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Glucose deprivation causes oxidative stress and stimulates aggresome formation and autophagy in cultured cardiac myocytes

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ABSTRACT

Aggresomes are dynamic structures formed when the ubiquitin–proteasome system is overwhelmed with aggregation-prone proteins. In this process, small protein aggregates are actively transported towards the microtubule-organizing center. A functional role for autophagy in the clearance of aggresomes has also been proposed. In the present work we investigated the molecular mechanisms involved on aggresome formation in cultured rat cardiac myocytes exposed to glucose deprivation. Confocal microscopy showed that small aggregates of polyubiquitinated proteins were formed in cells exposed to glucose deprivation for 6 h. However, at longer times (18 h), aggregates formed large perinuclear inclusions (aggresomes) which colocalized with γ-tubulin (a microtubule-organizing center marker) and Hsp70. The microtubule disrupting agent vinblastine prevented the formation of these inclusions. Both small aggregates and aggresomes colocalized with autophagy markers such as GFP-LC3 and Rab24. Glucose deprivation stimulates reactive oxygen species (ROS) production and decreases intracellular glutathione levels. ROS inhibition by N-acetylcysteine or by the adenoviral overexpression of catalase or superoxide dismutase disrupted aggresome formation and autophagy induced by glucose deprivation. In conclusion, glucose deprivation induces oxidative stress which is associated with aggresome formation and activation of autophagy in cultured cardiac myocytes.

1. Introduction

Protein degradation in the cell is catalyzed by the ubiquitin–proteasome system (UPS). The UPS plays key functions in protein quality control by monitoring and eliminating potentially toxic misfolded or damaged proteins [1]. When the capacity of this proteolytic system is exceeded, misfolded protein substrates aggregate and are assembled through an active and regulated process [1]. Overexpression of different cellular proteins as well as the inhibition of proteasomal activity has been associated with the formation of stable, polyubiquitinated aggregates termed aggresomes [2]. These inclusions are characterized by their localization at the centrosome and by the redistribution of intermediate filaments like vimentin as a cage surrounding the aggresome [2,3]. Aggresome formation requires an energy-dependent intracellular transport mechanism because the micro-aggregates produced in the peripheral cytoplasm are transported to the centrosome through the microtubular cytoskeleton via dynein/dynactin complexes [2]. Aggresome formation works like a cell defense mechanism against high concentrations of unfolded or unwanted proteins, oxidative stress, proteasomal impairment and other cellular insults [2].

Macroautophagy (herein referred to as autophagy) plays an essential role in differentiation and development as well as in cell response to stress. This process is activated during nutrient deprivation and has been associated with neurodegenerative diseases, cancer, pathogen infections and myopathies [4,5]. In the past decade, the molecular mechanisms underlying autophagy have been extensively investigated and the genes participating in this process, denoted *Atg* (autophagy-related genes) [6], were found to be conserved from yeast to man [7,8]. Autophagy is a degradation process by which eukaryotic cells breakdown and recycle macromolecules and organelles. Autophagy

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involves the formation of double-membrane-bounded structures known as autophagosomes or immature autophagic vacuoles. These vacuoles fuse with lysosomes to form autophagolysosomes and their contents are then degraded by acidic lysosomal hydrolases [9].

A growing body of evidence supports the hypothesis that autophagy is a primary mechanism through which mammalian cells may capture and degrade protein aggregates. Polyglutamine-expansion mutations such as those observed in mutant huntingtin and ataxin 3 (causing spinocerebellar ataxia type 3) [10], mutant forms of α-synuclein (which cause familial Parkinson disease) [11], and different forms of tau (including mutations causing frontotemporal dementia) are strongly dependent on autophagy for their clearance [12]. Pharmacological or genetic interference in this degradative pathway slows the clearance of these substrates and increases the levels of aggregates and toxicity. Indeed, inhibition of autophagy by gene knockout leads to the formation of polyubiquitinated protein aggregates in various tissues [13,14].

The contribution of the UPS and autophagy to heart muscle degeneration has largely remained obscure, with the exception of those reports investigating failing human hearts and Danon's cardiomyopathy [15,16]. Evidence is growing that cardiomyocyte degeneration is one of the most prominent phenomena in failing human myocardium. Kostin et al. demonstrated that cardiac myocytes not only die by apoptosis but also as a consequence of slow degenerative processes involving the ubiquitin/proteasomal pathway [15]. This group was the first to analyze molecules involved in the ubiquitin conjugation/proteasomal degradation cascade in human myocardium and to identify functional defects in this system responsible for cell death that ultimately contributes to cardiomyocyte loss [15]. Recent studies have also implicated the UPS in stressed cardiac phenotypes, including heart failure [17]. Cardiac autophagy is stimulated under stress conditions such as myocardial ischemia and hypertension [18,19]. However, the causative role of autophagy in the survival of cardiac myocytes and the underlying signaling mechanisms are poorly understood. Also glucose deprivation (GD), which mimics myocardial ischemia, induces autophagy in cultured cardiac myocytes [18]. Interestingly, Tannous et al. have proposed that protein aggregation is a proximal trigger of cardiac myocyte autophagy [19].

In this study we investigate the autophagy associated to aggresome formation in GD primary cultured neonatal rat cardiac myocytes. Our data show that GD induces aggresomes, characterized as aggregates of polyubiquitinated proteins, and activates autophagy in cultured cardiac myocytes. Aggresomes colocalize with markers of autophagy such as GFP-LC3, Rab24, and LC3-I processing. Our data also show that GD causes oxidative stress which is associated to autophagy induction and aggresome formation in cultured neonatal rat cardiac myocytes.

