*Original Research*

# The reduced autophagic response by oxidative stress in angiotensin II-induced hypertrophic H9C2 cells causes more apoptotic cell death

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## Abstract

Autophagy is an important process in the pathogenesis of cardiovascular diseases, and angiotensin II (Ang II) plays a causative role in the induction of cardiomyocyte autophagy. The purpose of this study was to explore whether, under conditions of oxidative stress, levels and types of cell death were different in untreated and Ang II-treated cardiomyocytes (H9C2 cells). Treatment with 20 μM Ang II induced cardiac hypertrophy in H9C2 cells, with increased expression of the hypertrophic markers c-Fos, ß-myosin heavy chain, atrial natriuretic factor (ANF), and brain natriuretic factor (BNF). Under normal conditions, there was no difference in the levels of autophagic vacuoles and apoptotic bodies in untreated and Ang II-treated H9C2 cells. However, oxidative stress generated by 100 μM $H_2O_2$ triggered autophagy in untreated control cells, but had a reduced effect in Ang II-induced hypertrophic cells, resulting in more cell death, and this was associated with a decrease in connexin 43 expression. Blocking this autophagic response with 3-methyladenine resulted in a significant increase in cell death and apoptosis of H9C2 cells but did not significantly affect the response of Ang II-treated cells. The autophagic response to 100 μM $H_2O_2$ provides a survival advantage for cells and this is reduced by Ang II treatment.

**Keywords:** Angiotensin, hypertrophy, autophagy, apoptosis, oxidative stress

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## Introduction

Cardiac hypertrophy is the dominant cellular response to the pathological stresses such as hemodynamic overload and myocardial injury.[1] Prolong pathological cardiac hypertrophy increases cardiomyocyte loss (apoptosis, autophagy, and necrosis), induces cardiomyopathy, depresses cardiac function, and eventually causes heart failure.[1] Multiple molecular mechanisms underlying cardiac hypertrophy transition into heart failure have been identified, including several signaling pathways (such as tumor necrosis factor-α, calcineurin, redox), epigenetic modification, and fetal gene program.[2–5] However, the precise mechanism causing cardiac dysfunction remains unclear.

Autophagy is an intracellular degradation process that involved the destruction of long-lived proteins and organelles to provide a survival advantage for cells undergoing nutrient deprivation or other stress. In addition, more recently, it has itself been linked to the death process. Thus, apoptosis is not the sole means by which a cell can undergo a genetically programmed regulated process leading to self-elimination; cell death can occur by several mechanisms and the phenotypic changes that accompany cell death can vary depending on the stimulus and cell setting.[6]

Recently, autophagy has been recognized as important in the turnover of cytoplasmic constituents in the heart. Autophagy activity is commonly increased in the heart under conditions that involve upregulation of the renin-angiotensin system.[7] High levels of autophagy are seen in the heart after acute and chronic ischemia, heart failure, and neonatal starvation.[8] There is evidence that pressure overload, a major risk factor for cardiac hypertrophy and heart failure, is associated with a change in autophagy.[9–11]

Angiotensin II (Ang II) is an octapeptide that exerts inotropic, hypertrophic, and apoptotic effects on cardiomyocyte.[12] Thus, Ang II is central to any process involved in controlling hypertrophy and heart failure. In addition to its own effects, Ang II participates in tissue-specific networks and cross-talk that control cardiac growth, cell function, and cell death in a more complex way.[13]

Many cellular stresses, such as endoplasmic reticulum stress or mitochondrial dysfunction, can induce autophagy.[14] Oxidative stress has been shown to induce autophagy under conditions of starvation or ischemia/reperfusion.[15,16] Under oxidative stress conditions, reactive oxygen species (ROS), including free radicals, such as superoxide ($O_2^{\cdot -}$), hydroxyl radical ($HO^{\cdot}$), and hydrogen peroxide ($H_2O_2$), are generated at high levels, inducing cellular damage and cell death.[17,18] This cell death often involves induction of apoptosis through caspase activation. Blockade of caspase activation causes degradation of catalase and increased ROS generation, leading to cell death. This degradation of catalase is mediated by autophagy, indicating a role for autophagy in caspase-independent cell death.[19] Under starvation conditions, ROS production is also increased and induces autophagy.[16]

Because oxidative stress is a key mechanism contributing to the development of various heart diseases, to elucidate the distinct response of hypertrophic cariomyocytes from normal cardiomyocytes when exposing to oxidative stress might clarify the mechanism of cardiac hypertrophy transition into heart failure. The purpose of this study was to explore whether levels and types of cell death were different in normal and Ang II-induced hypertrophic cardiomyocytes under conditions of oxidative stress generated by 100 μM $H_2O_2$. In addition, we investigated whether crosstalk between autophagy and apoptosis controlled cardiomyocyte death.

## Materials and methods

### Cell culture and treatment

H9C2, a myoblastic cell line derived from embryonic BD1X rat heart, was obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Hyclone, Auckland, *NZ*), 2 mM L-glutamine, 0.1 mM nonessential amino acids (Gibco BRL), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (Gibco BRL) at 37°C in a humidified chamber with 5% $CO_2$. After the subcultured cells had been incubated for 24 h and had become attached to the plate, they were left untreated or were treated with 1 or 20 μM Ang II (Sigma-Aldrich, St. Louis, MO, USA) for 24 h and then with 100 μM $H_2O_2$ in the continued presence of Ang II for 24 h. To regulate autophagy, 3-methyladenine (3-MA, 5 mM), bafilomycin A1 (BafA, 50 nM), or rapamycin (0.5 μM) was added for 2 h before the addition of 100 μM $H_2O_2$ and incubation for 24 h. After treatment, the cells were harvested for analysis.

### Cell size and viability

The cells were trypsinized, suspended in DMEM containing 20% FBS, and centrifuged at 800 *g* for 15 min at 4°C and then were resuspended in DMEM, smeared on a slide, observed on an inverted microscope at ×400 magnification, and photographed. Cell size was measured using Image Pro Plus software (version 4.5; MediaCybernetics, Silver Spring, MD, USA), a minimum of 100 cells per sample being evaluated in each case. For the viability assay, the cells were incubated for 2 h with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich), and then the absorbance of the sample at 450 nm was measured using a microplater reader. Experiments were repeated three times.

