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Basal autophagy protects cardiomyocytes from doxorubicin-induced toxicity

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ABSTRACT

Doxorubicin (Doxo) is one of the most effective anti-neoplastic agents but its cardiotoxicity has been an important clinical limitation. The major mechanism of Doxo-induced cardiotoxicity is associated to its oxidative capacity. However, other processes are also involved with significant consequences for the cardiomyocyte. In recent years, a number of studies have investigated the role of autophagy on Doxo-induced cardiotoxicity but to date it is not clear how Doxo alters that process and its consequence on cardiomyocytes viability. Here we investigated the effect of Doxo 1 uM for 24 h of stimulation on cultured neonatal rat cardiomyocytes. We showed that Doxo inhibits basal autophagy. This inhibition is due to both Akt/mTOR signaling pathway activation and Beclin 1 level decrease. To assess the role of autophagy on Doxo-induced cardiomyocyte death, we evaluated the effects 3-methyladenine (3-MA), bafilomycin A1 (BafA), siRNA Beclin 1 (siBeclin 1) and rapamycin (Rapa) on cell viability. Inhibition of autophagy with 3-MA, BafA and siBeclin 1 increased lactate dehydrogenase (LDH) release but, when autophagy was induced by Rapa, Doxo-induced cardiomyocyte death was decreased. These results suggest that Doxo inhibits basal autophagy and contributes to cardiomyocyte death. Activation of autophagy could be used as a strategy to protect the heart against Doxo toxicity.

1. Introduction

Developments in the oncology armamentarium have resulted in a significant improvement in cancer patient survival. However, many of the drugs that have shown beneficial effects on cancers can also produce untoward effects on the cardiovascular system. Accordingly, cardio-oncology units provide effective care of cancer patients as well as aiming to understand cardiotoxicity associated with the use of antineoplastic drugs. Although anthracyclines have long been used to treat a wide variety of cancers, cardiotoxicity has been an important clinical limitation (Zbinden et al., 1978). Doxorubicin (Doxo), the most widely used anthracycline, can produce both acute and chronic cardiotoxicity (Minotti et al., 2004). Although the underlying mechanisms involved in Doxo-induced cardiotoxicity are not completely understood, the production of reactive oxygen species (ROS), mitochondrial dysfunction, disruption of catalytic cycle of DNA-bound topoisomerase 2 and Ca^{2+} deregulation are purported mechanisms (Ghigo et al., 2016).

Macroautophagy (hereafter referred to as autophagy) is a cellular process involved in the degradation and recycling of cellular components via the lysosomal pathway (Klionsky et al., 2016). Specific autophagy-related (ATG) proteins regulate the four steps of the autophagy process: (i) induction, (ii) assembly of the phagophore (or also known as nucleation of the autophagosome precursor), (iii) membrane expansion and maturation of the autophagosome vacuole and (iv) fusion of the autophagosome vacuole with the lysosome, and recycling of the degraded cargo.

In the heart, autophagy activation seems to be beneficial or detrimental depending on the cellular context (Lavandero et al.,

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2013). Recently, a number of studies have investigated the role of autophagy on Doxo-induced cardiotoxicity. Most of the studies initilially showed that Doxo upregulates cardiac autophagy and contributes to Doxo-dependent cardiotoxicity (Chen et al., 2011; Kobayashi et al., 2010; Lu et al., 2009; Xu et al., 2012). By contrast, others have reported that activation of autophagy protects against Doxo cardiotoxicity (Katamura et al., 2014; Li et al., 2014). However, recent reports show that Doxo impaired autophagy (Kawaguchi et al., 2012; Sishi et al., 2013). A recent work by Hill's group supports these findings, showing that Doxo blocks lysosomal acidification, thus disrupting the autophagy flux (Li et al., 2016).

Currently, the role of autophagy in Doxo cardiotoxicity is still a matter of debate (see Table 1) as it is unclear: (a) whether Doxo induces or disrupts the cardiac autophagic process and (b) if cardiomyocyte autophagy modulation is beneficial o detrimental. In the present study, therefore, we investigated both controversial issues using both inhibition and activation approaches of the cardiomyocyte autophagy and assesment of the different types of cardiomyocyte death.

2. Methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), Triton X-100, EDTA, rapamycin (Rapa), 3-methyladenine (3-MA), bafilomycin-A1 (BafA), and GAPDH antibody were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Secondary anti-mouse and rabbit antibodies were obtained from Calbiochem (Burlington, ON, Canada). Fetal Bovine Serum (FBS), Optimem and trypsin were purchased from GIBCO (Paisley, Scotland, UK). Oligofectamine, Hoechst, Beclin1 and scramble siRNA were purchased from Probes-Invitrogen (Carlsbad, CA, USA). Antibodies for p62 Abcam (Cambridge, MA), LC3, Beclin 1, Caspase 3, Akt and p70S6K total and phosphorylated form were from Cell Signaling Technology (Danvers, MA, USA). Doxorubicin was from Pfizer Laboratories (New York, NY, USA). All the materials for SDS-PAGE, nitrocellulose membranes were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Table 1
Summary of current report of Doxorubicin effects in cardiac autophagy.