2. Methods

2.1. Materials

Antibodies against Hsp-70 were purchased from Chemicon (Temecula, CA) whereas the anti-ubiquitin was from Upstate (Billerica, MA). FBS and Lysotracker Green were from Invitrogen (Carlsbad, CA). Antibody against LC3 was purchase from Cell Signaling. Antibody against γ-tubulin, TRITC conjugated anti-IgG mouse polyclonal antibody, FITC conjugated anti-IgG rabbit, propidium iodide (PI), Dulbecco's modified Eagle's medium (DMEM), M199 medium, vinblastine, lactacystin, 3-methyladenine (3-MA), dihydrorhodamine 123, N-acetylcysteine and other reagents were purchased from Sigma-Aldrich Corp (St Louis, MO). Protein assay reagents were from Bio-Rad. RPMI 1649 medium was from Gibco BRL (Carlsbad, CA). CellTiter-Glo® was from Promega. MG132 was from Calbiochem (San Diego, CA). N-Suc-Leu-Leu-Val-Tyr-7-amido-4-methyl coumarin was from Biomol Research Laboratories (Plymouth Meeting, PA).

2.2. Culture of cardiac myocytes

Rats were bred in the Animal Breeding Facility from the Facultad Ciencias Quimicas y Farmaceuticas, Universidad de Chile (Santiago, Chile). This investigation conforms to the "Guide for the care and use of laboratory animals" published by the U.S. National Institutes of Health (NIH publication No. 85-23, revised 1985). We performed all studies with the approval of our Institutional Bioethical Committee. Cardiac myocytes were prepared from hearts of 1–3-day-old Sprague Dawley rats as described previously [20]. Briefly, ventricles were trisected, pooled, and myocytes dissociated in a solution of collagenase and pancreatin. After enzymatic dissociation, the cells were plated in gelatin-coated plastic Petri dishes and cultured in DMEM/M199 (4:1) containing 10% (v/v) FBS, 5% (v/v) FCS, penicillin and streptomycin (100 U/mL). Cardiomyocytes were 95% pure after 24 h plating period, assessed with an anti β-myosin heavy chain antibody. To prevent the overgrowth of fibroblasts and smooth muscle cells, bromodeoxyuridine (10 μM) was used in the cell culture media.

2.3. Stress conditions

Nutritional stress was induced by GD. Cells were incubated with RPMI 1640 medium for different times in the presence or the absence of chemical inhibitors.

2.4. Proteosome activity assay

Proteosome activity was measured fluorometrically by following the hydrolysis of N-Suc-LLVY-AMC (Biomol) as described previously [21].

2.5. Electron microscopy

Electron microscopy analysis was performed at our microscopy facility. Briefly, cells with or without GD were prefixed in 3% glutaraldehyde and postfixed in 2% osmium tetraoxide. After dehydration, they were embedded in epoxy resins. Ultrathin sections were cut, contrasted with uranyl acetate and lead citrate, and studied under a Zeiss EM-109 electron microscope (Zeiss, Germany).

2.6. Evaluation of GFP-LC3 and Rab24 positive autophagic vacuoles and degradation

Cultured cardiac myocytes were transduced with the adenovirus GFP-LC3 at a multiplicity of infection (MOI) of 60 for 24 h [22]. GFP-LC3 transduced cells were grown on coverslips and exposed to the different experimental conditions and fixed with PBS containing 4% paraformaldehyde. Samples were evaluated in a scanning confocal microscope. Rab24 was studied by immunofluorescence microscopy using specific antibodies. For FACS analysis, the level of GFP fluorescence intensity in each treatment was normalized to the level of control sample (cells incubated in complete medium). The level of control sample was set to 100%.

2.7. Incorporation of Lysotracker Green

Cells were incubated with Lysotracker Green DND-26 (50 nM) in control media or exposed to GD for 30 min. Cells were analyzed by a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Data were evaluated using FACS DIVA software (Becton Dickinson, San Jose, CA).

2.8. Visualization of aggresomes by immunocytochemistry

Cells grown on coverslips were fixed with PBS containing 4% paraformaldehyde and incubated in ice-cold 0.3% Triton X-100 for permeabilization. Nonspecific sites were blocked with 5% BSA in PBS

for 1 h. Cells were then incubated with ubiquitin, HSP-70 or γ-tubulin antibodies. Secondary antibodies were anti-mouse IgG-TRITC and anti-rabbit IgG-FITC.

2.9. Measurement of ROS production and intracellular glutathione (GSH) levels

Cultured cardiac myocytes incubated in control or GD media were incubated for 20 min with dihydrorhodamine 123 (25 μM). Cells were analyzed by a FACScan flow cytometer. Data were evaluated using FACS DIVA software. Total intracellular GSH levels were determined using the 5,5′-dithiobis-2-nitrobenzoic acid -dependent recycling assay as described by Anderson and Meister [23].

2.10. Cell viability and apoptosis assays

The integrity of the plasma membrane of control and GD cardiac myocytes was assessed by the ability of cells to exclude PI. Cells were collected by centrifugation, washed once with PBS, and resuspended in PBS containing 0.1 mg/mL PI. The levels of PI incorporation were quantified on a FACScan flow cytometer. Cell size was evaluated by forward-angle light scattering (FSC). PI-negative cells of normal size were considered alive [24]. Sub-G1 population was determined in cardiac myocytes. The cells were collected, pooled, permeabilized with methanol for 24 h, treated with RNase for 1 h and then 2 μL PI (25 μg/mL) was added prior to flow cytometry analysis. A total of five thousand cells per sample were analyzed.

2.11. ATP content determination

ATP content was determined using luciferin/luciferase assay as described [25].

2.12. Expression of results and statistical analysis

Values are shown as mean ± SEM. of the number of independent experiments indicated (n) or as examples of representative experiments performed on at least three separate occasions. Data were analyzed by ANOVA and comparisons between groups were performed using a protected Tukey's t test. A value of $p<0.05$ was set as the limit of statistical significance.