### Autophagy determination

Autophagy was measured by autophagosome and acidic vesicular organelles (AVOs). Autophagosomes were quantitated by saponin extraction FACS assay according to Eng *et al.*[20] LC3-I, nonautophagososme-associated form, is soluble, while the lapidated autophagosome-associated LC3II is membrane-bound and insoluble. Saponin extraction followed by intracellular staining for endogenous LC3 allows the formation of autophagosome to be quantified.[20] AVOs, both autophagosomes and autolysosomes,[21] were quantified by flow cytometry using acridine orange (AO) staining.[22] AO is a fluorescent weak base that accumulates in acidic spaces and emits bright red fluorescence, the intensity of which is proportional to the degree of acidity, allowing the formation of AVOs to be quantified. After treatment, the cell pellet was collected and the cells resuspended in phosphate-buffered saline (PBS), stained with 100 μg/ml of AO for 20 min at 25°C, washed twice with PBS, and resuspended in PBS for analysis on a FACS Calibur flow cytometer (Becton-Dickinson, Mountain View, CA).

### Early apoptosis and cell death determination

Double fluorescence staining with a PE-Annexin V/7-AAD kit (BD Bioscience, San Jose, CA, USA), followed by flow cytometry analysis, was used to measure early apoptosis and cell death of H9C2 cells, as reported by Wang *et al.*[23] Annexin V is useful for identifying apoptotic cells with exposed phosphatidylserine, while 7-aminoactinomycin (7-AAD) is a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells. Cells that stain positive for PE-Annexin V and negative for 7-AAD are undergoing apoptosis, those that stain positive for both PE-Annexin V and 7-AAD are in the end stage of apoptosis, or undergoing necrosis, or are already dead, while those that stain negative for both are alive and not undergoing measureable apoptosis.

After treatment, the cells were incubated with PE-Annexin V and 7-AAD reagents at 37°C for 2 h according to the manufacturer's protocol and analyzed on a FACS Calibur flow cytometer (Becton-Dickinson).

### Determination of apoptosis

Apoptosis was analyzed by counting cells in the sub-G1 phase of the cell cycle, indicating DNA fragmentation, and by the terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay, indicating DNA breaks. Cells in the sub-G1 phase were counted by fixing the cells with ethanol, staining them with propidium iodide (Merck Schuchardt OHG, Hohenbrunn, Germany), and analyzing them on a FACS Calibur flow cytometer. The TUNEL assay was performed using an APO-BrdU™

TUNEL Assay Kit (Molecular Probes, Eugene, OR) according to the manufacturer's protocol and a FACS Calibur flow cytometer.

The flow cytometry data were analyzed using WINMDI software version 2.8 (Scripps Research Institute, La Jolla, CA, USA), a minimum of $1 \times 10^4$ cells per sample being evaluated in each case.

### Preparation of total RNA and reverse transcription

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA). RNA samples (2 μg) were reverse transcribed using random hexamer primers and M-MLV reverse transcriptase (Promega Corporation) and the cDNA used for the polymerase chain reaction (PCR).

### PCR analysis

PCR was performed using a multiplex PCR kit (MBI, So. San Francisco, USA), and cDNA amplification was performed to detect the expression of the hypertrophic markers c-Fos, ß-myosin heavy chain (ß-MHC), atrial natriuretic factor (ANF), and brain natriuretic factor (BNF) according to the manufacturer's procedure. The respective 5′ and 3′ primers used were as follows: for c-Fos, 5′-GAG CGGC AGA GCA TCG GCA G-3′ and 5′-AGG CTC CCA GTC TGC TGC ATA G-3′; for ß-MHC, 5′-CGC ACC CTC ACT TTG TAC GC-3′ and 5′-GCT TCA TCC ACG GCC AAT TC-3′; for ANF, 5′-ATG GGC TCC TTC TCC ATC AC-3′ and 5′-TGT TGC AGC CTA GTC CGC-3′; for BNF, 5′-CAG AAG CTG CTG GAG CTG ATA AG-3′ and 5′-TGT AGG GCC TTG GTC CTT TG-3′, and for GAPDH, 5′-GCA CCA CCA ACT GCT TAG C-3′ and 5′-TGA GTG GCA GTG ATG GCA T-3′. The amplified PCR products were separated by gel electrophoresis in 2% agarose and then stained with ethidium bromide.

### Western blotting

The cells were harvested and total cell lysates prepared using lysis buffer (20 mM Tris-HCl, pH 7.2, 2 mM EGTA, 5 mM EDTA, 500 μM sodium orthovanadate, 10 mM sodium fluoride, 1% Triton X-100, 0.1% SDS, and protease inhibitor cocktail). Protein concentrations were determined using Bio-Rad protein assay reagents (Bio-Rad, Hercules CA, USA). Forty to sixty micrograms of protein lysate was analyzed by SDS-polyacrylamide gel electrophoresis. After transfer of the proteins from the gel to a nitrocellulose membrane (Amersham Pharmacia Biotech, Freiburg, Germany), the membranes were blocked for 1 h at room temperature in PBS containing 0.05% Tween 20 (PBS-T) and 5% nonfat dry milk and then were incubated with monoclonal antibodies against Bcl-2 or mTOR-Pi (Cell Signaling Technology Inc., Hertfordshire, UK) or polyclonal antibodies against Beclin, LC3, p62, Bnip3, or ß-actin (Santa Cruz Biotechnology Inc., MA USA), followed by horseradish peroxidase-conjugated secondary antibodies (PharMingen, San Diego CA, USA). Immunoreactive bands were visualized using an enhanced chemiluminescence kit (Perkin-Elmer Life Sciences, Boston, MA, USA) and analyzed by QUANTITY ONE (Bio-Rad). β-actin was used as the internal control.

### Statistical analysis

All data are expressed as the mean ± SD. The significance of differences was determined by one-way ANOVA followed by Fisher's test. Statistical analyses were performed using SAS (version 6.011; SAS Institute Inc, Cary, NC. USA). A $p$ value $< 0.05$ was considered statistically significant.