Ref.	Model	Doxorubicin administration	Autophagy Flux	Autophagy inhibition	Autophagy Activation	Cell Death	Comments
Lu et al. (2009)	In vivo: normal rat In vitro: primary cultured neonatal rat cardiomyocytes	2.5 mg/Kg ip 6× in 2 wk Cum. dose 15 mg/Kg 1 mg/L for 24 h	ND	3-MA	ND	ND	↓ autophagy improves myocardial function.
Kobayashi et al. (2010)	In vitro: primary cultured neonatal rat cardiomyocytes	1 μM for 24 h	↑	3-MA sh-Beclin 1 BafA	Rapamycin Adenovirus Beclin 1 wt	↑	GATA4 protects against Doxo-induced cell death.
Chen et al. (2011)	In vitro: primary cultured neonatal rat cardiomyocytes	1 μM for 24 h	↑	3-MA sh-Beclin 1 BafA	ND	↑	Caloric restriction protects cardiomyocytes
Xu et al. (2012)	In vitro: primary cultured neonatal rat cardiomyocytes	1 μM for 24 h	↑	3-MA sh-Beclin 1	Adenovirus Beclin 1 wt	↑	Resveratrol protects from cell death.
Kawaguchi et al. (2012)	In vivo: wild type mice In vitro: primary cultured neonatal mouse cardiomyocytes	10 mg/Kg ip 2× in 3 days Cum.dose 20 mg/Kg 0.1 μM for 6 h	↓	CQ	Starvation	↓	Starvation restores the activation of AMPK and ULK-1
Li et al. (2014)	In vivo: ko mice Nrf2 In vitro: primary cultured neonatal rat cardiomyocytes	25 mg ip 1 × 1 μM for 24 h	=	CQ BafA	Ad-Atg5	ND	↑ Atg5 reduces dox-induced cardiomyocyte death.
Sishi et al. (2013)	In vivo: wild type mice In vitro: transformed myoblast cell line H9c2	10 mg/Kg ip 2× in 3 days Cum dose 20 mg/Kg 3 μM for 24 h	↓	BafA	Rapamycin	↓	H9c2 is a myoblast cell line (not cardiomyocytes)
Katamura et al. (2014)	In vivo: wild type mice In vitro: primary cultured neonatal rat cardiomyocytes	20 mg/Kg ip 1 × 1 μM for 24 h	ND	3-MA CQ	Curcumin	=	CQ ↓ improvement on myocardial function induced by curcumin
Li et al. (2016)	In vivo: haplo-insufficient mice for beclin In vitro: primary cultured neonatal rat cardiomyocytes In vitro: stem cell line H9	5 mg/Kg ip 4× in 4 wk Cum dose 20 mg/Kg 1 μM for 24 h 1 μM for 24 h	↓	Beclin-1 +/− Mice BafA	Cardiomyocyte specific expression of beclin1	ND	Dox reduces lysosome acidification in a mTOR independent manner. However, this work did not investigate whether this effect on lysosome acidification was linked with cell death or myocardial function. Relationship of autophagy with cell death was also not studied
Our present work	In vitro: primary cultured neonatal rat cardiomyocytes	1 μM for 24 h	↓	3-MA si-Beclin-1	Rapamycin	↓	Autophagy is protective of Doxo-induced cell death by necrosis. Dox reduced protein levels of Beclin-1, LC3 and p62 as a part of the mechanisms of DOX on autophagy.

ND: not determined; 3MA: 3-methyladenine; BafA: bafilomycin A1; CQ: chloroquine, Cum: cumulative; wk: weeks: ip: intraperitoneal; ×: times; wt: wild type.

2.2. Culture of cardiomyocytes

Rats were obtained from the Animal Breeding Facility of the Faculty of Chemistry and Pharmaceutical Sciences, University of Chile. All experiments conformed the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (8th Edition, 2011) and was approved by our Institutional Ethics Review Committee. Cardiomyocytes were prepared from hearts of 1–3 day-old Sprague Dawley rats as described previously (Foncea et al., 1997). In brief, rat ventricles were minced and cells dissociated in a solution of collagenase and pancreatin. After enzymatic digestion, the cells were plated in gelatin-coated plastic dishes and cultured in DMEM/M199 (4:1) containing 10% FBS, 5% FCS, and antibiotics (streptomycin and penicilin, 100 U/mL). To prevent the overgrowth of fibroblasts and smooth muscle cell, bromodeoxyuridine (10 mM) was used in the cell culture media. Cardiomyocytes were 96% pure after a 24-h plating period (evaluated with an anti-beta-myosin heavy chain antibody).

2.3. Evaluation of necrosis and apoptosis

Necrosis was determined by measuring lactate dehydrogenase (LDH) activity in cell and culture media using The CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega. The fraction of LDH released was determined by comparing LDH activity in culture medium relative to total LDH activity. Cardiomyocyte apoptosis was assessed by the cleavage of caspase 3 by Western blot analysis. Apoptosis/Necrosis was quantified by flow cytometry, using the Annexin-FITC Apoptosis Detection Kit, BD Pharmingen. The assay was performed according to the manufacturer's instructions. Positive cells for Annexin V-FITC were quantified by FACScan. Propidium iodide was not used because doxorubicin autofluorescence interferes with its detection.