3. Results

3.1. GD generates ubiquitin-labeled aggresomes in cultured cardiac myocytes

In the last few years evidence has accumulated connecting autophagy machinery with the ubiquitin/protein degradation system [15]. Therefore, we first analyzed by immunofluorescence the distribution of ubiquitinated proteins in cultured cardiac myocytes. As shown in Fig. 1A, cells under GD for 2–6 h, developed small aggregates immunoreactive to ubiquitin, whereas at longer incubation times (i.e. 18 h treatment), bigger perinuclear structures were observed. Cardiac myocytes with ubiquitin-immunoreactive aggregates increased from 7.0 ± 1.2% to 23.8 ± 1.4% ($p<0.01$ vs control) after 2 h of GD. Near 50% of the cells presented ubiquitin-immunoreactive aggregates after 18 h GD (Fig. 1B). As indicated before, misfolded proteins are deposited into large aggregates (i.e. aggresomes) close to the microtubule-organizing center and they become unsheathed in a cage of vimentin [3]. Thus, further characterization was undertaken to confirm that the observed inclusion bodies corresponded to aggresomes. Double immunofluorescence for the centrosome marker γ-tubulin and ubiquitin revealed that, like aggresomes, these cardiac myocytes inclusions assembled at the microtubule-organizing center (Fig. 1C, upper panel). We also tested another protein known to be associated with aggresomes, the heat shock protein Hsp70, which acts as a chaperone and is involved in the unfolded protein response (UPS) [26,27]. Fig. 1C (lower panel) shows the colocalization of polyubiquitinated proteins and Hsp70 in cardiac myocytes under GD for 18 h.

Proteosome activity had a biphasic behavior in cardiac myocytes exposed to GD (Fig 1D). Proteosome activity significantly increased after 2 h but at longer times its activity was similar to basal levels. These last results are consistent with an increase in aggresome formation in cardiac myocytes under GD for longer times (Fig. 1A).

Because aggresome formation depends on the transport along microtubules [3,28], we next addressed whether vinblastine, a drug that prevents microtubule polymerization, could affect the formation of the perinuclear structures. As expected, vinblastine treatment prevented the formation of the discrete perinuclear structure containing polyubiquitinated proteins in cardiac myocytes under GD and only smaller cytoplasmic structures distributed throughout the cell were observed (Fig. 1E). Thus, consistent with the properties of aggresomes [29], the aggregates formed in cardiomyocytes were dependent upon the microtubule-mediated intracellular transport system.

3.2. GD induces autophagy in cardiac myocytes

The loss of cardiac myocytes is an important factor in the development of many cardiac diseases. However, the mechanisms by which cardiac myocytes die and disappear from the tissue are not entirely clear. Kostin et al. described that in failing human hearts cardiac myocytes presented features associated to apoptosis, oncosis and autophagy [15]. We assessed if GD can induce autophagy in cultured cardiac myocytes. Modification of light chain 3 (LC3) I (apparent mobility, 18 kDa), (the mammalian homolog of yeast Atg8) with covalent attachment of phosphatidylethanolamine results in LC3-II (apparent mobility, 16 kDa), is a process essential for autophagy. The amount of LC3-II correlated with the number of autophagosome [30] and is a simple and quantitative method for determining autophagic activity of mammalian cells [31]. GD induced LC3-II formation in cultured cardiac myocytes, this marker was being detected from 2 up to 24 h after GD (Fig. 2A). This activation was similar to that obtained with rapamycin (100 nM), a well known autophagy activator [32]. LC3 was also the first mammalian protein localized to the autophagosome membrane and therefore, has been widely used as a marker for the detection of autophagosomes [33]. We examined the localization of LC3 in cardiac myocytes under GD. For this purpose, adenoviral transduced cells expressing GFP-LC3 were exposed to GD for 6 and 24 h and visualized by fluorescence microscopy. Fig. 2B shows that GD for 6 h induced the relocalization of GFP-LC3 from a diffuse to a punctuated pattern. Fig. 2C shows the quantification at 6 and 24 h of the cells with autophagic features induce by GD and rapamycin.

We next examined the ultrastructure of cardiac myocytes under GD to detect autophagy by transmission electron microscopy as indicated in Methods. The morphological characteristics demonstrated that GD induced autophagy. Fig. 2D depicts typical autophagic features in cardiac myocytes after GD for 18 h. Many small vesicles appeared in the cytoplasm and these compartments contained membranous structures. Higher magnification showed that most membrane vesicles possessed double or multiple membrane boundaries, with mitochondria or other cellular organelles inside. Taken together these results indicate that GD induces autophagy in cultured cardiac myocytes.

3.3. Aggresomes colocalize with autophagy markers

Aggregate-prone proteins are, in some instances, dependent upon autophagy for clearance [9,34]. Cytoplasmic inclusion bodies associated with neurodegenerative protein aggregates have shown to be susceptible to macroautophagic degradation [4,11,13,35,36]. To investigate whether cardiac myocytes with polyubiquitin aggregates are

