol 3-kinase, and Fyn kinase.2,16 It has been documented that Fyn kinase is capable of phosphorylating Nrf2 in the nucleus, thereby leading to nuclear export of the phosphorylated Nrf2 for its degradation in vascular smooth muscle cells.17 Yet, the pathophysiological significance of the Nrf2 regulation cascade in the heart remains to be explored.

In this study, we found that the pathophysiological consequence of Nrf2 activation is linked to the functional integrity of autophagy in the heart. Nrf2 is required for cardiac adaptive responses when autophagy is intact; however, it becomes a mediator of cardiac maladaptive remodeling and dysfunction when autophagy is impaired. Autophagy impairment most likely turns off nuclear Fyn-operated Nrf2 export, thus, activating Nrf2-driven Agt transcription, which exacerbates cardiac maladaptation leading to dysfunction.

Methods
Litters of wild-type (WT) and Nrf2 knockout (Nrf2−/−), or floxedAtg5 (Atg5fl/fl), αMHC-MerCreMer (MerCreMer), and MerCreMer−/−:Atg5fl/+ and Atg5fl/+ mice were generated by cross-breeding of heterozygous Nrf2+/− or MerCreMer−/− and Atg5fl/+ mice, respectively. TAC model, echocardiography, histopathology, immunohistochemistry, and biochemical assays were performed as previously described.4,7,8

Results
Knockout of Nrf2 impairs acute cardiac adaptation; however, it ameliorates the progression of cardiac maladaptive remodeling leading to heart failure after pressure overload.

In a murine TAC model, TAC-induced pressure overload initially results in an adaptive cardiac hypertrophy with preserved cardiac function (days 1–14) followed by maladaptive cardiac remodeling, which eventually causes chronic heart failure (days 14–28).4 Of note, ≤40% of WT mice die from acute heart failure within the first 2 weeks after TAC.4 In this TAC model, we have demonstrated that knockout of Nrf2 exacerbates cardiac pathological hypertrophy, fibrosis, and apoptosis, which results in cardiac dysfunction with increased mortality within the first 2 weeks.4 Thus, these findings indicate that Nrf2 is a critical mediator of cardiac adaptation.4 Also, Nrf2 may act as a negative regulator of cardiac maladaptive remodeling and dysfunction in response to...
sustained pressure overload. Accordingly, we further determined the effect of Nrf2 deficiency on cardiac adaptation, as well as cardiac maladaptive remodeling, and dysfunction after TAC. TAC-induced death rate was increased by knockout of Nrf2 (Figure 1A), presumably because of the increased acute heart failure as previously reported.4,18 Because cardiomyocyte necrosis is one of the causes for heart failure,19 we determined the impact of Nrf2 deficiency on TAC-induced myocardial necrosis. A time-course study revealed that TAC-induced myocardial necrosis appeared on day 1, peaked on day 3, and declined to basal levels after 1 week (Figure S1 in the online-only Data Supplement). Thus, we compared myocardial necrosis of WT and Nrf2−/− mice 3 days after TAC. As shown in Figure 1B, the loss of Nrf2 enhanced TAC-induced myocardial necrosis. These results indicate that Nrf2 is capable of protecting against TAC-induced acute heart failure via its ability to suppress myocardial necrosis during the initial stage of pressure overload–induced cardiac adaptation. These findings provide additional evidence to demonstrate a critical role of Nrf2 in mediating cardiac protection.

However, we found unexpectedly that knockout of Nrf2 attenuated cardiac hypertrophy and ameliorated progression of cardiac dysfunction by 8 weeks after TAC (Figure 1C and 1D; Table). In addition, knockout of Nrf2 significantly inhibited cardiomyocyte hypertrophy and the fetal gene reprogramming and the downregulation of sarco-endoplasmic reticulum calcium ATPase2a (SERCA2a) for pathological cardiac hypertrophy,20,21 as well as myocardial fibrosis, apoptosis, and oxidative stress at 4 weeks after TAC (Table S1; Figures S2–S4). These results clearly demonstrate a mediator role of Nrf2 in cardiac maladaptive remodeling and dysfunction in response to pressure overload.

Taken together, our findings reveal that Nrf2 is required for cardiac adaptation and paradoxically it becomes a mediator of...
cardiac maladaptive remodeling and dysfunction in response to pressure overload.

Pathophysiological Consequences of Nrf2 Activation Are Linked to the Functional Integrity of Autophagy in the Heart After Pressure Overload

Because autophagy impairment may be linked to the Nrf2-mediated adverse effects aforementioned, we postulated that there is an intimate relationship between autophagy function and Nrf2-mediated actions in the heart. To test this hypothesis, we examined the temporal autophagy functional states in the heart after TAC. We found that myocardial autophagy flux, a more accurate parameter reflecting autophagy function, was intact at 2 weeks, suppressed at 4 weeks, and blocked at 8 weeks after TAC (Figure 2). These results indicate that TAC eventually induces autophagy impairment in the heart. Given that the loss of Nrf2 exaggerated cardiac maladaptive remodeling and led to an onset of cardiac dysfunction at 2 weeks after TAC when the autophagic flux remains normal (Figure 2), these results indicate that TAC eventually induces autophagy impairment in the heart. Also, because the observed Nrf2-mediated cardiac adverse remodeling at 4 weeks after TAC (Figures S2–S4) was associated with insufficient autophagy in the heart (Figure 2) and the Nrf2-mediated cardiac pathological hypertrophy and dysfunction at 8 weeks after TAC (Table 1) was associated with impaired autophagy in the heart (Figure 2), it is conceivable that Nrf2 mediates TAC-induced cardiac pathological remodeling and dysfunction in a setting of autophagy impairment. Collectively, these findings in the heart highlight a crucial link between autophagy functional states and the Nrf2-mediated dual effects.

Nrf2 Expression and Activity Are Enhanced in the Failing Heart With Autophagy Impairment After Pressure Overload

To further investigate the interplay between autophagy and Nrf2 activation in cardiac dysfunction, we determined the impact of myocardial-specific autophagy impairment on Nrf2 expression and activity in the heart at 4 weeks after TAC using tamoxifen-inducible cardiomyocyte-restricted Atg5 knockout (Atg5−/−) mice. Tamoxifen (20 mg/kg per day, intraperitoneally) for 21 days ablated Atg5 expression in the heart of MerCreMer+::Atg5fl/fl mice (Figure 3A) without any adverse health problems as previously reported. The ablation of Atg5 induced accumulation of p62 and suppression of LC3-II expression in the heart (Figure 3A), indicating myocardial autophagy impairment because of the loss of Atg5. Echocardiography showed that TAC-induced cardiac dysfunction was worsened in the cardiac-specific Atg5-depleted and

Figure 2. The effect of transverse aortic arch constriction (TAC) on myocardial autophagy in mice. Male wild-type C57BL/6J mice at 9 to 10 wk of age were subjected to sham or TAC operation as indicated. A, Western blot analysis of myocardial LC3-I, LC3-II, and p62 expression. Left, Representative immunoblots from 4 separate experiments. Right, Densitometric analysis of LC3-II and p62 protein levels. n=4, * or #P<0.05 vs control (0). There are no differences of LC3-II or p62 expression between control (0) and sham groups at each time point as indicated. B, Myocardial autophagic flux after TAC. Upper, Representative immunoblots of LC3-I and LC3-II from 4 separate experiments. Middle, Densitometric analysis of LC3-II protein levels. Bottom, Quantified autophagic flux. n=4. CQ indicates chloroquine; and ns, nonsignificant. There are no differences of LC3-II expression between control (0) and sham groups at each time point as indicated.
autophagy-impaired mice, compared with the control groups of Atg5flo/flo and MerCreMer+ mice (data not shown) as previously described.24 Tamoxifen treatment per se did not affect TAC-induced cardiac hypertrophy and dysfunction and death in WT mice (Figure S5; Table S2). Of interest, TAC-induced expression of Nrf2 and its downstream target gene NAD(P)H dehydrogenase, quinone-1 (NQO1) were enhanced by the ablation of Atg5 (Figure 3B and 3C), indicating that myocardial autophagy impairment results in a further production of Nrf2 in the pressure-overloaded heart. Considering the Nrf2-mediated cardiac adverse remodeling and dysfunction in the autophagy-impaired heart (Table 1; Figures S2–S4), it is likely that autophagy impairment switches on this remodeling and dysfunction via a yet unknown mechanism in the pressure-overloaded heart.