2.4. Evaluation of autophagy flux

Cardiomyocytes were seeded in 35 mm dishes at a density of 1.5×10^6 cells/well in medium containing 2% FBS. To evaluate autophagy flux, the cells were incubated with Doxo 1 uM and Baf A 10 uM for 24 h. Cell lysates were analyzed by Western blotting with anti-LC3 and GAPDH antibodies. The autophagic flux was expressed in relation to untreated cultures with Baf A.

2.5. Experimental approaches to block or activate cardiomyocyte autophagy

a) Gene silencing with siRNA. For RNA interference, cells were seeded at a density of 1×10^6/well in 35 mm dishes. Lipofectamine 2000 transfection reagent was used to silence Beclin. A nonspecific siRNA was used as a control. A final concentration of 100 nM siRNA was transfected into cells by standard established protocols. Twenty-four hours after transfection, the cells were washed with phosphate buffered saline (PBS) and culture medium was replaced by DMEM containing 2% FBS and mantained for 24 h. Then, the cells were stimulated with Doxo 1 uM for 24 h for the experiments. The efficiency of Beclin protein silencing was analyzed by Western blot at 24 h.

b) Activation of autophagy by Rapa. Cardiomyocytes were incubated with Rapa 0.1 uM, two hours previous to Doxo 1 uM stimulus and then both were mantained for 24 h. Cell death was determined by measuring lactate dehydrogenase (LDH) activity into the cell and culture medium as previously described.

2.6. Preparation of cell extract and Western blot analysis

Following the administration of the indicated treatments, the culture medium was removed and the cells were washed with cold PBS and scraped into 60 μl of cold lysis buffer. The protein content was determined by Bradford assay. Aliquots of the extracted proteins (approx. 30 μg/lane) were separed according to molecular weight on a mono-dimensional SDS-PAGE, 10% (for Beclin, Akt, p70S6K) and 12% (for LC3 and Caspase 3) and were transferred to nitrocellulose membrane. Non-specific binding sites were blocked with 5% (w/v) non-fat milk in Tris-buffered saline (pH 7.6) containing 0.1% (v/v) Tween 20, for 1 h at room temperature. Nitrocellulose membrane was incubated with primary antibodies (caspase 3, LC3, Beclin 1, anti-phosphorylated and total form of Akt and p70S6K) overnight at 4 °C. GAPDH was used a as loading control. After an additional incubation period with secondary HRP-coupled antibody for 1 h at room temperature, the blots were developed by chemiluminescence using the ECL system and were quantified by scanning densitometry (UN-SCAN-IT gel 6.1).

2.7. Statistical analysis

Data are shown as mean ± SEM of the number of independent experiments indicated (n) and represent experiments performed on at least three separate attempts with similar outcomes. Data were analyzed by Student's *t*-test or ANOVA and comparisons between groups were performed using a protected Dunnett or Tukey's test. GraphPad Prism 6 statistical program was used and a P value less than 0.05 was considered to represent a statistically significant difference.

3. Results

3.1. Doxorubicin inhibits autophagy in cardiomyocytes

The effect of Doxo on cardiomyocyte autophagy is controversial, showing either induction or inhibition of autophagy (Dirks-Naylor, 2013). An increase in LC3-II levels can result not only from augmented autophagosome formation (positive flux), but also from decreased lysosomal degradation of autophagosomes (impaired autophagic flux). To discriminate between these two possibilities, the currently validated strategies consist of measuring autophagic parameters in the presence of lysosomal inhibitors (Klionsky et al., 2016). We used bafilomycin-A (BafA), a lysosomal H-ATPase inhibitor that impairs autophagosome -lysosome fusion. As expected, BafA increased LC3-II content in cardiomyocytes. However, Doxo treatment for 24 h decreased BafA-induced LC3-II accumulation (Fig. 1). The same result was observed when LC3-I + LC3-II levels were analyzed (Fig. 1). These results suggest that Doxo inhibits autophagic flux in cardiomyocytes.

3.2. Doxo activates the Akt/mTOR pathway

The Akt/mTOR pathway negatively regulates autophagy (Levine and Klionsky, 2004). Mutations causing Akt activation or inactivation cause supression and induction of autophagy, respectively (Datta et al., 1997). We found that Doxo robustly induced Akt activation, as shown by the increased phosphorylation in Ser-473 at 18–24 h of Doxo treatment (Fig. 2A). As expected, Doxo treatment also led to an increased phosphorylation of p70S6 K, a downstream target of mTOR (Fig. 2B). These results suggest that Doxo downregulates autophagy through Akt/mTOR pathway activation.