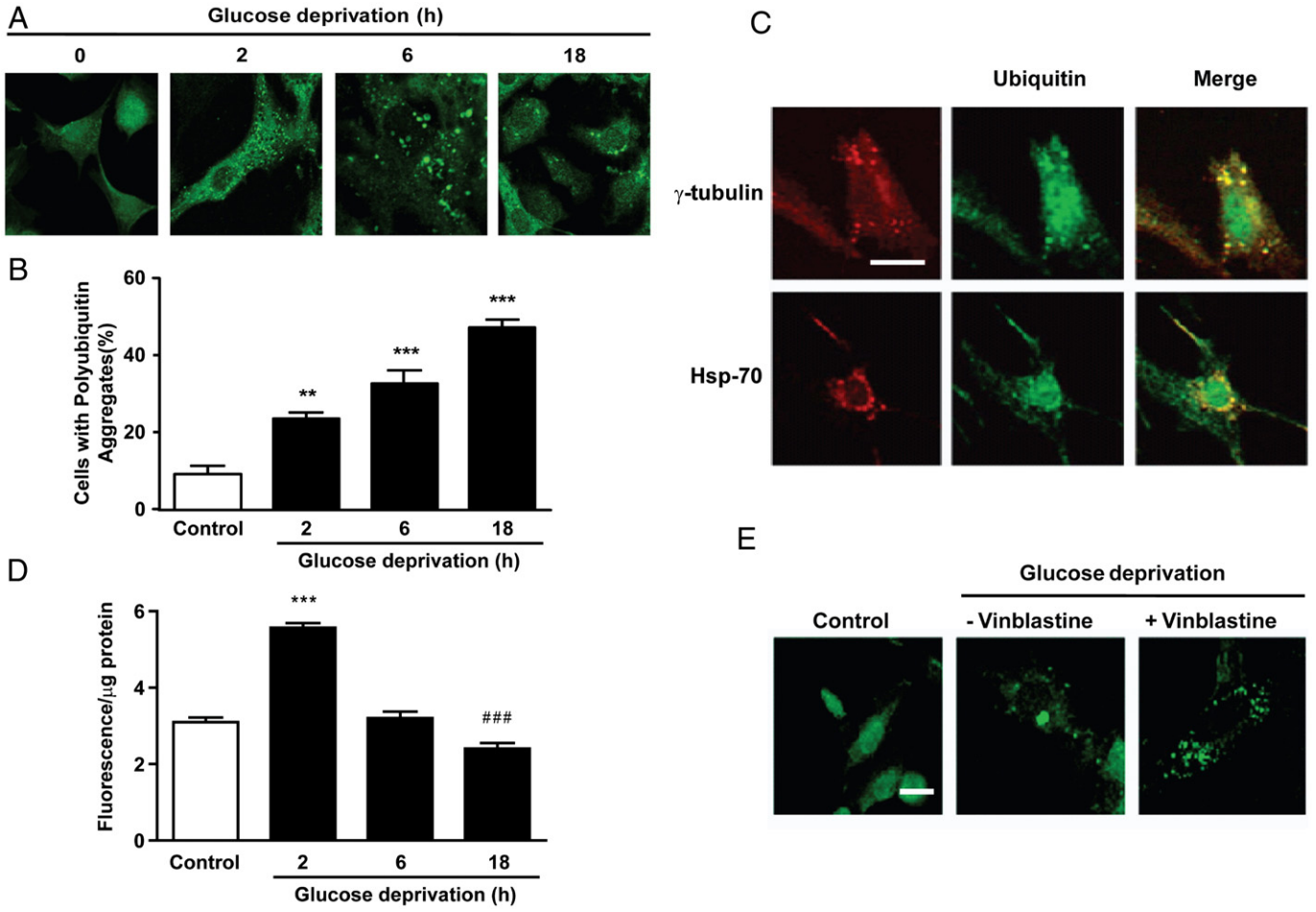

Fig. 1. Glucose deprivation (GD) stimulates aggresome formation in cultured cardiac myocytes. (A) Polyubiquitinated proteins were detected by immunofluorescence in cultured cardiac myocytes exposed to glucose withdrawal for 2–18 h. Short times of stimulus (2 to 6 h) induced small punctate structures while cytoplasmic bodies are formed with longer times of treatment (18 h). (B) Quantification of cells containing polyubiquitinated immunoreactive aggregates. Data are mean ± SEM of 3 independent experiments. In each condition, 150 to 250 cells were analyzed. $^{**}p<0.01$ and $^{***}p<0.001$ vs control. (C) Double immunofluorescence for the centrosome marker γ-tubulin or the heat shock protein Hsp70 (markers of aggresomes) and ubiquitin was studied by confocal microscopy in cardiac myocytes exposed to GD for 18 h. (D) Cardiac myocytes under GD for 18 h were incubated with Suc-LLVY-AMC fluorogenic substrate and then fluorescence was determined. Data are mean ± SEM. of 3 independent experiments. $^{***}p<0.001$ vs control and $^{\#\#\#}p<0.01$ vs GD for 2 h. (E) Cardiac myocytes under GD were treated or not with vinblastine (50 μM) and then polyubiquitin aggregates were visualized by immunofluorescence and confocal microscopy.

linked to the autophagic pathway, we performed double labeling for ubiquitin and autophagy markers. We examined by immunofluorescence the colocalization of ubiquitin and LC3 in cardiac myocytes under GD. For this purpose, adenoviral transduced cells expressing GFP-LC3 were exposed to GD for 18 h and ubiquitin was detected by indirect immunofluorescence. Fig. 3A shows that ubiquitin-labeled aggresomes colocalized with GFP-LC3 in nutrient-stressed cardiac myocytes. Rab GTPases are ubiquitous components of vesicle trafficking machinery, with different Rab proteins regulating traffic between different intracellular compartments [37]. Although the physiological relevance of Rab24 remains unknown, this GTPase might be involved in autophagy-related processes directing misfolded proteins from the ER to degradative pathways [38]. Furthermore, exogenously overexpressed GFP Rab24 translocated to autophagosomes in response to starvation and colocalized with GFP-LC3, suggesting that Rab24 is involved in the autophagy pathway [39,40]. To assess the participation of Rab24 in our system, endogenous Rab24 was immunodetected in cardiac myocytes exposed to GD (Fig. 3B). A remarkable increase in Rab24 and ubiquitin-immunoreactive punctuated pattern was observed in cardiac myocytes under GD. Ubiquitin and Rab24 colocalize in punctate structures after 6 h and both proteins colocalized in cytoplasmic bodies at longer ties (i.e. 18 h treatment), thus suggesting a link between ubiquitin accumulation and autophagy.