Nrf2 Drives Agt Expression in the Pressure-Overloaded Heart With Functional Insufficiency of Autophagy

A recent study has documented that Nrf2-operated upregulation of Agt is causative for renal damage associated with type 1 diabetes mellitus,25 a state which induces autophagy impairment.26,27 Because it is well established that the upregulation of Agt is a primary cause of cardiac pathological remodeling and dysfunction,28,29 we postulated that Nrf2 drives the upregulation of Agt in the autophagy insufficient heart, thereby leading to cardiac pathological remodeling and dysfunction in response to pressure overload. Thus, we first performed a temporal study of Nrf2 activation and Agt expression in the heart of WT mice after TAC. We found that Agt expression was upregulated by 2 weeks and remained so at 4 weeks after TAC; however, Nrf2 expression and activation were apparent at 1 week, peaked at 2 weeks, and then Nrf2 mRNA expression contrastingly declined closer to the basal level while NQO1 mRNA expression (reflecting Nrf2 transcriptional activity) was largely retained by 4 weeks after TAC (Figure 4A). These expression patterns indicate that Nrf2 activation per se may not be able to upregulate Agt expression in the heart, and Nrf2-mediated upregulation of Agt is dependent on a yet unknown mechanism in the pressure-overloaded heart. In addition, it is likely that posttranscriptional as opposed to transcriptional activation of Nrf2 plays a critical role in the upregulation of Agt in the pressure-overloaded heart. Given that the myocardial autophagy function is intact within 2 weeks and then declines and becomes insufficient at 4 weeks after TAC (Figure 2), we questioned whether

**Figure 3.** The effect of cardiomyocyte-restricted knockout of Atg5 on transverse aortic arch constriction (TAC)–induced activation of nuclear factor erythroid-2–related factor 2 (Nrf2) in the murine heart. Six-week-old male mice with different genotypes as indicated were intraperitoneally injected with tamoxifen (20 mg/kg per day) for 3 weeks. After 2 wk, for washing out tamoxifen from the body, these mice were subjected to sham or TAC operation for 4 wk. A, The efficacy of tamoxifen-induced knockout of Atg5 in the heart. Representative immunoblots of myocardial Atg5, p62, LC3-I, and LC3-II from 8 separate experiments. B, Quantitative polymerase chain reaction analysis of myocardial Nrf2 and NQO1 mRNAs in the heart 4 wk after sham (−) and TAC. *P<0.05 vs sham (−) in the same group. C, Western blot analysis of myocardial Nrf2 and NQO1 proteins in the heart 4 wk after sham and TAC. n=3 for each sham group and n=4 to 5 for each TAC group. *P<0.05 vs sham (−) in the same group. WT indicates wild-type.
Autophagy function is critical for Nrf2-operated upregulation of Agt in the heart. We found that ablation of Atg5 in cardiomyocytes dramatically enhanced 4 weeks TAC-induced Agt expression (Figure 4B), whereas knockout of Nrf2 blocked 4 weeks TAC-induced Agt expression in the heart (Figure 4C). Considering that TAC-induced upregulation of LC3-II in the heart is mostly blocked in cardiomyocyte-restricted Atg5−/− mice,24 the loss of Atg5 in cardiomyocytes impairs autophagy in the pressure-overloaded heart. In addition, we have demonstrated that Nrf2 does not directly regulate autophagy per se and may facilitate autophagy-mediated clearance in the myocardium.

Autophagy impairment suppresses TAC-induced activation of Jak2/Fyn pathway, which operates Nrf2 nuclear export for degradation in the pressure-overloaded heart.

To explore the signaling mechanism by which Nrf2 upregulates Agt in the autophagy-deficient heart after TAC, we determined the effect of Nrf2 knockout on TAC-induced myocardial activation of Jak2/Stat3 pathway, which has been shown to control Agt expression in cardiomyocytes.30 As shown in Figure 5A, phosphorylation of Jak2 and Stat3 was enhanced in the heart of control Atg5+/+ and MerCreMer+ mice at 4 weeks after TAC when myocardial autophagy is insufficient (Figure 2). Compared with the control, 4 weeks TAC-induced enhancement of Jak2 and Stat3 activities was blocked in cardiomyocyte-specific Atg5−/− mice (Figure 5A). These results demonstrate that autophagy is required for TAC-induced activation of Jak2/Stat3 pathway in the heart as observed in other cell types.31 However, knockout of Nrf2 minimally regulated the 4 weeks TAC-induced activation of Jak2/Stat3 pathway (Figure 5B). These results indicate that Nrf2 mediates TAC-induced upregulation of Agt in the heart with autophagy insufficiency via a mechanism independent of Jak2/Stat3 pathway or dependent on a yet unappreciated signaling mechanism because of Jak2 inhibition.

Considering the observed link between post-transcriptional activation of Nrf2 and Agt upregulation (Figure 4), we postulated that Jak2-dependent post-transcriptional regulation of Nrf2 is critical for the upregulation of Agt in the autophagy-impaired heart. It has been reported that nuclear Fyn kinase, a downstream substrate of Jak2,32 once activated, is capable of causing Nrf2 nuclear export for its degradation.17 Therefore, we hypothesized that autophagy insufficiency leads to suppression of Jak2-dependent Fyn phosphorylation, as well as the subsequent Nrf2 nuclear export and degradation, thereby resulting in increased nuclear accumulation of Nrf2 to activate Agt expression in the heart. To test this hypothesis, we determined the impact of Nrf2 and Atg5 knockout on angiotensin II (Ang II)–induced activation of Jak2/Stat3 and Fyn kinases, as well as the protein expression of Agt, in cultured rat neonatal cardiomyocytes. As shown in Figure 6A and 6B, Ang II upregulated LC3-II expression (without affecting bafilomycin A1-induced accumulation of LC3-II; data not shown), induced phosphorylation of Jak2, Stat3, and Fyn, and upregulated Agt protein expression in the control group. These results indicate that the Ang II–induced upregulation

Figure 4. Nuclear factor erythroid-2–related factor 2 (Nrf2)–mediated upregulation of angiotensinogen (Agt) in the autophagy-impaired heart after transverse aortic arch constriction (TAC). A, TAC-induced expression of Agt, Nrf2, and NQO-1 in the heart. Male wild-type (WT) mice at 9 wk of age were subjected to sham or TAC operation. Left ventricles of mice before and after 1, 2, and 4 wk post surgery were subjected to quantitative polymerase chain reaction (qPCR) analysis of Agt, Nrf2, and NQO-1 mRNA expression. *P<0.05 vs sham. Sample numbers for each group are indicated in the figure. B, The effect of cardiomyocyte-restricted knockout of Atg5 on Agt expression in the heart in 4 weeks after sham (−) or TAC (+). Male mice with different genotypes were treated as described in Figure 3. Top, qPCR analysis of angiotensinogen (Agt) mRNA expression in the heart. Middle, Representative immunoblots. Bottom, Densitometric analysis of Agt protein in the heart. n=4, *P<0.05 vs sham (−) in the same group. C, Effect of Nrf2 knockout on Agt expression in the heart 4 weeks after sham (−) or TAC (+). Six-week-old littermates of WT and Nrf2 received the same treatment as described in Figure 3. Top, qPCR analysis of Agt mRNA expression in the heart. Middle, Representative immunoblots. Bottom, Densitometric analysis of Agt protein in the heart. n=4, *P<0.05 vs WT sham (−).