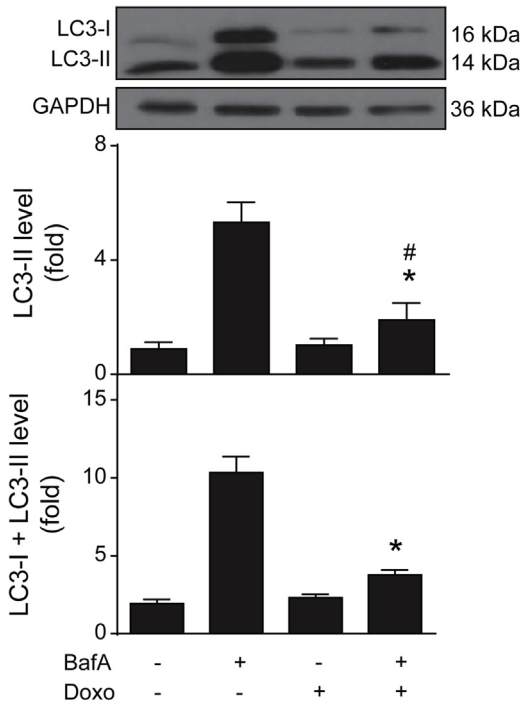

Fig. 1. Doxo inhibits basal autophagy. Cultured neonatal cardiomyocytes were exposed to Doxo 1 μM for 24 h. Total protein extracts were obtained and LC3-I and LC3-II protein levels were determined by Western blot. GAPDH was used as loading control. Upper panel, representative Western blot. Middle panel, LC3-II level quantification. Lower panel, total LC3 (LC3-I + LC3-II) level quantification. Values are the average of 4 independent experiments ± SEM. *$p < 0.05$ vs control; #$p < 0.05$ vs BafA.

3.3. Treatment with Doxo decreases autophagy protein levels

Beclin 1, LC3 and p62 are involved in the autophagy process (Klionsky et al., 2016). Doxo decreased Beclin 1 levels significatively at 24 h of exposure (Fig. 3A). In addition, the same effects were observed in p62 and total LC3 (LC3-I + LC3-II) protein levels (Fig. 3B). Taken together, the decrease in these proteins in addition to the activation of Akt/mTOR pathway, support the role of Doxo as an inhibitor of basal autophagy in cardiomyocytes.

3.4. Autophagy reduces Doxo-induced cardiomyocyte death

Cardiomyocyte death plays an important role in the pathogenesis of Doxo-induced myocardial injury. Anthracycline treatment has been shown to induce cell death in a concentration-dependent manner (Arola et al., 2000; Sawyer et al., 1999) including apoptosis and necrosis (Sawyer et al., 1999). We found that Doxo 1 μM induced cardiomyocyte apoptosis as visualised by caspase 3 activation at 3 h (Fig. 4A) and Annexin V-FITC binding at 18 h (Fig. 4B). Doxo 1 uM also induced necrosis as determined by a 50% increase of LDH release at 24 h (Fig. 4C).

Autophagy could be either protective or detrimental depending on several factors, especially the nature and intensity of the stimuli (Kang and Avery, 2008). Accordingly, to assess the role of autophagy on Doxo-induced cardiomyocyte death, we evaluated the effects of 3-MA and BafA. Inhibition of autophagy with both 3-MA and BafA increased LDH release (Fig. 5A, B). The same result was obtained when reducing Beclin1 protein level by 70% using a siRNA (Fig. 5C, D). Moreover, when autophagy was induced by Rapa – an mTOR inhibitor – Doxo-induced cardiomyocyte death was decreased (Fig. 5E). These results suggest that autophagy protects against Doxo-induced cardiomyocyte death. In this study we confirmed that Doxo 1 uM caused cell death by apoptosis and necrosis in a 24-h period.

4. Discussion

Anthracyclines are drugs which are commonly used to treat hematological malignancies and solid tumors. Although highly effective, they may cause persistent and progressive cardiovascular damage (Lipshultz et al., 1991). Despite extensive research, the exact mechanism by which anthracyclines produce cardiotoxicity has not been fully explained. In the present study, we have shown that Doxo – one of the most representative anthracyclines – inhibits autophagy in cardiomyocites via an Akt/mTOR pathway and that autophagy can protect against Doxo-induced cardiomyocite necrosis and apoptosis.

Under physiologic conditions, autophagy is a protective process, whereas under pathological conditions autophagy can be either protective or detrimental and thus contribute to death cell (Lavandero et al., 2013). It is important noting that patients receiving Doxo are usually not under a pathological condition related to the functioning of the heart. In fact, normal values of