## Results

### Ang II induces hypertrophy of H9C2 cells

To examine whether Ang II induced H9C2 cell hypertrophy, the cells were treated with 1 or 20 μM Ang II, and then levels of mRNAs for hypertrophic markers were measured by RT-PCR every 6 h for 48 h, while cell size was measured at 24 and 48 h. Treatment with 1 μM Ang II did not affect H9C2 cell viability (Figure 1(a)) and the cells did not become hypertrophic even after 48 h of treatment. In contrast, using 20 μM Ang II, significant increases were seen in c-Fos expression after 6 h of treatment, in ß-MHC expression at 12 h, and in ANP and BNP expression at 18 h compared to the untreated control (data not shown) and maximal levels were seen at 24 h (Figure 1(b)). Furthermore, cell size showed a significant increase after 24 or 48 h treatment with 20 μM Ang I, with no significant difference between the two times (Figure 1(c)). These results show that 20 μM Ang II treatment causes H9C2 cells to become hypertrophic.

### Hypertrophic H9C2 cells reduce oxidative stress-induced autophagy

Several studies have reported that cardiac hypertrophy is associated with a change in autophagy.[10,11] To examine whether autophagy was changed by Ang II treatment, H9C2 cells were treated with 1 or 20 μM Ang II for 24 h and then were incubated for 24 h in serum-free medium for starvation stress or with 100 μM $H_2O_2$ for oxidative stress in the continued presence of Ang II. After treatment, autophagy was determined by estimating the number of autophagosome and acidic vesicle organelles (AVOs) by flow cytometer. Figure 2(a) shows that Ang II had no effect on autophagosome formation in control cells, while, under conditions of $H_2O_2$ stress, levels of autophagosome were reduced by Ang II treatment. Figure 2(b) shows that Ang II had no significant effect on levels of AVOs in control cells, while, under conditions of starvation or $H_2O_2$ stress, levels of AVOs were reduced, but not significantly, by 1 μM Ang II treatment and were significantly reduced by 20 μM Ang II treatment. A lysosomal inhibitor, BafA, was used to clarify whether Ang II-induced hypertrophic H9C2 cells undergoing oxidative stress decreased the autophagic flux. BafA treatment increased the autophagosome formation in control and Ang II-treated cells. With BafA treatment, Ang II-treated cells under $H_2O_2$ stress had a lower level of autophagosomes than the hypertrophic control (Figure 2(a)). In addition, BafA suppressed the level of AVOs in control cells, and hypertrophic cells with/without $H_2O_2$ stress (Figure 2(b)). These results give evidence that

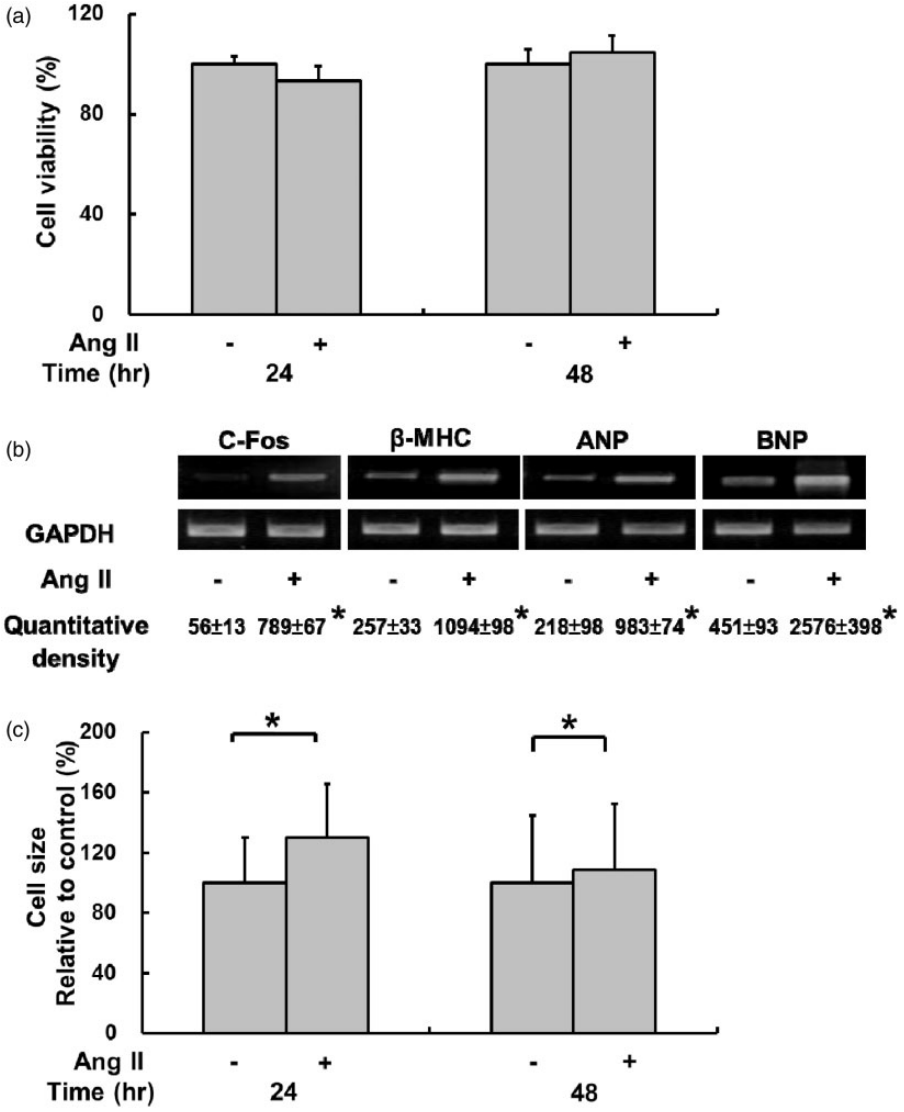


**Figure 1** Ang II induces hypertrophy of H9C2 cells. (a) Cell viability was measured after 20 μM Ang II treatment for 24 or 48 h. H9C2 cells were left untreated or were treated with 20 μM Ang II for 24 h and then (b) levels of mRNAs for the hypertrophic markers c-Fos, ß-MHC, ANP, and BNP were measured by RT-PCR, with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the internal control. (c) Cell size was measured after 20 μM Ang II treatment for 24 or 48 h. The results are expressed relative to the control value and are the mean ± SD for three independent experiments, with a minimum of 100 cells per sample being evaluated in each case. *$p < 0.05$ comparing the indicated groups

Ang II treatment reduced autophagy under conditions of starvation or oxidative stress.