Figure 5. Autophagy function is critical for Nrf2-operated upregulation of Agt in the heart. A, TAC-induced expression of Agt, Nrf2, and NQO-1 in the heart. Male wild-type (WT) mice at 9 wk of age were subjected to sham or TAC operation. Left ventricles of mice before and after 1, 2, and 4 wk post surgery were subjected to quantitative polymerase chain reaction (qPCR) analysis of Agt, Nrf2, and NQO-1 mRNA expression. *P<0.05 vs sham. Sample numbers for each group are indicated in the figure. B, The effect of cardiomyocyte-restricted knockout of Atg5 on Agt expression in the heart 4 weeks after sham (−) or TAC (+). Male mice with different genotypes were treated as described in Figure 3. Top, qPCR analysis of angiotensinogen (Agt) mRNA expression in the heart. Middle, Representative immunoblots. Bottom, Densitometric analysis of Agt protein in the heart. n=4, *P<0.05 vs sham (−).

Figure 6. Nuclear factor erythroid-2–related factor 2 (Nrf2)–mediated upregulation of angiotensinogen (Agt) in the autophagy-impaired heart after transverse aortic arch constriction (TAC). A, TAC-induced expression of Agt, Nrf2, and NQO-1 in the heart. Male wild-type (WT) mice at 9 wk of age were subjected to sham or TAC operation. Left ventricles of mice before and after 1, 2, and 4 wk post surgery were subjected to quantitative polymerase chain reaction (qPCR) analysis of Agt, Nrf2, and NQO-1 mRNA expression. *P<0.05 vs sham. Sample numbers for each group are indicated in the figure. B, The effect of cardiomyocyte-restricted knockout of Atg5 on Agt expression in the heart 4 weeks after sham (−) or TAC (+). Male mice with different genotypes were treated as described in Figure 3. Top, qPCR analysis of angiotensinogen (Agt) mRNA expression in the heart. Middle, Representative immunoblots. Bottom, Densitometric analysis of Agt protein in the heart. n=4, *P<0.05 vs sham (−).
of Agt associates with the activation of autophagy, as well as Jak2/Stat3 and Fyn kinases, in cardiomyocytes. Knockdown of Atg5 alone resulted in downregulation of LC3-II expression and upregulation of p62 (Figure 6), indicating that the knockdown of Atg5 impairs autophagy in cardiomyocytes as observed in vivo (Figure 3A). The Atg5 knockdown-induced autophagy impairment blocked the Ang II–induced activation of Jak2, Stat3, and Fyn kinases, whereas dramatically enhancing Ang II–induced Agt expression (Figure 6), demonstrating that autophagy impairment suppresses the activation of Jak2/Stat3 pathway and Fyn kinase but facilitates upregulation of Agt in cardiomyocytes as we observed in the heart (Figures 4B and 5A). Knockdown of Nrf2 alone had minimal impact on the Ang II–induced activation of autophagy, Jak2/Stat3, and Fyn kinases but suppressed the Ang II–induced upregulation of Agt (Figure 6). Although knockdown of Nrf2 did not affect the Atg5 deficiency–induced inactivation of Jak2/Stat3 and Fyn kinases, it blocked the Atg5 deficiency–induced enhancement of Agt expression (Figure 6). These results demonstrate that Nrf2 is a critical mediator of Agt expression in cardiomyocytes regardless of autophagy functional status. Importantly, these findings also strongly support the aforementioned notion that Jak2/Fyn signaling plays a key role in the control of Nrf2-mediated upregulation of Agt in autophagy-impaired cardiomyocytes. To further establish a causative link between Jak2, Fyn kinases, and Nrf2-mediated Agt expression, we used Jak2 and Fyn kinase inhibitors, AG490 and PP2, in rat neonatal cardiomyocytes. A time-course study showed that Ang II activated Nrf2-driven NQO1 expression and Agt transcription, reaching a peak at 6 to 12 hours and declining to the basal levels by 48 hours (Figure 7A). Both AG490 and PP2 enhanced Ang II–induced upregulation of NQO1 and Agt (Figure 7B). These results reveal an intimate interaction between Jak2–Fyn kinases, Nrf2 activation, and Agt expression in cardiomyocytes, in which Jak2 and Fyn kinases serve as negative feedback regulators of Nrf2 activation and Agt expression when autophagy is intact. Notably, knockdown of Atg5 dramatically enhanced Ang II–induced mRNA expression of NQO1 and Agt and this enhancement could not be further increased by inhibiting Fyn kinase; whereas, both the Fyn kinase inhibitor PP2-potentiated and Atg5 deficiency–heightened Agt expression were blocked by knockdown of Nrf2 (Figure 7C). These results indicate that Fyn kinase suppresses Agt expression by inactivating Nrf2, serving as a negative feedback mechanism for the control of Agt expression in autophagy intact cardiomyocytes; however, autophagy impairment could turn off the negative feedback regulation thus exaggerating Agt expression in cardiomyocytes. To explore the pathophysiological relevance of Fyn–Nrf2 axis, we studied the nuclear location of Fyn kinase and Nrf2, as well as the role of Nrf2, in driving Agt expression in the heart after TAC. Immunohistochemical staining showed that there is no detectable level of nuclear phosphorylated (p)-Fyn or Nrf2 in 4-week sham hearts of Atg5fl/fl, MerCreMer+, and Atg5−/− mice (data not shown). However,
4-week TAC led to increased levels of nuclear p-Fyn in the heart of control Agt5−/− and MerCreMer+ mice but not in the heart of Atg5−/− mice (Figure 8A). Also, 4-week TAC led to increased levels of nuclear Nrf2 in the heart of control Agt5fl/fl and MerCreMer+ mice, and it was more dramatic in the heart of Atg5−/− mice (Figure 8A). This reciprocal relationship between nuclear p-Fyn and Nrf2, as well as the enhanced expression and transcriptional activity of Nrf2 in Atg5−/− hearts suggest that the inactivation of Fyn is responsible for the nuclear accumulation of Nrf2 leading to upregulation of Agt expression in myocardium. Indeed, knockout of Nrf2 blocked the TAC-induced upregulation of Agt expression in autophagy-impaired hearts in which Atg5 is specifically knocked out in cardiomyocytes (Figure 8B).