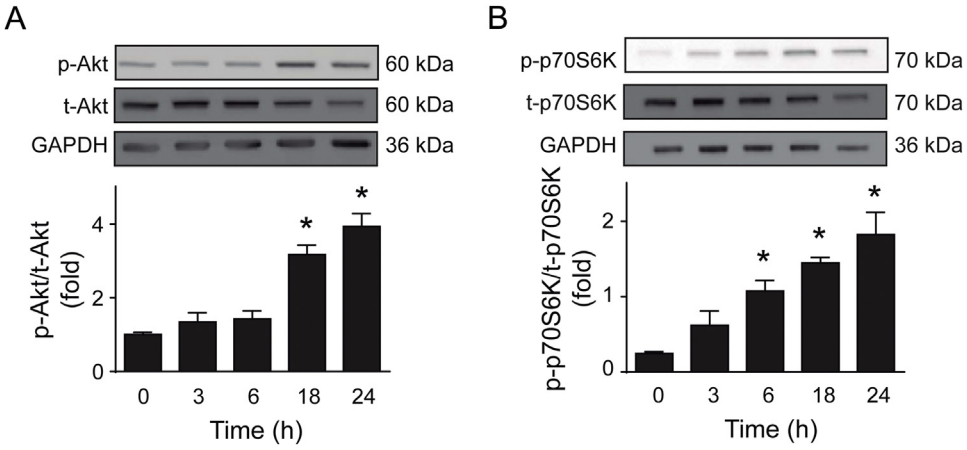

Fig. 2. Doxo activates Akt/mTOR pathway. Cultured neonatal cardiomyocytes were exposed to Doxo 1 μM for 0 to 24 h. Total protein extracts were obtained and (A) Ser-473 phospho-Akt (p-Akt), total Akt (t-Akt), and (B) phospho-p70S6K (*p*-p70S6K), total p70S6 K (t-−70S6 K) levels were determined by Western blot. GAPDH was used as a loading control. Values are the average of 4 independent experiments ± SEM. *$p < 0.05$ vs 0 h.

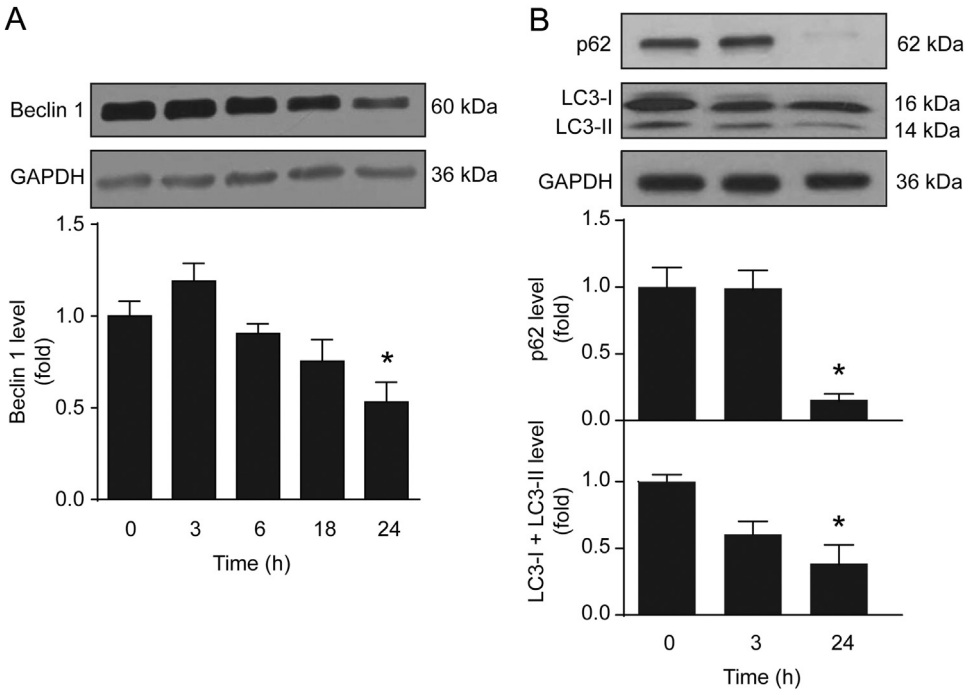

Fig. 3. Doxo decreases Beclin 1 protein levels. Cultured neonatal cardiomyocytes were exposed to Doxo 1 μM for 0 to 24 h. (A) Total protein extracts were obtained and Beclin 1 protein levels were determined by Western blot. GAPDH was used as a loading control. Values are the average of 3 independent experiments ± SEM. *$p < 0.05$ vs 0 h. (B) Total protein extracts were obtained and p62 and LC3-I y LC3-II protein levels were determined by Western blot. GAPDH was used as a loading control. Values are the average of 3 independent experiments ± SEM. *$p < 0.05$ vs 0 h.

cardiac function are required previous to Doxo initiation and changes in these parameters are used to identify cardiotoxicity. Basal autophagy is important for turnover of organelles and removal of protein aggregates; this occurs at low basal levels under normal conditions (Levine and Klionsky, 2004), and represents an important survival mechanism. Indeed, autophagy principally serves an adaptive role to protect organisms against diverse pathologies (Mizushima et al., 2008). Our study suggests that autophagy can protect from Doxo-induced cell death, yet the same Doxo is capable of inhibiting this process. In agreement with our findings, the level of autophagy in the heart has shown to be altered in several pathological conditions, such as in ischaemia/reperfusion injury (Hamacher-Brady et al., 2006; Matsui et al., 2007), hypertrophy (Dammrich and Pfeifer, 1983), heart failure (Nishino et al., 2000; Shimomura et al., 2001), and in the presence of external stressors, like starvation, hormonal imbalance, and oxidative stress. Taken together, this underpins the key role of autophagy in normal cardiac physiology and its potential role in protecting from drug-induced damage.