### Ang II-induced hypertrophic H9C2 cells undergoing oxidative stress show increased apoptotic cell death

In order to investigate the status of cell death and apoptosis under conditions of oxidative stress caused by 100 μM $H_2O_2$, double fluorescence staining with Annexin V-PE/7-AAD was used to detect cell death and early apoptosis. Figure 3(a) shows that, after 24 h pretreatment with 1 or 20 μM Ang II, followed by treatment or no treatment with 100 μM $H_2O_2$ for 24 h, both cell death (all 7-AAD-positive cells) and early apoptosis (7-AAD-negative and PE Annexin V-positive cells) were induced by 100 μM $H_2O_2$ alone (compare bottom and top panels) and this effect was increased in the 1 and 20 μM Ang II-pretreated cells (bottom panels). Ang II had no effect on the results in the absence of $H_2O_2$ (top panels). Using sub-G1 phase measurements (Figure 3(b)) and TUNEL staining (Figure 3(c)) to detect apoptosis, similar results were obtained showing that apoptosis was significantly increased in 1 or 20 μM Ang II-pretreated cells stressed by 100 μM $H_2O_2$.

### Expression of autophagy- and apoptosis-related proteins

To determine whether the expression of autophagy- and apoptosis-related proteins was altered in line with the Ang II-induced changes in autophagy and apoptosis, H9C2 cells were treated with 1 or 20 μM Ang II for 24 h and then with or without 100 μM $H_2O_2$ for a further 24 h and total proteins were extracted for Western blotting. Figure 4 shows that Ang II treatment or $H_2O_2$ stress alone

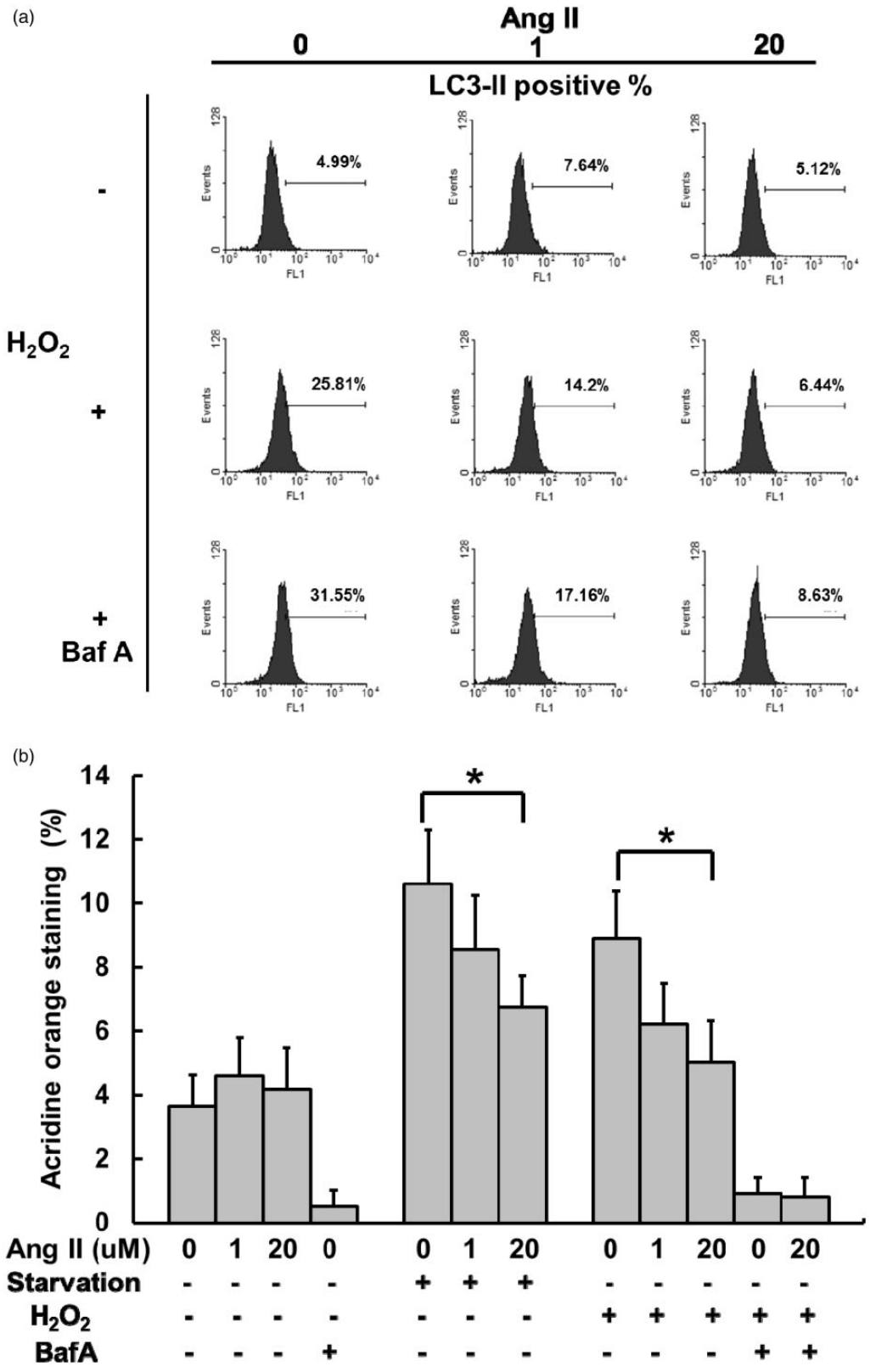


**Figure 2** Lower autophagy in Ang II-induced hypertrophic cells under conditions of oxidative stress. H9C2 cells were left untreated or were treated with 1 or 20 μM Ang II for 24 h and then were incubated in the continued presence of Ang II without further treatment, were starved by serum deprivation, or were treated with 100 μM $H_2O_2$ for a further 24 h; then autophagy was estimated by (a) autophagosome formation (LC3 II-positive) or (b) acridine orange staining of acidic vesicle organelles and flow cytometry analysis. The data are the mean ± SD for three independent experiments. *$p < 0.05$ comparing the indicated groups