Figure 6. The effect of nuclear factor erythroid-2-related factor 2 (Nrf2) and Atg5 knockdown on angiotensin II (Ang II)–induced activation of the Jak2/Fyn/Agt signaling axis in cardiomyocytes. Rat neonatal cardiomyocytes infected with adenovirus of control scramble shRNA (Ad-shCtr) and Ad-shNrf2 were further transfected with control siRNA (siCtr) and siAtg5 before Ang II treatment as described in the online-only Data Supplement. A, Representative immunoblots from 4 separate experiments. B, Densitometric analysis. n=4, *P<0.05 vs the control (vehicle treated Ad-shCtr+siCtr cells). C, The efficacy of Nrf2 and Atg knockdown. Top, Quantitative polymerase chain reaction analysis of Nrf2 mRNA expression and Western blot analysis of Nrf2 target gene NQO1 in rat neonatal cardiomyocytes infected with Ad-shCtr and Ad-shNrf2. n=4, *P<0.05 vs Ad-shCtr infected cells. Bottom, Representative immunoblot of LC3 and Atg5 in the rat neonatal cardiomyocytes transfected with siCtr and siAtg5 from 4 separate experiments.

Figure 7. The effect of Fyn kinase inhibition on angiotensin II (Ang II)–induced Nrf2 activation and angiotensinogen (Agt) expression in cardiomyocytes with a setting of autophagy impairment. A, A time-course study of Ang II–induced mRNA expression of NQO1 and Agt. Serum starved rat neonatal cardiomyocytes were treated with Ang II (1 µmol/L) in serum-free DMEM as indicated. n=4, *P<0.05 vs Ang II (0 h) in the same group. B, The effect of Jak2 inhibitor AG490 and Fyn kinase inhibitor PP2 on Ang II–induced mRNA expression of NQO1 and Agt. Serum starved rat neonatal cardiomyocytes were treated with Ang II (1 µmol/L), AG490 (1 µmol/L), and PP2 (1 µmol/L) in serum-free DMEM as indicated for 48 h. n=4, *P<0.05 vs vehicle, PP2, AG490, or Ang II alone treated groups. C, The effect of PP2 on Ang II–induced mRNA expression of NQO1 and Agt in the settings of Nrf2 and Atg5 knockdown. Rat neonatal cardiomyocytes were treated as in Figure 6 and stimulated with Ang II and PP2 as in Figure 7B. n=4, *P<0.05 vs Ang II–treated Ad-shCtr+siCtr group. ns indicates nonsignificant.
Discussion

In this study, there are several novel findings which address the contradictory roles of Nrf2 in cardioprotection and cardiac dysfunction as follows: (1) Nrf2 activation is cardioprotective when myocardial autophagy function is sufficient; however, it exacerbates cardiac maladaptive remodeling and dysfunction when myocardial autophagy function is insufficient; (2) Nrf2 is capable of inhibiting myocardial necrosis in the heart with a sufficient function of autophagy, whereas it could enhance Ang II signaling in the heart with cardiomyocyte-specific knockout of Atg5 at 4 weeks after TAC. (3) Nrf2 nuclear accumulation and the subsequent activation of Nrf2-driven transcription of Agt in cardiomyocytes, most likely because of the inactivation of Jak2/Fyn signaling for Nrf2 export and degradation. Our findings uncover for the first time that autophagy plays a critical role in controlling the pathophysiological consequences of Nrf2 activation, unveiling the nature of the Nrf2-mediated controversial actions in the heart.

The heart is capable of initiating a rapid adaptation to compensate for a large variety of mechanical, hemodynamic, hormonal, and pathological stimuli, which are characterized by cardiac hypertrophy with preserved left ventricle function. However, the cardiac compensation is limited and, as a result, the adaptive responses become maladaptive and left ventricle dysfunction and heart failure death ensue if the stress persists.33 The molecular mechanism of cardiac compensation and decompensation has only been partly understood. Our previous studies indicate that, in a setting of pressure overload, Nrf2 is critical for the initial cardiac adaptation most likely via its ability to suppress oxidative stress, as well as its antioxidant independent capability to facilitate clearance of toxic ubiquitinated proteins in cardiomyocytes.4,8 Considering a critical role of Nrf2 in inhibiting cardiomyocyte necrosis,7,8 it was not surprising to find that Nrf2 knockout enhances myocardial necrosis during the cardiac adaptation process. These results collectively indicate that Nrf2 is a crucial mediator of cardiac compensation and serves as an inhibitor of the transition from cardiac compensated to maladaptive remodeling and dysfunction. Also, Nrf2 may play a subsequent critical role in the decompensation, which leads to heart failure. However, the suppression of cardiac maladaptive remodeling and dysfunction in Nrf2 knockout mice is intriguing. Given that Nrf2 activation was cardioprotective during the cardiac adaptation phase while being detrimental to the heart during the decompensation phase in the same TAC model, it is conceivable that there must be a yet unappreciated mechanism that could turn off Nrf2-mediated cardioprotection and switch on Nrf2-operated cardiac maladaptation in the pathogenesis.
of pressure overload–induced heart failure. In this regard, our findings support a notion that myocardial autophagy impairment is responsible for switching on Nrf2-mediated cardiac maladaptive remodeling and dysfunction in response to pressure overload. At the molecular level, autophagy impairment switches off Jak2/Fyn signaling for Nrf2 export and degradation, thus leading to nuclear accumulation of Nrf2 to drive Agt expression in the heart. Because the upregulation of myocardial Agt ineffectively elevates the local levels of Ang II, in turn, activation of Ang II–Ang II receptor type 1 axis, which causes cardiac adverse remodeling and dysfunction.34,35 It is predictable that the activation of Nrf2–Agt axis eventually leads to cardiac adverse remodeling and dysfunction. Taken together, these results suggest that autophagy impairment is a contributing mechanism for initiating cardiac decompensation in which Nrf2 activates Agt–Ang II signaling.

There are several issues requiring future studies. First, the precise mechanism by which Fyn suppresses Nrf2-mediated Agt expression in the heart has not been completely delineated in this study. A plausible explanation is likely that the phosphorylated Nrf2 via Fyn36 could not bind to the promoter of Agt and activate Agt expression when autophagy is intact; however, autophagy impairment blocks the Fyn-mediated phosphorylation of Nrf2, which in turn enable Nrf2-driven expression of Agt. Second, the Nrf2–Agt axis may not be the sole mechanism for driving cardiac maladaptive remodeling and dysfunction in a setting of autophagy impairment. We have observed that autophagy impairment compromises ubiquitin–proteasome system performance in a p62/SQSTM1-dependent manner in cardiomyocytes.37 Because p62 is a target gene of proteasome system performance in a p62/SQSTM1-dependent manner,37,38 it is predictable that the activation of Nrf2–Agt axis eventually leads to cardiac adverse remodeling and dysfunction. Taken together, these results suggest that autophagy impairment is a contributing mechanism for initiating cardiac decompensation in which Nrf2 activates Agt–Ang II signaling.

As summarized in Figure 8C, Nrf2 activation exacerbates maladaptive remodeling and dysfunction in a setting of autophagy impairment. At the molecular level, autophagy impairment suppresses the activation of Jak2/Fyn pathway, which facilitates nuclear export of Nrf2 for degradation, leading to nuclear accumulation of Nrf2 and activation of Nrf2-driven expression of Agt to enhance Ang II formation in the heart. Subsequently, the activation of Ang II signaling contributes to the progression of cardiac maladaptive remodeling and dysfunction. Our findings highlight a unique coupling between Nrf2 signaling and autophagy function in the pathogenesis of cardiac dysfunction and also suggest that the simultaneous targeting of Nrf2 and autophagy may be a novel therapeutic approach for the prevention of adverse cardiac remodeling and dysfunction associated with hypertensive heart disease.