Table 1 summarizes the literature related to Doxo and cardiac autophagy and depicts the broad discrepancy of the reported results. In this table, we describe the experimental models employed, Doxo effects on autophagy flux, the tools used to inhibit or induce autophagy and the relationship between autophagy and cardiac death. Doxo effects on autophagy can be separated into two time periods. The first one (between 2009 and 2012) started with Lu et al. (2009) reporting autophagy stimulation by Doxo, *in vitro* and *in vivo*. However, this early work only studied autophagy but it did not evaluate autophagy flux. Lu et al. (2009) determined the number of autophagic vacuoles as a measure of autophagy. The number of autophagic vacuoles can be increased by inducing autophagy, as well as by inhibiting their degradation, for example, by blocking fusion with lysosomes. Therefore, to evaluate the induction of autophagy, it is essential to study the autophagic flux (Klionsky et al., 2016). Later, Liang's group published three papers describing that Doxo stimulates autophagy flux and autophagy inhibition decreased Doxo-induced cardiomyocyte death (Chen et al., 2011; Kobayashi et al., 2010; Xu et al., 2012). Also, they described that caloric restriction, which induces autophagy, surprisingly also reduced Doxo-induced cell death (Chen et al., 2011). Moreover, they showed that resveratrol prevented Doxo-dependent cardiomyocyte death and reduced Doxo-induced autophagy flux (Xu et al., 2012). Nevertheless, they also showed that resveratrol reduced Doxo-induced p70S6K phosphorylation, a downstream target of mTOR (Xu et al., 2012). Because mTOR is an inhibitor of autophagy, a decrease of p70S6K phosphorylation would suggest autophagy activation by resveratrol. However, Xu et al. (2012) did not observed effects of resveratrol on autophagy flux. Additionally, Dimitrakis et al. showed that Doxo 10 μM, but not Doxo 1 μM, increased autophagy markers associated with decreased proteosomal activity in cultured adult cardiomyocytes. However, autophagic flux was not assessed (Dimitrakis et al., 2012). In summary, during this first period, the evidence provided suggests that Doxo induces cardiac autophagy and inhibition of autophagy reduced Doxo-induced cell death. However, two of the five studies did not determine autophagic flux. Paradoxically, they also described that strategies that also induced autophagy, i.e. caloric restriction or probably resveratrol, also reduced Doxo-induced cardiomyocyte death. In these cases, these results could not be replicated by either our group or others. The second period (between 2012 until now) started when Kawaguchi et al. (2012) reported that Doxo impaired autophagy flux. In mice they showed that starvation, before Doxo treatment, promotes autophagic flux and reduces cardiotoxicity (Kawaguchi et al., 2012). Later, Sishi et al. (2013) working with mice and the H9c2 myoblast cell line, showed that Doxo disrupted autophagy and the autophagy activator rapamycin protects against apoptosis. However H9c2 is not an appropriate *in vitro* model, because this is not a cardiomyocyte cell line. H9c2 is a subclone of the original clonal cell line derived from embryonic BD1X rat heart