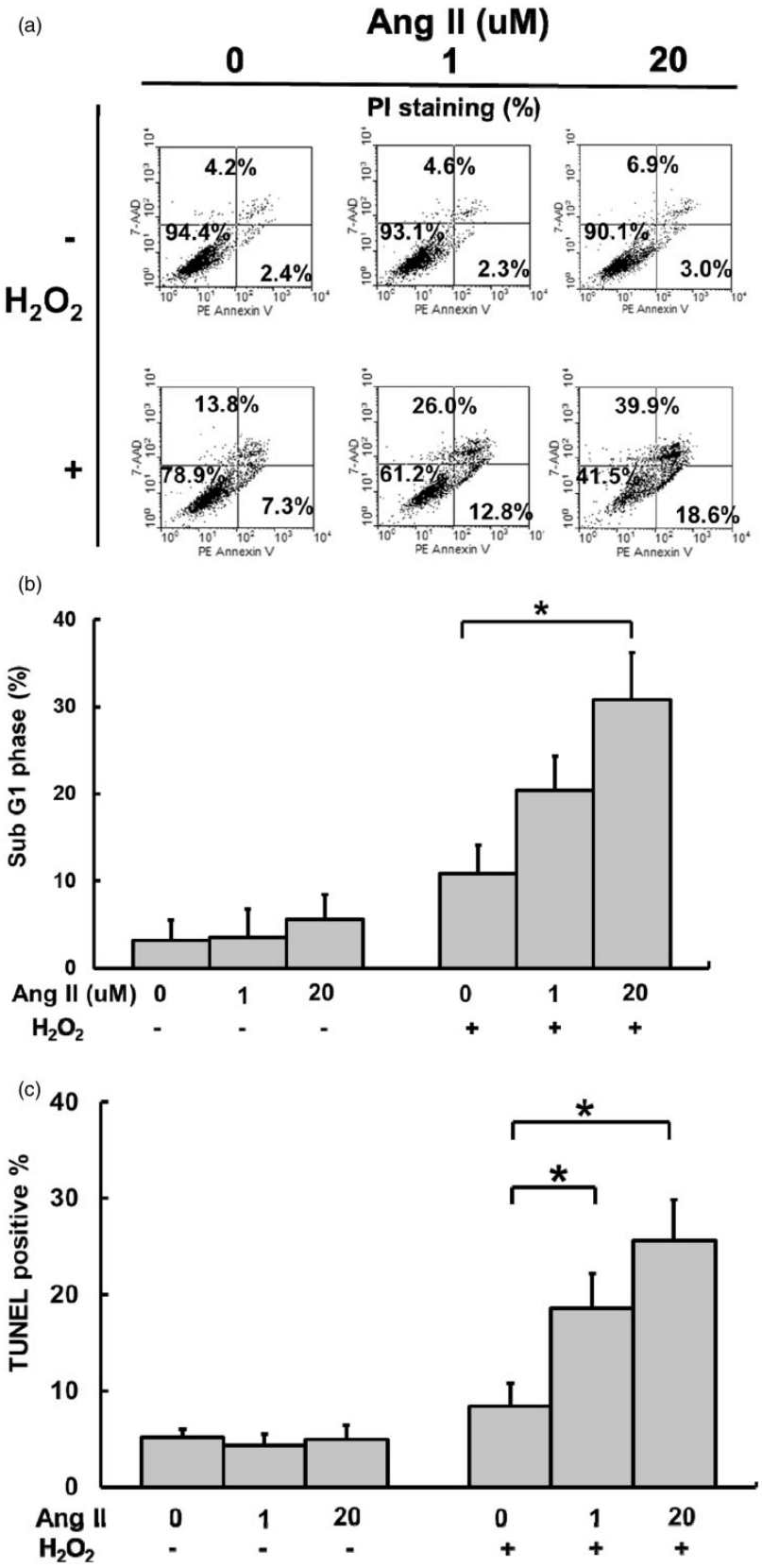


**Figure 3** $H_2O_2$ induces more cell death and apoptosis in Ang II-treated H9C2 cells than in untreated controls. H9C2 cells were treated as in Figure 2 and then (a) cell death and early apoptosis were measured by double fluorescence staining with Annexin V-PE/7-AAD or (b and c) apoptosis was measured by (b) counting sub-G1 phase cells using propidium iodide staining or (c) the TUNEL assay. The data are the mean ± SD for three independent experiments. *$p < 0.05$ comparing the indicated groups

did not affect Beclin 1 levels. Ang II treatment alone increased levels of LC3-I, an active cytosolic LC3 form, and of LC-3 II, an autophagosome membrane-bound form, but did not change the LC-3II/LC3-I ratio compared to the untreated control. Consistent with the observations on the level of autophagy as estimated by the presence of AVOs in Figure 2(b), the LC-3II/LC3-I ratio was significantly increased by 100 μM $H_2O_2$ stress alone and this effect was significantly reduced by Ang II treatment, while levels of Bnip 3 (Bcl-2/E1B-19 kDa interacting protein 3), an up-regulator of autophagy, were reduced. As expected, levels of the activated/phosphorylated mammalian target of rapamycin (mTOR), a negative regulator of autophagy, were increased by either Ang II or 100 μM $H_2O_2$ stress alone or 1 μM Ang II plus $H_2O_2$ and were extremely high in the 20 μM Ang II-pretreated $H_2O_2$-stressed group. The p62, an ubiquitin-associated protein, interacted with LC3 to recruit autophagosome formation, deficiency of autophagy resulted in p62 accumulation,[24] and therefore, p62 was applied as a marker for autophagic clearance. Ang II had no significant effect on levels of p62 in control cells, while, under conditions of $H_2O_2$ stress, levels of p62 tended to be increased by Ang II treatment ($p = 0.12$). These results further support the evidence that $H_2O_2$ stress reduced autophagy in hypertrophic cells, which in turn increased p62 accumulation.

Levels of the anti-apoptotic protein Bcl-2 were not changed by Ang II treatment alone but were markedly reduced in cells treated with $H_2O_2$ alone, and this effect was overcome by Ang II treatment. Levels of the pro-apoptotic protein Bax were significantly reduced by Ang II treatment alone, while treatment with 100 μM $H_2O_2$ stress alone resulted in an increase in Bax levels, which was unaffected by addition of 1 μM Ang II, but significantly increased by 20 μm Ang II. Thus, Bcl-2 levels did not correlate with the level of apoptosis caused by $H_2O_2$ stress (Figure 3), whereas Bax levels showed a similar trend to apoptosis.