Sources of Funding
This research was supported by grants from NIH NCCAM PO20 GM103641 and 2PO1AT003961-06A1, the National Natural Science Foundation of China (No. 81370267), and the Shandong University National Qianren Scholar Fund and Taishan Scholar Fund.

Disclosures
None.

References
The findings of this study suggest that simultaneously targeting of Nrf2 and autophagy may be a novel approach for the prevention of cardiac maladaptive remodeling and dysfunction.

Nrf2 activation is cardioprotective when myocardial autophagy is intact, whereas it becomes detrimental to the heart when myocardial autophagy is impaired. Autophagy impairment most likely turns cardiomyocytes toward apoptosis in diabetic heart failure. It is the first report to demonstrate that autophagy impairment switches cardiomyocyte death to apoptosis, which exacerbates cardiac maladaptation leading to heart failure.

Nrf2, Autophagy, and Cardiac Dysfunction

What Is Relevant?

- this is the first report to document that autophagy impairment switches on nuclear factor erythroid-2-related factor 2 (Nrf2)–mediated cardiac maladaptive remodeling and dysfunction secondary to pressure overload in mice.

What Is New?

- A catalytically active Jak2 is required for the angiotensin II-dependent activation of Fyn.
- A catalytically active Jak2 is required for the angiotensin II-dependent activation of Fyn.

Novelty and Significance

Summary

Nrf2 activation is cardioprotective when myocardial autophagy is intact, whereas it becomes detrimental to the heart when myocardial autophagy is impaired. Autophagy impairment most likely turns off nuclear Fyn–operated Nrf2 export and degradation thus activating Nrf2–driven angiotsinogen transcription, which exacerbates cardiac maladaptation leading to heart failure.
Nrf2-Mediated Cardiac Maladaptive Remodeling and Dysfunction in a Setting of Autophagy Insufficiency
Qingyun Qin, Chen Qu, Ting Niu, Huimei Zang, Lei Qi, Linmao Lyu, Xuejun Wang, Mitzi Nagarkatti, Prakash Nagarkatti, Joseph S. Janicki, Xing Li Wang and Taixing Cui

Hypertension. published online November 16, 2015;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2015/11/16/HYPERTENSIONAHA.115.06062

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2015/11/16/HYPERTENSIONAHA.115.06062.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
Nrf2-mediated cardiac maladaptive remodeling and dysfunction in a setting of autophagy insufficiency

Qingyun Qin¹, Chen Qu¹, Ting Niu¹, Huimei Zang¹, Lei Qi¹, Linmao Lyu¹, Xuejun Wang², Mitzi Nagarkatti³, Prakash Nagarkatti³, Joseph S Janicki³, Xing Li Wang¹, Taixing Cui¹,⁴

Running Title: Nrf2, autophagy, and cardiac dysfunction

¹Shandong University Qilu Hospital Research Center for Cell Therapy, Key Laboratory of Cardiovascular Remodeling and Function Research, Qilu Hospital of Shandong University, Jinan, Shandong 250012, China
²Sanford School of Medicine, University of South Dakota, Vermillion, SD 57069, USA
³Department of Pathology, Microbiology and Immunology, ⁴Department of Cell Biology and Anatomy, University of South Carolina School of Medicine, Columbia, SC 29208, USA

ONLINE SUPPLEMENTARY MATERIALS

I. Full Description of Methods
II. Supplementary Tables, Figures and Legends
I. Full Description of Methods

Animals
All animals were kept on a 12-h light/dark cycle in a temperature-controlled room with ad libitum access to food and water. All animal were treated in compliance with the USA National Institute of Health Guideline for Care and Use of Laboratory Animals. The use of animals and all animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Shandong University, China, and the University of South Carolina, USA. The breeding pairs of Nrf2 heterozygote knockout (Nrf2+/−) and floxed Atg5 (Atg5fl/fl) mice in a C57BL/6J background were purchased from Riken BioResource Center, Japan. The breeding pairs of a transgenic mouse strain harboring a tamoxifen-inducible Cre-fusion protein (MerCreMer) under the control of the cardiomyocyte-specific α-myosin heavy-chain promoter (Myh6 or αMHC) in a C57BL/6J background were purchased from JAX. Littermates of wild type (WT) and Nrf2−/− mice were generated by breeding pairs of Nrf2+/− mice as previously described. Littermates of MerCreMer+, Atg5fl/fl, and MerCreMer+:Atg5fl/fl mice were obtained using the breeding pairs of MerCreMer+:Atg5fl/+ and Atg5fl/+ which were generated by crossing MerCreMer+ with Atg5fl/fl mice. Double Nrf2 and cardiomyocyte-restricted Atg5 knockout (Nrf2−/−:Atg5−/−) mice were generated by crossing Nrf2−/− mice with MerCreMer+:Atg5fl/fl mice. Genotyping information is provided in the inserted figure and in supplementary Table S1.

Induction and Quantification of Recombination
Tamoxifen (Cat#: T5648, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in warm sunflower seed oil at a concentration of 10 mg/ml and injected intraperitoneally (i.p.) at 20 mg/kg body weight daily for 3 wks to avoid the cardiac Cre toxicity as previously reported. The induction of recombination with tamoxifen was started in male mice at 6 wks of age. After a time period of 2 wks for washing out the tamoxifen, the efficacy of
recombination was determined by Western blot analysis of Atg5 expression in WT, 
MerCreMer\(^+\), MerCreMer\(^+\)::Atg5\(^{fl/+}\), and MerCreMer\(^+\)::Atg5\(^{fl/fl}\) mice which received the 
tamoxifen induction.

**Transverse Aortic Arch Constriction (TAC)**

Male littermates of WT and Nrf2\(^{-/-}\) mice at ages of 11 wks were subjected to sham or TAC 
operation for 4 and 8 wks. Male littermates of WT, MerCreMer\(^+\), Atg5\(^{fl/fl}\), and 
MerCreMer\(^+\)::Atg5\(^{fl/fl}\) mice which received tamoxifen induction as described above were 
subjected to sham or TAC operation at ages of 11-12 wks and sacrificed 4 wks later. The 
sham or TAC operation in mice was performed under deep anesthesia as previously 
described.  

Briefly, mice were anesthetized by i.p. injection of ketamine (80 mg/kg) 
and xylazine (5 mg/kg). The use of a horizontal incision at the level of the suprasternal 
notch allows direct visualization of the transverse aorta without entering the pleural 
space and thus obviates the need for mechanical ventilation. The transverse aorta was 
banded between the right innominate and left carotid arteries to a 27-gauge needle using 
a 6-0 nylon silk suture. Sham operation on mice were similar but without actual aortic 
banding and these mice served as a control group for all experimental groups. Cardiac 
hypertrophy was determined by heart weight–to–tibial length (HW/TIBIA) ratio, heart 
weight–to–body weight (HW/BW) ratio and expression levels of cardiac hypertrophy 
marker genes including atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), 
alpha-myosin heavy chain (\(\alpha\)-MHC), beta-myosin heavy chain (\(\beta\)-MHC), 
sarco-endoplasmic reticulum calcium ATPase2a (SERCA2a).

**Echocardiographic Analysis**

Echocardiography was performed on anesthetized mice, using the Vevo 2100 
High-Resolution Imaging System (VisualSonics Inc.) with a 30-MHz high-frequency 
linear transducer, as previously described. 