Autophagy is activated by class III phosphatidylinositol 3-kinases (PI3Ks). Thus, we used 3-methyladenine (3-MA), an inhibitor of PI3Ks to block autophagosome formation [36,41,42]. Inhibition of autophagy with 3-MA alone (10 mM) for 18 h increased the percentage of cardiac myocytes presenting the accumulation of ubiquitin-immunoreactive aggregates from 12.0 ± 3.0% to 33.1 ± 2.6% ($p<0.05$ vs control) (Fig. 3C). GD for 18 h increased the ubiquitin-positive cells to 47.5 ± 1.7% ($p<0.001$ vs control) but treatment with 3-MA (10 mM) did not further induced ubiquitin-positive aggregates (Fig 3C). However, incubation with the autophagy inductor rapamycin (100 nM) decreased the percentage of cardiac myocytes containing ubiquitin aggregates to 27.1 ± 4.6% ($p<0.05$ vs GD) (Fig. 3C). These results suggest that ubiquitin-immunoreactive aggregates are accumulated as a consequence of autophagy inhibition and decrease in cardiac myocytes exposed to GD when autophagy is activated.

3.4. GD increases ROS levels which are associated to aggresome formation and autophagy induction

Imbalance due to either, increased ROS production or a decrease in ROS degradation, can cause ROS accumulation and cell damage. ROS damage has shown to cause various types of cell death [22,24], but whether these reactive species play a role in autophagy cardiac

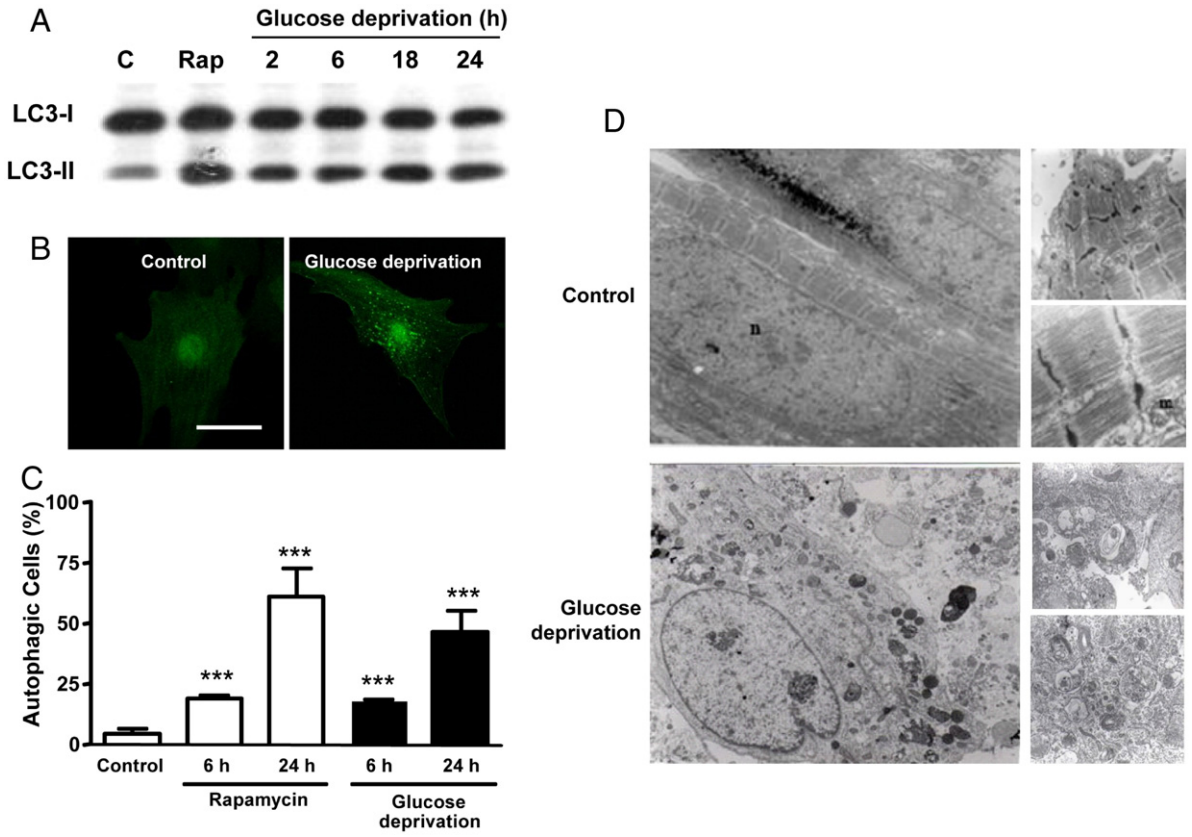

Fig. 2. Glucose deprivation (GD) induces autophagy in cultured cardiac myocytes. (A) Conversion of LC3-I into LC3-II. Cells were exposed to GD for 2–24 h and LC3 protein levels were determined by Western blot. Rapamycin (100 nM) was used as positive control. Figure shows a representative image from three independent experiments. (B) Cardiac myocytes were transduced with adenovirus GFP-LC3 with MOI = 60 for 24 h. Then, cells were exposed to a complete medium (control) or GD for 6 h. Representative pictures obtained by fluorescence microscopy are displayed. Bar corresponds to 20 μm. (C) Quantification of autophagic cells. Data are mean ± SEM. of 3 independent experiments. In each experimental condition, 150 to 250 cells were analyzed. $^{***}p<0.001$ vs control. (D) Ultrastructural characterization of cardiac myocytes under GD. Electron microscopic images were obtained from cells exposed to complete medium (control) or GD for 18 h. Then cells were prefixed in 3% glutaraldehyde and postfixed in 2% osmium tetraoxide. Typical autophagic features, membrane vesicles possessed double or multiple membrane boundaries, with mitochondria or other cellular organelles inside were observed.

myocyte has not been evaluated [43]. To test whether ROS participate in aggresome formation in nutrient-stressed cardiac myocytes, ROS production was measured in cells exposed to GD. For this purpose, we used dihydrorhodamine 123 which reacts with H_2O_2 forming a fluorescent compound [44].