### Blockade of autophagy causes more apoptotic cell death under oxidative stress conditions

Upon 100 μM $H_2O_2$ stress, both autophagy and apoptosis were triggered and were affected by Ang II, so we hypothesized that there might be cross-talk between autophagy and apoptosis under these conditions. To examine this, untreated or Ang II-treated H9C2 cells were treated with the autophagy inhibitor 3-MA (5 mM) for 2 h before and during $H_2O_2$ treatment and then cell death and apoptosis were examined. The results showed that, in the untreated control cells, 3-MA had no effect on early apoptosis (Figure 5(a)) or apoptosis measured by the sub-G1

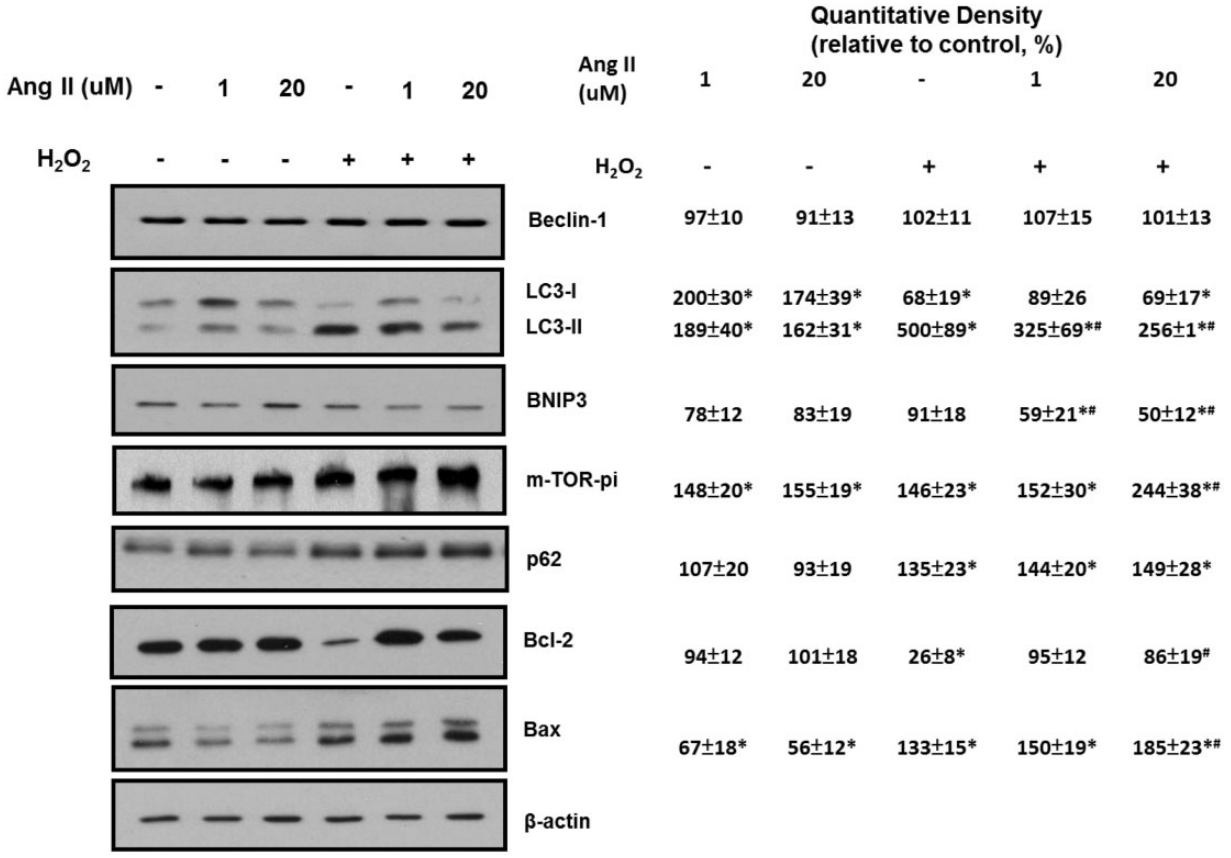


**Figure 4** Expression of autophagy- and apoptosis-related proteins. H9C2 cells were treated as in Figure 2 and then were harvested and proteins extracted for Western blotting using antibodies against the indicated proteins, with ß-actin as the internal control. For the quantitative density results, the intensity of the band of interest was divided by that of the β-actin band and this value expressed relative to that in the untreated control; the results are the mean ± SD for three separate experiments. *$p < 0.05$ compared to the untreated control; #$p < 0.05$ compared to the $H_2O_2$-treated group

phase cells (Figure 5(b)) and TUNEL assay (Figure 5(c)). Upon 100 μM $H_2O_2$ stress, inhibition of autophagy by 3-MA resulted in more cell death and early apoptosis (Figure 5(a)) and in a higher percentage of apoptosis-positive cells (Figure 5(b) and (c)) in the Ang II-untreated cells, but not in the 1 or 20 μM Ang II-treated cells. Rapamycin, an autophagy inducer, was applied to check whether activating autophagy rescued $H_2O_2$-induced apoptosis in hypertrophic cells. Our results showed that rapamycin tended to decrease the percentage of apoptosis-positive cells in Ang II-treated cells under $H_2O_2$ stress (Figure 5(b) and (c), $p = 0.13$).

### Ang II decreases connexin 43 expression under conditions of oxidative stress

Intracellular and endogenous connexin 43 (CX-43) colocalizes with autophagosomes[25] and it has been proposed that it might be associated with the process of autophagy. We therefore examined whether CX-43 expression was altered by Ang II treatment that reduced autophagy under conditions of oxidative stress. As shown in Figure 6, CX-43 levels were significantly increased by addition of 100 μM $H_2O_2$ and this effect was significantly decreased by 1 or 20 μM Ang II pretreatment, while Ang II treatment had no effect in nonstressed cells.