Briefly, mice were anesthetized with 3% 
isoflurane and maintained with 1.5% isoflurane in room air supplemented with 100% O\(_2\). 
After the anterior chest was shaved, the animals were placed on a warming pad to 
maintain normothermia. The echocardiographic gel was warmed before use to avoid 
hypothermia. Care was taken to avoid excessive pressure on the thorax, which can 
induce bradycardia. Two-dimensional (2D) long axis images of the left ventricle (LV) 
were obtained at the plane of the aortic and mitral valves where the LV cavity is largest 
and visualization of the LV apex is adequate, and a short-axis image was recorded at the 
level of the papillary muscles. A 2D guided M-mode echocardiogram was recorded 
through the anterior and posterior LV walls at 21 frames/s. Images were obtained at the 
level of the papillary muscle tips, and measurements were then performed to obtain the 
LV internal dimension (LVID; in mm), interventricular septum thickness (IVS), and LV 
posterior wall thickness (LVPW; in mm). LV percent fractional shortening FS (%) was 
calculated via VisualSonics Measurement Software.
Pathology
Mice were anesthetized and the hearts perfused via the abdominal aorta with a saline solution to remove the blood from the heart tissue. Then, the heart was dried on gauze, weighed, dissected, and frozen. Lungs and tibias were also dissected. Lungs were dried on gauze and weighed. The length of the tibia from the condyles to the tip of the medial malleolus was measured by micrometer calipers.

Evens Blue Labelling
Evans blue dye becomes intensely red fluorescent when conjugated to albumin in the circulation. The dye/albumin complexes are excluded from cells with intact plasma membranes while accumulating in damaged myofibers when the muscle cell membrane is broken, thus providing a dye-exclusion viability test. The red auto-fluorescence accumulated in myocardium has been used as a histopathological sign of cardiomyocyte necrosis. Briefly, mice were subjected to a single intraperitoneal injection of Evans blue (100 mg/kg, Solarbio science & Technology Co., Ltd., Beijing, China) 18 h prior to harvesting tissues. Harvested hearts were fixed in 4% paraformaldehyde and then embedded in paraffin. Paraffin sections were prepared (5 µm, Leica, rotary microtome) and stored at room temperature until staining. Myocardial cellular membranes were stained with Wheat Germ Agglutinin, Alexa Fluor® 488 Conjugate (Invitrogen Corp. Carlsbad, CA). Sections were observed using a fluorescence microscope (Nikon Eclipse 80i; Nikon Instruments Inc. Tokyo, Japan) at 200× magnification. Eight fields of each section were randomly photographed using NIS-Elements F 4.0 imaging software (Nikon Instruments Inc. Tokyo, Japan) and the percentage of Evans blue positive areas were measured using Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD, USA). At least two section of each heart were analyzed. Evans blue dye-positive area (red) indicates cardiomyocyte necrosis.

Histological and Immunochemical Analysis
Hearts were cannulated via the LV apex, cleared of blood by perfusion with normal saline at 90 mmHg, arrested in diastole with 60 mM KCl, fixed by perfusion with 4% paraformaldehyde, and embedded in paraffin. Paraffin sections were prepared (5 µm, Leica RM2235, rotary microtome) and stored at room temperature until staining. For LV cardiomyocyte cross-sectional area, coronal sections were deparaffinized and the cardiomyocyte membranes were stained with Alexa Fluor 488 conjugated wheat germ agglutinin (WGA) (Invitrogen Corp., Carlsbad, CA) and observed using a fluorescence microscope (Nikon Eclipse 80i; Nikon Instruments Inc. Tokyo, Japan) at 400× magnification. Twenty fields of each section were randomly photographed using NIS-Elements F 4.0 imaging software (Nikon Instruments Inc. Tokyo, Japan) and cross sectional areas of 1000-1400 circular cardiomyocytes per heart was measured using Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD, USA). For myocardial fibrosis, coronal sections were stained for collagen with a Masson's
Trichrome Kit (Poly Scientific, Bay Shore, NY, USA) according to the protocol provided by the manufacturer. Sections were observed under a light microscope (Nikon Eclipse 80i; Nikon Instruments Inc. Tokyo, Japan) at 200 × magnification. Twenty fields of each section were randomly photographed using NIS-Elements F 4.0 imaging software (Nikon Instruments Inc. Tokyo, Japan). The percentage of fibrosis (the blue stained area) was measured by Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD, USA). At least two sections of each heart were analyzed for the measurements of cardiomyocyte cross sectional areas and cardiac fibrosis. Apoptosis was measured by TUNEL assays on tissue sections using In Situ Cell Death Detection Kit, TMR red (Roche Applied Science, Indianapolis, IN, USA) according to the protocol provided by the manufacturer. Briefly, the sections were deparaffinized, rehydrated, microwaved in 0.01 M citrate buffer for 30 mins, and incubated in 0.3% triton X-100 in PBS for 30 mins. They were then incubated with 50 μl of TUNEL Reaction Mixture at 37ºC for 1 h and mounted with Prolong Gold Antifade Reagent and DAPI (Invitrogen Corp., Carlsbad, CA). A positive control section was digested with DNase (RNase-Free DNase Set, QIAGEN Inc., Valencia CA) for 30 min and a negative control section was only incubated with labeling solution (without enzyme solution). The apoptotic nuclei were labeled with TUNEL (red) all nuclei were counterstained with DAPI (blue). TUNEL-positive cells were quantified in all nuclei of a LV section. At least two sections from each heart were analyzed.

Myocardial oxidative stress was assessed by staining of 8-hydroxydeoxyguanosine (8-OHdG), a marker of DNA oxidation, with a mouse anti 8-OHdG antibody (Cat#: sc-660369, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA). Numbers of 8-OHdG positive nuclei were counted in 8 randomly chosen fields of each tissue section. Two sections from each heart were analyzed. Sub-cellular locations of p-Erk, p-Fyn, and Nrf2 were analyzed by immunochemical staining using anti-p-Erk (cat#: 9101, Cell Signaling Technology, Inc., Danvers, Massachusetts, USA), anti-p-Fyn (cat#: sc-16848, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), and anti-Nrf2 (cat#: sc-722, 1:200, Santa Cruz Biotechnology Inc., Dallas, Texas, USA). Two sections from each heart were analyzed.