GD for 2–24 h increased >2 fold ROS levels (Fig. 4A) and decreased GSH levels (Fig. 4B). N-acetylcysteine (NAC, 5 mM) reduces ROS formation and increases intracellular GSH levels in cultured cardiac myocytes under GD for 24 h (Fig. 4A–B). NAC also inhibited aggresome formation in cardiac myocytes under GD (Fig. 4C) and reduced Lysotracker Green-positive cells (Fig. 4D). Because GFP-LC3 specifically localizes in autophagosomes, this protein is in part degraded as autophagy take places. Therefore, variation in the total content of GFP-LC3 can be used as a direct marker for measuring autophagy occurrence. NAC also inhibited GFP-LC3 degradation, hence autophagy (Fig. 4E). Also, we assessed the effects of adenoviral overexpression of SOD and catalase on aggresome formation in cardiac myocytes exposed to GD. The results depict that overexpression of SOD1, SOD2 and catalase decreased the formation of aggresomes (supplementary Fig. 1). In addition, the antioxidant enzyme overexpression inhibits the Rab24 subcellular redistribution induced by GD (supplementary Fig. 1). Taken together these results imply that the increase in ROS levels induced by GD participates in both aggresome formation and activation of autophagy in cardiac myocytes.

Because Szegezdi et al. [45] have shown that simulated ischemia by serum, glucose and oxygen deprivation induces endoplasmic reticulum (ER) stress and the activation of the unfolded protein response (UPR) activation in neonatal rat cardiac myocyte cultures, we evaluated if GD stimulates these stress responses in our experimental model. The levels of BIP and CHOP, an ER stress and UPR markers, respectively, did not change in cultured cardiac myocytes exposed to GD. However, the ER stress inductor tunicamicyn used as a positive control increased CHOP levels (supplementary Fig. 2).

3.5. GD decreases ATP levels and induces cell death

Because neonatal cardiac myocytes utilize glucose and fatty acid as preferred sources for ATP production [46], we determined if GD alters total ATP content in cultured cardiac myocytes. GD for 24 h induced a 40% reduction in total ATP content (Fig. 5A). Treatment with 3-MA (10 mM) further decreased the total ATP content by 64% (Fig. 5A).

GD for 24 h did not induce cardiac myocyte apoptosis as determined by the presence of Sub-G1 population (Fig. 5B) and caspase 3 activation (data not shown). However, this stress condition induced a 3.3-fold increase in cardiac myocyte death ($p<0.05$ vs control), determined by propidium iodide incorporation in non-permeabilized cells by flow cytometry. Inhibition of autophagy with 3-MA further increased by 2 fold GD-induced cell death ($p<0.05$ vs GD cells) (Fig. 5C). Collectively, these results indicate that GD induces a non-apoptotic cardiac myocyte death and autophagy is a protective mechanism.

To examine the possible relationship among GD, ROS and cell death, cardiac myocytes were exposed to GD for 24 h in the presence or absence of NAC. Under this condition NAC increased 1.5 fold GD-induced cardiac myocyte death (Fig. 5D). These results together with those obtained in Fig. 4 show that ROS induces protection by activating autophagy and aggresome formation.