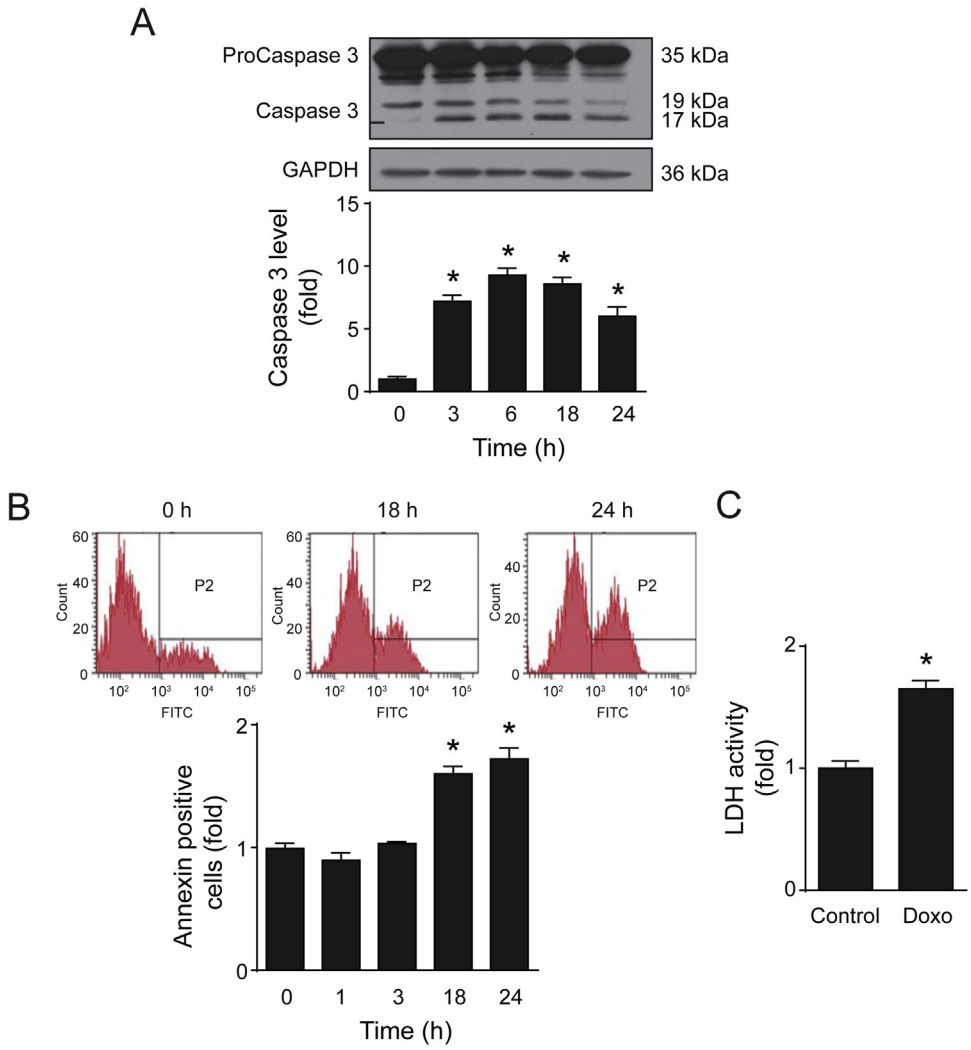

Fig. 4. Doxo induces cardiomyocyte cell death. (A) Cultured neonatal cardiomyocytes were exposed to Doxo 1 μM for 0 to 24 h. Total protein extracts were obtained and caspase 3 protein levels were determined by Western blot. GAPDH was used as a loading control. Values are the average of 6 independent experiments ± SEM. ***$p < 0.001$ vs 0 h. (B) Cultured neonatal cardiomyocytes were exposed to Doxo 1 μM for 0 to 24 h. Cells were detached using trypsin, incubated with Annexin V-FITC and analyzed by flow cytometry. Annexin positive cells were determined as P2 population. Values are the average of 4 independent experiments ± SEM. *$p < 0.05$ vs 0 h. (C) Cultured neonatal cardiomyocytes were exposed to Doxo 1 μM for 24 h. LDH activity was determined in the culture media by spectrophotometry. Values are the average of 4 independent experiments ± SEM. *$p < 0.05$ vs control.

tissue and exhibits many of the properties of skeletal muscle (see www.attc.org, CRL-1446). Moreover, adenoviral ATG5 overexpresion and curcumin prevented cell death and improved myocardial function by activating autophagy, respectively (Katamura et al., 2014; Li et al., 2014). A recent study shows *in vivo* that blocking autophagic flux improves myocardial function (Li et al., 2016). This study also adds novel information by proposing that Doxo blocks lysosomal acidification and disrupts autophagic flux. However, the relationship between autophagy and cell death was not studied (Li et al., 2016). In conclusion, all the more recent evidence available suggests that Doxo disrupts cardiomyocyte autophagy. Our data are in agreement with these latest findings by showing that Doxo (1 μM for 24 h) reduces basal autophagic flux in cultured neonatal cardiomyocytes. Our data also showed that the Doxo-mediated inhibitory effect on autophagy can be also explained by Akt/mTOR pathway activation and decreases in Beclin-1, LC3 and p62 protein levels. We also found that inhibition of autophagy by Doxo contributes to cell death by necrosis, because after blocking autophagy with 3-MA, Baf A or a siRNA to Beclin 1, cardiomyocyte death increased in a synergistic fashion with Doxo. Conversely, the activation of autophagy with rapamycin decreased necrosis.

There is abundant evidence supporting a cardioprotective role for Akt activation in several cellular types (de Jonge et al., 2006; Kumar et al., 2002; Matsui et al., 2003, 2001; Matsui and Rosenzweig 2005; McGowan et al., 2003). Akt activation reduced apoptotic cardiomyocyte death in response to ischaemia-reperfusion injury (Armstrong 2004; Bae and Zhang, 2005), pressure overload challenge (Ceci et al., 2007), and oxidative stress (Aikawa et al., 2000). It is unclear, though, whether the persistent activation of Akt could lead to deleterious effects, hence promoting cell death. Perhaps, it might be necessary to consider the different Akt isoforms and their effects on cell viability. For example, Taniyama et al. described Akt3 upregulation in heart samples from patients suffering from hypertrophyc cardiomyophaty, DCM and chronic miocarditis (Taniyama et al., 2005).

Anthracycline-induced cardiotoxicity is primarily dependent on total dose accumulation and few strategies have proven beneficial to reduce the damage but reducing the amount of drug administered and hence compromising the anti-tumoral effect (Volkova and Russell, 2011). Our findings can have important clinical implications, as testing of targeted therapies to tackle Akt/mTOR pathway when using anthracyclines could potentially