Cell Cultures, Adenoviral Infection, Oligo and Plasmid Transfection
Rat neonatal cardiac myocytes were isolated and cultured as previously described. Adenovirus of control scramble shRNA (Ad-shCtr) and rat Nrf2 shRNA (Ad-shNrf2) were generated as previously reported. Rat neonatal cardiomyocytes were infected with Ad-shCtr (20 MOI) and Ad-shNrf2 (20 MOI) in serum-free DMEM for 6 h and the cultured with full growth medium (1 g/L glucose DMEM supplemented with 8% horse serum (HS) and 5% newborn calf serum (NCS) for additional 24 h. The infected cardiomyocytes were further transfected with scramble siRNA (siCtr, sense, 5’-UCUCCGAACGUCAACUGUGA -3’; antisense, 5’- AAGAGGCUCAGCAUCAGUGGCA -3’, purchased from Invitrogen Corp., Carlsbad, CA), Agt5 siRNA (siAtg5, sense 5’-
GCGGATTCCAACGTGCTTTA -3'; antisense, 5'- TAAAGCACGTTGGAATCCGC -3' purchased from Ribobio, Guangzhou, China using Lipofectamine 2000 (Cat#: 12566-014, Thermo Fisher Scientific, Waltham, USA) for 6 h and then cultured with full growth medium aforementioned for 24 h. The transfection was repeated once and then the cells were serum starved for 48 h prior to the treatment with Ang II (1 µM, Cat#: RAB0010, Sigma-Aldrich, St. Louis, MO, USA) and PP2 (1 µM, Fyn kinase inhibitor, Cat#: P0042, Sigma-Aldrich, St. Louis, MO, USA) in serum free DMEM. Based on our pilot study that Ang II stimulation induced phosphorylation of Jak2, Stat3, and Fyn, and upregulation of LC3-II at 24 h while enhancing protein expression of Agt at 48 h in rat neonatal cardiomyocytes (data not shown), we treated the cardiomyocytes with Ang II for 24 h prior to Western blot analysis of Jak2, Stat3, and Fyn, and for 48 h prior to Western blot analysis of Agt protein expression. For qPCR analysis of the effect of PP2 (1 µM) and AG490 (a Jak2 inhibitor, 1 µM, Cat#: T3434, Sigma-Aldrich, St. Louis, MO, USA) on Ang II-induced mRNA expression of NQO1 and Agt, we treated serum starved cardiomyocytes in serum free DMEM as indicated for 48 h. These experiments were repeated for 4 times.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Quantitative Real Time (qPCR)
The total RNA from the LV was extracted using RNeasy Fibrous Tissue Mini kit (Qiagen Inc., Valencia, CA), and the reverse transcription reaction was performed with 1 µg of total RNA using a RevertAid™ First Strand cDNA Synthesis Kit (Cat#: K1622, Thermo Scientific). qPCR was carried out using the Bio-Red CFX96™ Real-Time system (C1000™ Thermal Cycler, Bio-Red Laboratories, Inc. Hercules, CA, US). Expression levels of target genes were normalized by concurrent measurement of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. Primers that were used for qPCR are summarized in supplementary Table S1. Genomic DNAs were also extracted from mouse tails and subjected to PCR for genotyping of transgene mice.

Autophagy Flux Assay
A cohort of male WT mice (C57BL/6J) at ages of 9-10 wks were subjected to sham or TAC operations. It has been reported that intraperitoneal (i.p.) injection of chloroquine (CQ, 10 mg/kg), or rapamycin (2 mg/kg), or rapamycin plus chloroquine for 4 h induce accumulation of mCheery-LC3 in the heart of transgenic mice expressing mCherry-LC3. These results suggest that the differences between the basal and CQ-induced or the rapamycin-induced and rapamycin plus CQ-induced LC3 protein expression could be quantified as autophagic flux, a more accurate parameter reflecting autophagic activity. We performed preliminary experiments to verify the efficacy of these treatments on LC3 expression in the murine heart. We found that the myocardial expression level of LC3 was hardly affected by 4 h of i.p. injection of (CQ, 10 mg/kg) or rapamycin (2 mg/kg), respectively (data not shown). However, i.p. injection of rapamycin (2 mg/kg) plus CQ
(10 mg/kg) for 4 h induced a clear increase in LC3 protein expression in the heart. Hence, the mice before (0 wk) and at 1, 2, 4, 8 wks after operation were i.p. injected with rapamycin (2 mg/kg) with vehicle or chloroquine (CQ, 10 mg/kg) for 4 h prior to the autophagy flux assay. LV lysates were subjected to Western blot analysis of LC3-I and LC3-II protein levels. Autophagic flux is defined as the amount of CQ-induced accumulation of LC3-II which is calculated by a formula of (rapamycin + CQ)-induced LC3-II density – (rapamycin-induced) LC3-II density.

Western Blot Analysis
LV tissues were lysed in a homogenization buffer (RIPA) contained 150 mM NaCl, 1% NP-40, 50 mM Tris (pH 8.0), 0.5% sodium deoxycholate (C_{24}H_{39}NaO_{4}), 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate (Na_{3}VO_{4}), and proteinase inhibitor cocktail (Roche, Basel, Switzerland). Immunoblotting was conducted as described elsewhere. Antibodies used included anti-APG5L/ATG5 monoclonal antibody (Cat#: 3447-1, Abcam, Cambridge, UK), anti-LC3B polyclonal antibody (Cat#: L7543, Sigma-Aldrich, St. Louis, MO, USA), anti-phospho-Jak2 (Cat#: 3776, Cell Signaling Technology, Inc., Danvers, Massachusetts, USA), anti-Jak2 (Cat#: 3230, Cell Signaling Technology, Inc., Danvers, Massachusetts, USA), anti-phospho-Stat3 (Cat#: 9145, Cell Signaling Technology, Inc., Danvers, Massachusetts, USA), anti-Stat3 (Cat#: 4904, Cell Signaling Technology, Inc., Danvers, Massachusetts, USA), anti-phospho-Fyn (cat#: sc-16848, Santa Cruz Biotechnology Inc., Dallas, Texas, USA), anti-Fyn (cat#: 4023, Cell Signaling Technology, Inc., Danvers, Massachusetts, USA), anti-angiotensinogen (Cat#: sc-7419, Santa Cruz Biotechnology Inc., Dallas, Texas, USA), anti-p62 (Cat#: ab91526, Abcam, Cambridge, UK), anti-NQO1 monoclonal antibody (Cat#: sc-376023, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), anti-Nrf2 polyclonal antibody (Cat#: sc-722, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), anti-GAPDH polyclonal antibody (Cat#: G9545, Sigma-Aldrich, St. Louis, MO, USA), peroxidase-conjugated AffiniPure goat anti-Mouse IgG (H+L) (Cat#: ZB2305, ZSGB-BIO, Beijing, China) and peroxidase-conjugated AffiniPure rabbit anti-goat IgG (H+L) (Cat#: ZB2306, ZSGB-BIO, Beijing, China).

Statistics
Data are shown as mean ± SEM. Differences between 2 groups were evaluated for statistical significance using the Student t test when the sample size was appropriate and the population was distributed normally. When differences among > 3 groups were evaluated, results were compared by one-way ANOVA with Bonferroni test for multiple comparisons. Survival rate between experimental groups after TAC was analyzed using Kaplan Meier test. Differences were considered significant at p < 0.05.