## Discussion

In this study, we found that treatment with 20 μM Ang II induced hypertrophy of H9C2 cells. Under the oxidative stress caused by 100 μM $H_2O_2$, the Ang II-induced hypertrophic cells had a low capacity for autophagy and showed more apoptotic cell death compared to cells without Ang II treatment, although there were no differences between cells treated or not treated with Ang II in terms of levels of autophagic vacuoles and apoptotic bodies in the absence of stress. Consistent with this oxidative stress-induced autophagic response, levels of LC3-II, an autophagosome component, levels of Bnip3, an upregulator of autophagy, and levels of CX-43, which colocalizes with autophagosomes,[25] were lower in Ang II-treated cells. Bcl-2 levels did not reflect the status of apoptosis, whereas Bax levels did. Blocking autophagy using 3-MA significantly increased apoptotic cell death in H9C2 cells without Ang II treatment but not in Ang II-treated cells. These findings suggest that the autophagic response to the oxidative stress caused by 100 μM $H_2O_2$ provides a survival advantage for cells which is reduced by Ang II treatment.

A study by Chen *et al.*[26] showed that the use of $H_2O_2$ and 2-methoxy-estradiol (2-ME) to induce oxidative stress causes autophagy-induced cell death in the transformed cell line HEJ293 and the cancer cell lines U87 and HeLa and that blocking autophagosome accumulation using chemical inhibitors or knocking down autophagy-related genes blocks oxidative stress-induced cell death. In contrast, $H_2O_2$ or 2-ME failed to significantly increase autophagy in mouse primary astrocytes, but cell death was higher than that in the similarly treated transformed and cancer cells. At first sight, these results seem to be inconsistent with those in the present study; however, they differ in the concentrations of the oxidative stress inducer, $H_2O_2$, and the type of cell used. The $H_2O_2$ concentration used in our study was 10 times lower (100 μM vs. 1 mM). Cell death

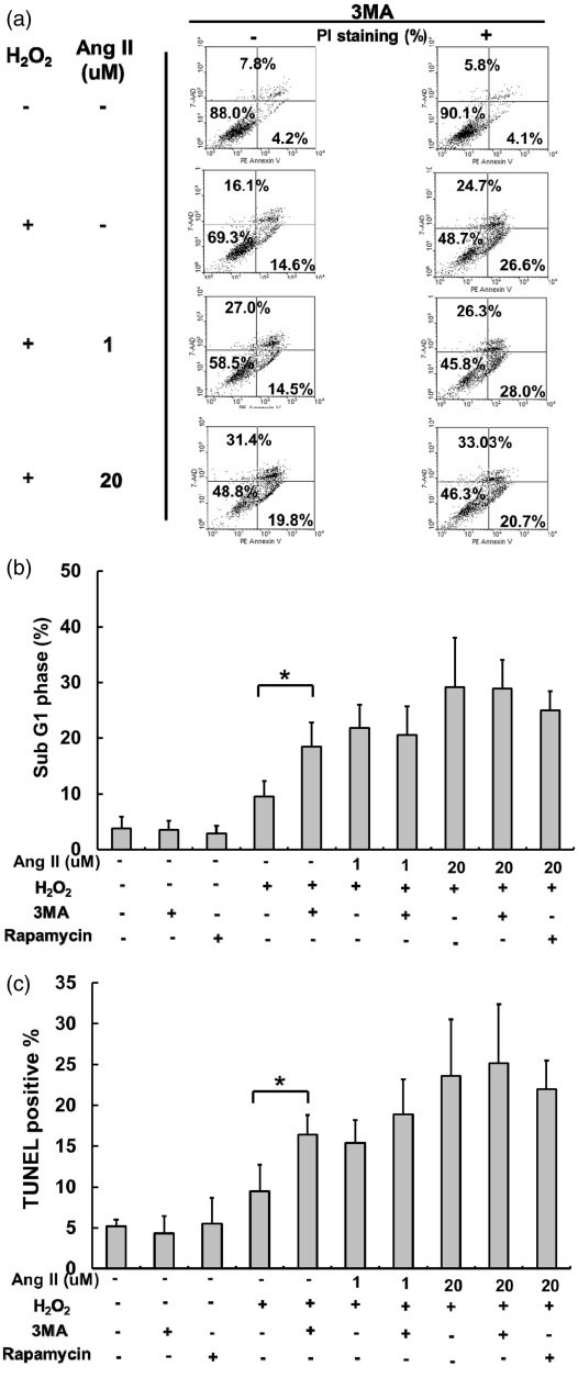


**Figure 5** Blockade of autophagy causes more cell death under oxidative stress. H9C2 cells were left untreated or were treated with 1 or 20 μM Ang II for 22 h and then incubated for 2 h with or without autophagy modulator, followed by treatment with 100 μM $H_2O_2$ in the continued presence of Ang II and/or autophagy modulator for a further 24 h. After treatment, (a) cell death and early apoptosis were measured by double fluorescence staining with Annexin V-PE/7-AAD or (b and c) apoptosis was measured by (b) counting sub-G1 phase cells using propidium iodide staining and (c) the TUNEL assay. The data are the mean ± SD for three independent experiments. *$p < 0.05$ comparing the indicated groups