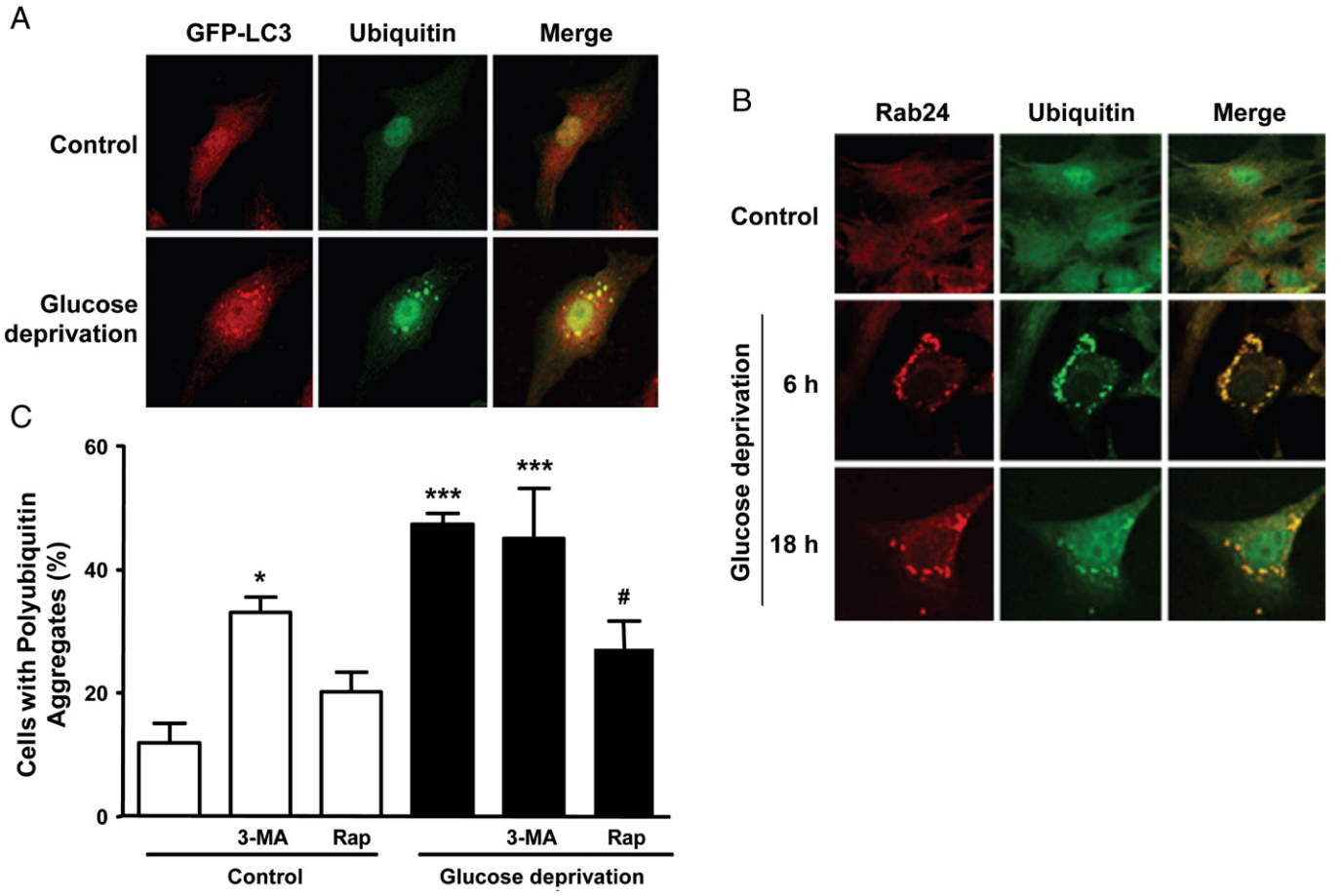

Fig. 3. Effect of autophagy modulators on aggresome formation induced by glucose deprivation (GD) in cultured cardiac myocytes. Autophagy markers were used to determine colocalization with aggresomes. (A) Cardiac myocytes were transduced for 24 h with adenovirus GFP-LC3 (Ad GFP-LC3) and then exposed to GD for 18 h. Ubiquitin was detected by indirect immunofluorescence. (B) Control or GD cells were stained for ubiquitin (green) or Rab24 (red) and then visualized by confocal microscopy. Colocalization of autophagy markers with ubiquitin increased in stressed cells. (C) Quantification of polyubiquitinated protein aggregates. Cardiac myocytes were exposed to complete medium or GD for 18 h in the presence or absence of 3-MA (10 mM), rapamycin (100 nM) or DMSO (vehicle). After treatment, cells were fixed and labeled with ubiquitin antibody. For each condition, 150 to 250 cells were counted in 3 independent experiments. Data are mean ± SEM. $^{*}p<0.05$ and $^{***}p<0.001$ vs control; $^{\#}p<0.05$ vs GD.

4. Discussion

Cumulative evidence indicate that autophagy is involved in many diseases [4,5,14,47], and recently, the participation in the most important cardiovascular pathologies (i.e. myocardial ischemia, heart hypertrophy, cardiomyopathies and hypertension) has also been reported [32]. In the present study we studied the formation of protein aggregates and their relationship with autophagy in primary cardiac myocyte cultures. Our results show that conditions that stimulates autophagy such GD leads to the formation of ubiquitin-labeled aggresomes. The close relationship between the ubiquitin/proteasome degradation system and autophagy in human hearts with chronic cardiomyopathy has been clearly demonstrated in early studies [15]. However, our results indicate that even at short times of treatment with the stressors (i.e. 2 h) the small aggregates, formed prior to the biogenesis of the large aggresomes, clearly colocalized with autophagy markers. These results indicate that besides the UPS, the autophagy pathway likely participates early on in protein aggregates clearance in cardiac myocytes.

Our results also show that in cells under GD, aggregates were strongly labeled by the protein Rab24. Although the function of Rab24 in the autophagic pathway has not been defined, it has been shown that this small GTPase partly colocalize with LC3 both in CHO culture cells subject to starvation [48] and in nerve-injured hypoglossal motor neurons of rats [39]. In injured neurons as well as in differentiated PC12 cells treated with the proteasome inhibitor MG132, the levels of expression of both Rab24 mRNA and LC3 were induced. Furthermore MG132 treatment caused an increase in the accumulation of the processed form of LC3 (i.e. LC3-II) indicating that autophagy-related events are activated in those situations. Indeed, autophagy induction and activation of the lysosomal pathway in response to impaired UPS activity has also been conveyed in others reports [35,49]. It is tempting to speculate that Rab24 may facilitate the transport of protein aggregates towards autophagic vacuoles via the microtubule network. Indeed, an interaction of Rab24 and tubulin has been reported by the yeast two-hybrid system [50].

The link between the UPS and autophagy has been described in many systems, but in general it has been postulated that the accumulation of protein aggregates subsequently activates the autophagy response [11,14,51]. Our data point that in our system both processes are likely activated in parallel, at very early times after the exposure to GD. We have also evidence that as early at 15 min several proteins are ubiquitinated (data not shown), indicating that both processes are rapidly activated in cardiac myocytes exposed to nutrient stress conditions.

Depending on cell type and aggregating protein, aggresomes may be cytoprotective or may contribute to cell death [52]. Indeed, the aggresome levels do not always correlate with disease. Adult cardiac myocytes face enormous challenges to correctly fold nascent polypeptides and to keep mature proteins from denaturing. The UPS is responsible for the degradation of short-lived proteins, such as cytosolic, nuclear, and myofibrillar proteins, whereas autophagy regulates