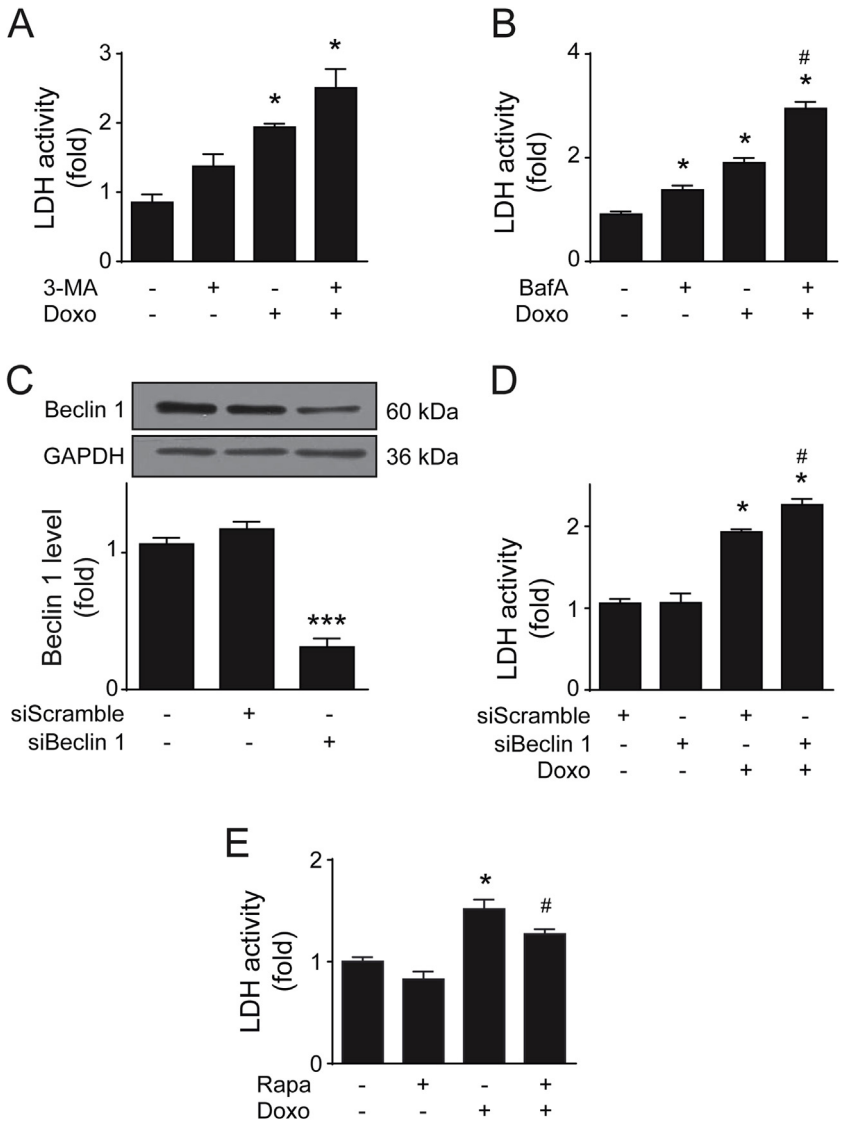

Fig. 5. Autophagy protects against Doxo-induced cardiomyocyte cell death. (A) Cultured neonatal cardiomyocytes were exposed to Doxo 1 μM for 24 h in the presence of 3-methyladenine (3-MA, 10 μM). LDH activity was determined in the culture media by spectrophotometry. Values are average of 3 independent experiments ± SEM. $^{*}p < 0.05$, vs control; $^{\#}p < 0.05$ vs Doxo. (B) Cultured neonatal cardiomyocytes were exposed to Doxo 1 μM for 24 h in the presence of Bafilomycin A (BafA, 10 μM). LDH activity was determined in the culture media by spectrophotometry. Values are the average of 6 independent experiments ± SEM. $^{*}p < 0.05$ vs control; $^{\#}p < 0.05$ vs Doxo. (C) Cultured neonatal cardiomyocytes were treated with siRNA Beclin 1 (siBeclin 1) or siRNA scramble (siScramble) as control for 24 h. Beclin 1 level was determined by Western blot. Upper panel, representative Western blot. Lower panel, Beclin 1 protein level quantification. Values are the average of 4 independent experiments ± SEM. $^{***}p < 0.001$ vs control. (D) Cultured neonatal cardiomyocytes were treated with siBeclin 1 or siScramble for 24 h. Then cardiomyocytes were exposed to Doxo 1 μM for 24 h. LDH activity was determined in the culture media by spectrophotometry. Values are the average of 3 independent experiments ± SEM. $^{*}p < 0.05$ vs control; $^{\#}p < 0.05$ vs Doxo. (E) Cultured neonatal cardiomyocytes were preincubated with rapamycin (Rapa, 0.1 μM) for 2 h and then exposed to Doxo 1 μM for 24 h. LDH activity was determined in the culture media by spectrophotometry. Values are the average of 5 independent experiments ± SEM. $^{*}p < 0.05$ vs control; $^{\#}p < 0.05$ vs Doxo.

reduce their deleterious effects without compromising clinical efficacy. Likewise, strategies to induce basal autophagy could also prove useful in this respect.

In conclusion, the present study provides solid evidence for the key role of autophagy to protect from Doxo-induced cardiac damage as well as presenting a mechanistic insight to explain its cardiotoxicity. Testing targeted interventions can potentially benefit patients receiving anthracyclines in the future.

Conflicts of interests

None declared.

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