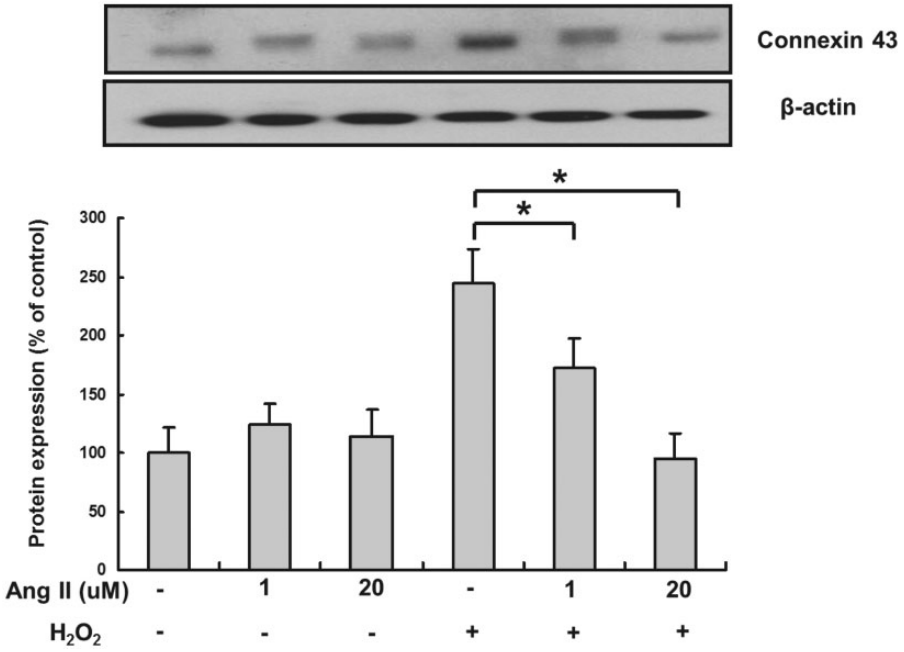


**Figure 6** Ang II treatment decreases Connexin 43 expression under oxidative stress. H9C2 cells were treated as in Figure 2 and then were harvested and proteins extracted for Western blotting using antibody against connexin 43, with ß-actin as the internal control. Quantitative density analysis was performed as in Figure 4 and the results are the mean ± SD for three separate experiments. $^*p < 0.05$ comparing the indicated groups

can occur by several mechanisms and the phenotypic changes that accompany cell death can vary depending on the stimulus and cell setting. Furthermore, apoptosis and autophagy are not mutually exclusive pathways, as they have been shown to act in synergy and also to counter each other.[6] We suggest that the high concentration of 1 mM $H_2O_2$ used in study of Chen *et al*.[26] may have been higher than the threshold at which autophagy can rescue cells from death and thus failed to increase autophagy, but resulted in another phenotypic death in mouse primary astrocytes, while it significantly increased autophagic cell death in transformed cell lines and cancer cell lines. In contrast, the concentration of 100 μM $H_2O_2$ used in our study was able to trigger autophagy to partially rescue H9C2 cells from death, and this rescue was attenuated in Ang-II induced hypertrophic H9C2 cells and accompanied by increased apoptotic cell death. Similar to our findings, a study on chronic ischemic swine myocardium found that areas of the heart showing increased autophagy displayed fewer apoptotic cells and suggested that induction of autophagy might have prevented apoptosis.[27]

In addition to the ability of clearance of defective organelles, autophagy is essential to maintain energy production and cellular homeostasis.[28] In response to the cardiac hypertrophy, various cellular functions have been changed, including a shift in energy substrate utilization. Glucose uptake and glycolysis are significantly increased while β-oxidation of fatty acid is decreased.[29] Autophagy activation in cardiac hypertrophy not only removes the damaged macromolecules and organelles, but it also provides essential nutrients which contribute to energy and biomaterial production.[28] In addition, autophagy is necessary for regression of cardiac hypertrophy during unloading of neurohumoral and hemodynamic stress.[30] Under oxidative stress, a limited autophagic response in hypertrophic cardiomyocytes might not efficiently supply nutrients or clear the damaged molecules, therefore partially contributes to apoptotic cell death. The above findings indicate that, under low levels of oxidative stress, autophagy provides a survival advantage for cells and any blocking or reduction of autophagy may lead to an increase in apoptotic and necrotic cell death.

Autophagy and apoptosis share many of the same molecular regulators and thus it is not possible to predict the outcome of inhibition or activation of one death program without considering its effect on the other. The Bcl-2 family proteins are known to be important regulators of apoptosis, but there is increasing evidence that they can also regulate autophagy. The autophagy protein Beclin 1 was initially identified in a yeast two-hybrid system as a Bcl-2 interacting protein, and it was later reported that binding of Bcl-2 to Beclin 1 disrupts autophagy.[31] In addition, in transgenic mice, overexpression of Bcl-2 resulted in reduced levels of autophagy.[31] Bcl-2 is therefore regarded as a negative regulator of autophagy, acting by inhibiting Beclin 1.[32] In contrast, in a study using the HL-1 cardiac myocyte cell line, overexpression of wild-type Bcl-2 did not have any effect on starvation-induced autophagic flux.[33] Our results showed that, under conditions of oxidative stress that triggered the cell survival-promoting effect of autophagy, Bcl-2 levels did not correlate with the status of apoptosis, whereas Bax levels did. We propose that, in additional to its role in apoptosis, Bcl-2 might play a role in the autophagic process. These findings suggest that the interaction between Beclin-1 and Bcl-2 might provide a potential convergence point for apoptosis and autophagy.

Further studies are needed to clarify how Bcl-2 can act in opposing manners, but it is possible that the regulatory effects of Bcl-2 on autophagy differ depending on the cell type and/or stimulus.

Gap junctions allow ions and small molecules to be exchanged between the cytoplasm of adjacent cells. The principle gap junction protein expressed in mammalian heart ventricles is CX-43. Studies have reported that CX-43 is downregulated under high glucose conditions[34] or by aldosterone.[35] In addition, a heterogeneous reduction in CX-43 expression and disordering of gap junction distribution have been reported in human ventricular disease.[36] Recently, it was reported that intracellular and endogenous CX-43 colocalizes with autophagosomes,[25] and it was proposed that CX-43 negatively modulated autophagy during nutritional deprivation.[37] Moreover, CX-43-dependent inhibition of autophagy does not require functional gap junction communication.[37] Interestingly, a different association between autophagy and CX-43 was observed in our study, CX-43 expression was positively associated with the status of autophagy. The possible explanation might be due to the stimulus and the types of cells. Nutritional deprivation was applied in the previous study, while Ang II and $H_2O_2$ were applied in our study. In addition, the previous study used several cell types, including mouse embryonic fibroblasts, mouse osteoblasts, normal rat kidney cells, and HeLa cells, while we used H9C2 cardioblasts. The regulatory effect of CX-43 on autophagy might differ depending on the cell type and/or stimulus; as a result, further studies are needed to clarify how they interact and the factors involved.

In conclusion, under low levels of oxidative stress, autophagy of H9C2 cells is triggered to rescue the cell from death, and this autophagic function is reduced in Ang-II-induced hypertrophic H9C2 cells, leading to increased cell death.

**Author contributions:** C-YC carried out the studies and data analyses and drafted the manuscript. H-CH conceived of the study, participated in its design and coordination, and helped to draft the manuscript. M-FC obtained funding and supervised the study. All authors read and approved the final manuscript.

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