References


<table>
<thead>
<tr>
<th>Primers</th>
<th>Gene access #</th>
<th>Forward (5’—3’ )</th>
<th>Reverse(5’—3’ )</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>qPCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANF</td>
<td>NM_008725.2</td>
<td>CATCACCTGGGCTTCTTCCT</td>
<td>TGGGCTCCAATCCTGTCAATC</td>
<td>405</td>
</tr>
<tr>
<td>BNP</td>
<td>NM_008726.4</td>
<td>GCGGCATGGATCTCTGAAGGGCTCCAATCCT</td>
<td>CCCAGGCAGAGTCAGAAACTG</td>
<td>418</td>
</tr>
<tr>
<td>α-MHC</td>
<td>NM_010856.3</td>
<td>CCAATGAGTACCGCTGAAGGCTCCAATCCT</td>
<td>ACAGTCATGGCCGGAGATGAT</td>
<td>254</td>
</tr>
<tr>
<td>β-MHC</td>
<td>NM_080728.2</td>
<td>ATGTGCCGGACCTTTGGAA</td>
<td>CCTCGGGTTAGCTGAGAGATCA</td>
<td>170</td>
</tr>
<tr>
<td>SERCA2a</td>
<td>NM_009722.3</td>
<td>CCATCTGGCTTGCTCACTGCCTCAACACA</td>
<td>CAAATGGTTTAGAGAAGTTG</td>
<td>213</td>
</tr>
<tr>
<td>Nrf2</td>
<td>NM_010902.3</td>
<td>ATGATGGACCTTGGAGTTGCC</td>
<td>TCCTGGTTCCTTCTGAGTTG</td>
<td>200</td>
</tr>
<tr>
<td>NQO-1</td>
<td>NM_008706.5</td>
<td>CGGTATTACGATCCTCCTCAACACA</td>
<td>AGCCTCTACAGGACCCGTTCCTCAT</td>
<td>120</td>
</tr>
<tr>
<td>Agt</td>
<td>NM_007428.3</td>
<td>TTGTCTAGGTTGGAGCTGTTGA</td>
<td>GGGTGGATGTATACGCCGTC</td>
<td>143</td>
</tr>
<tr>
<td>GAPDH</td>
<td>XM_001479322</td>
<td>ATGTTCCAGTATGACTCCACTCACG</td>
<td>GAAGACACCAGTAACTCCACGACA</td>
<td>171</td>
</tr>
<tr>
<td>Genotyping</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cre</td>
<td></td>
<td>AGGTGGACCTGATCTAGGGAG</td>
<td>ATACCCGAGATCATGCAAGC</td>
<td>440</td>
</tr>
<tr>
<td>Myh6- MerCreMer+</td>
<td>NW_001030719.1</td>
<td>TAGGTTGGAAATTCGTAGCATCATCC</td>
<td>TTAGGCCACAGATCTGAAAGATCT</td>
<td>324</td>
</tr>
<tr>
<td>floxedAtg5 (Atg5&lt;sup&gt;fl/fl&lt;/sup&gt;)</td>
<td>flox</td>
<td>ACAACGTCGAGCAGCAGCT</td>
<td>GTACTGCATAATGGTTTAACTTGCT</td>
<td>700</td>
</tr>
<tr>
<td>Nrf2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>NT_039492.8 (WT)</td>
<td>GAATATGAAGGCACCCCTGAAATGG</td>
<td>ATACCCGAGATCATGCAAGC</td>
<td>440</td>
</tr>
<tr>
<td></td>
<td>LacZ(KO)</td>
<td>GGGATTGACTATTGGAAGGCTG</td>
<td>GGGATGGATAGGTTTAACTTGCT</td>
<td>700</td>
</tr>
<tr>
<td></td>
<td>NM-010902.3</td>
<td>TGGACGGGACTAATGGAGCT</td>
<td>GCCGCTTTTCTCGAGATGG</td>
<td>734</td>
</tr>
</tbody>
</table>

Int CTL: internal control (interleukin-2); ANF, atrial natriuretic factor; BNP, B-type natriuretic peptide; α-MHC, alpha-myosin heavy chain; β-MHC, beta-myosin heavy chain;
SERCA2a, sarcoplasmic reticulum calcium ATPase; NQO-1, NAD(P)H dehydrogenase, quinone-1; Agt, angiotensinogen.

**Table S2. Echocardiography and pathology of WT mice treated with or without tamoxifen at 4 weeks after TAC.**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Tamoxifen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>TAC</td>
</tr>
<tr>
<td>Echocardiography (n)</td>
<td>(10)</td>
<td>(9)</td>
</tr>
<tr>
<td>IVS;d (mm)</td>
<td>0.75±0.04</td>
<td>1.03±0.06&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>LVID;d (mm)</td>
<td>4.40±0.24</td>
<td>4.42±0.22</td>
</tr>
<tr>
<td>LVPW;d (mm)</td>
<td>0.66±0.03</td>
<td>1.04±0.07&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>EF%</td>
<td>69.8±5.56</td>
<td>33.86±3.92&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>FS%</td>
<td>34.65±2.29</td>
<td>17.35±2.41&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pathology (n)</td>
<td>(10)</td>
<td>(9)</td>
</tr>
<tr>
<td>BW (g)</td>
<td>28.47±1.48</td>
<td>28.26±2.59</td>
</tr>
<tr>
<td>HW (mg)</td>
<td>142.2±8.33</td>
<td>273.8±33.35&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>LW (mg)</td>
<td>178.9±19.65</td>
<td>400±77.09&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>TIBIA (mm)</td>
<td>17.81±0.28</td>
<td>17.93±0.31</td>
</tr>
<tr>
<td>HW/TIBIA (mg/mm)</td>
<td>8±0.41</td>
<td>15.24±1.86&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>5.01±0.18</td>
<td>10.14±1.70&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>LW/TIBIA (mg/mm)</td>
<td>10.06±1.80</td>
<td>22.27±4.32&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Male WT C57BL/6J mice at 6 wks of age were injected i.p. with tamoxifen (20 mg/kg/d) for 21 days, followed with 2 wks for washing out tamoxifen, and then subjected to sham or TAC operation for 4 wks. LVPW;d, left ventricular posterior wall diastolic; FS, fractional shortening; EF, ejection fraction. BW, body weight; HW, heart weight; LW, lung weight; TIBIA, tibia length; HW/TIBIA, heart weight/tibia length ratio; HW/BW, heart
weight/body weight ratio; LW/TIBIA, lung weight/tibia length ratio. \(^A\) or \(^B\), \(p<0.05\), TAC vs. sham.
Figure S1. Role of Nrf2 in TAC-induced myocardial necrosis. Male WT mice at 9 wks of age were subjected to sham or TAC operation. Mice were treated with a single i.p. injection of Evans blue dye (EBD, 100 mg/kg) 18 h prior to harvesting hearts as indicated. Green indicates the cardiomyocyte membrane (stained with WGA). Red indicates the necrotic myocardium. n=4, *p<0.05 vs. the control (0 time).
Figure S2. The effect of Nrf2 knockout on TAC-induced cardiac hypertrophy. Male littermates of WT and Nrf2⁻/⁻ mice at 9 wks of age were subjected to sham or TAC operation for 4 wks and then cardiomyocyte cross-sectional areas (MC CSA) of the WT and Nrf2⁻/⁻ mice were quantified. Upper panel: representative WGA staining. Lower panel: quantified MC CSA. *p<0.05 vs. Sham in the same group.
Figure S3. The effect of Nrf2 knockout on TAC-induced expression of fetal genes and SERCA2a in the heart. Male littermates of WT and Nrf2−/− mice at 9 wks of age were subjected to sham or TAC operation for 4 wks and then myocardial expression of fetal genes and SERCA2a was analyzed by qPCR. *p<0.05 vs. Sham in the same group.
Figure S4. The effect of Nrf2 knockout on TAC-induced myocardial fibrosis, apoptosis, and oxidative stress. Male littermates of WT and Nrf2−/− mice at 9 wks of age were subjected to sham or TAC operation for 4 wks and then myocardial fibrosis, apoptosis, and oxidative stress was analyzed as described in “Method”. *p<0.05 vs. WT. #p<0.05 Sham in the same group.
Figure S5. The effect of tamoxifen on TAC-induced death in mice. Male WT C57BL/6J mice at 6 wks of age were injected i.p. with tamoxifen (20 mg/kg/d) for 21 days, followed by a 2 wk period for washing out tamoxifen, and then subjected to sham or TAC operation for 4 wks. The number of dead mice was counted daily.