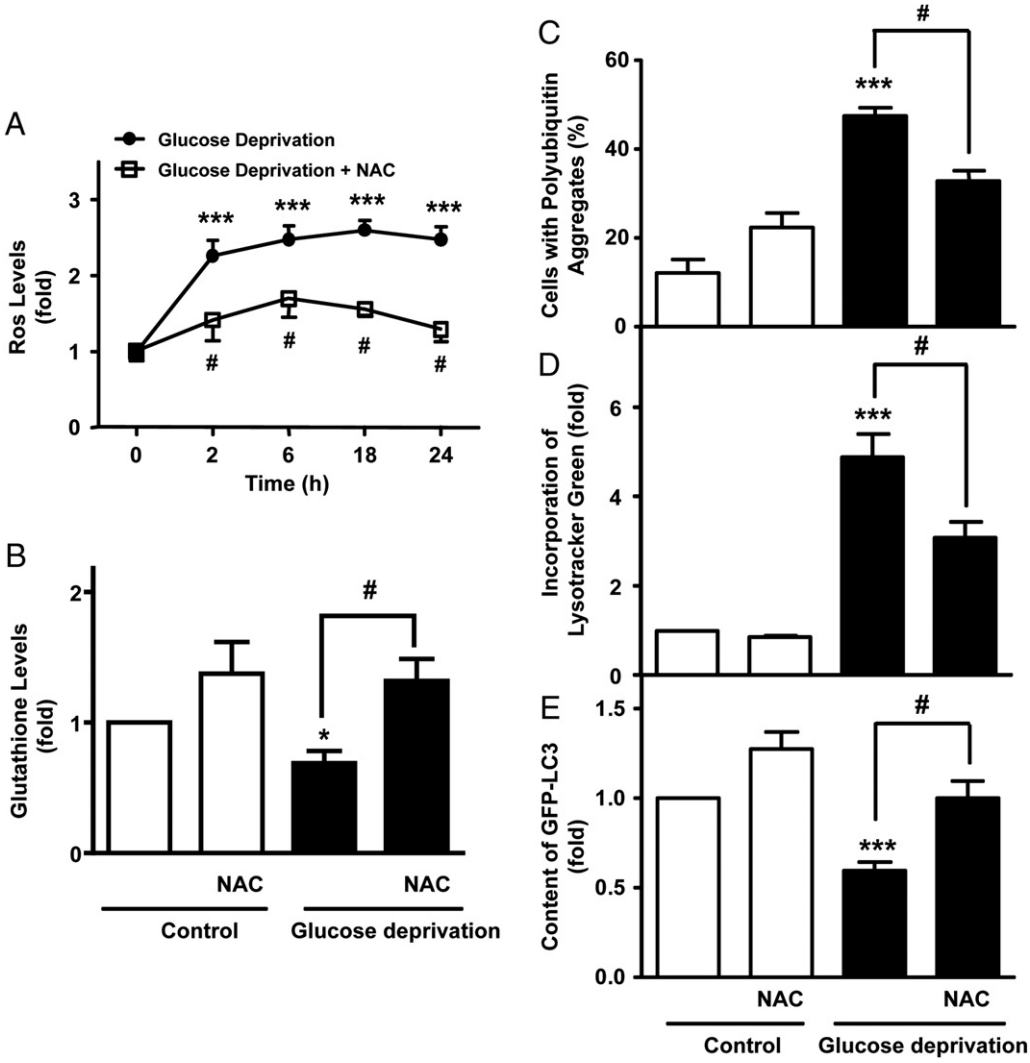

Fig. 4. N-acetylcysteine (NAC) attenuates the increase in ROS levels and the decrease in GSH levels induced by glucose deprivation (GD) in cultured cardiac myocytes. Cells were preincubated with or without NAC (5 mM) and then exposed to complete medium (control) or GD for 24 h. (A) Dihydrorhodamine 123 sensor (25 μM) was used to detect ROS levels by flow cytometry. (B) Total GSH intracellular levels were determined. (C) Cells were then fixed and polyubiquitinated proteins were detected by immunofluorescence. (D) Cells were incubated with the probe Lysotracker Green (100 nM) and fluorescence was analyzed by flow cytometry. (E) Cells were transduced for 24 h with adenovirus GFP-LC3 before exposure to NAC and/or GD for 24 h. GFP fluorescence degradation was analyzed by flow cytometry. Graphs are representative results obtained by flow cytometry. Data are mean ± S.E.M. of 3–5 independent experiments. $^{*}p<0.05$ and $^{***}p<0.001$ vs control alone or time 0; $^{\#}p<0.05$ vs GD.

levels of long-lived proteins and organelles [53]. The UPS and autophagy are generally considered to be two separate degradation pathways but recent findings have demonstrated that existence of a cross-talk between both degradative pathways. Cardiac myocytes with deficient autophagy have increased levels of ubiquitinated proteins in autophagosomes, indicating that autophagy may work in parallel with the UPS to turnover cellular proteins [53]. Moreover, cardiac-specific deficiency of Atg5 leads to cardiac hypertrophy, left ventricular dilatation and contractile dysfunction in adult mice [54]. Atg5-deficient hearts show increased levels of ubiquitination, disorganized sarcomeres and mitochondria l aggregation [54]. Given that the production of polyubiquitinated proteins is often increased during cardiomyopathy and chronic heart failure [55], it is likely that autophagy provides protection by degrading misfolded proteins and aberrant protein aggregates that may be toxic to cardiac myocytes. Indeed, pressure overload promotes accumulation of polyubiquitinated protein aggregates in the left ventricle, development of aggresome-like structures, and a corresponding induction of autophagy [19]. In addition, Sanbe et al. have shown that blocking aggresome formation results in increased levels of toxic amyloid oligomer and decreased cardiac myocyte viability [56]. Recently, Kanamori et al. show that ubiquitin and polyubiquitinated substrates in the adult heart were increased by starvation and furthermore, augmented by the treatment with an inhibitor of lysosomal degradation [57]. In our system we present evidence that aggresome accumulation and autophagy were induced by GD and these aggresomes colocalized with GFP-LC3 and Rab24, both markers for autophagy.

Our data showed that GD causes oxidative stress because induces ROS and decreases intracellular GSH levels. We have not attempted to identify the sites of ROS generation in this study. However ROS can be formed in the heart by several mechanisms; they can be produced by xanthine oxidase, NAD(P)H oxidases, cytochrome P450; by autooxidation of catecholamines; and by uncoupling of NO synthase [58–62]. In other cellular models, GD seems to induce ROS by different mechanisms. In cerebellar granule neurons, Isaev *et al*. reported that inhibitors of mitochondrial electron transport significantly decreased neuronal ROS production induced by GD [63]. This effect was accompanied by a progressive decrease in the mitochondrial membrane potential and an increase in free cytosolic Ca^{2+}. These results lead to conclude that GD-induced ROS production in neurons is related to potential-dependent mitochondrial Ca^{2+} overload. However GD for 48 h markedly increased H_2O_2 level in astrocytes that was in part

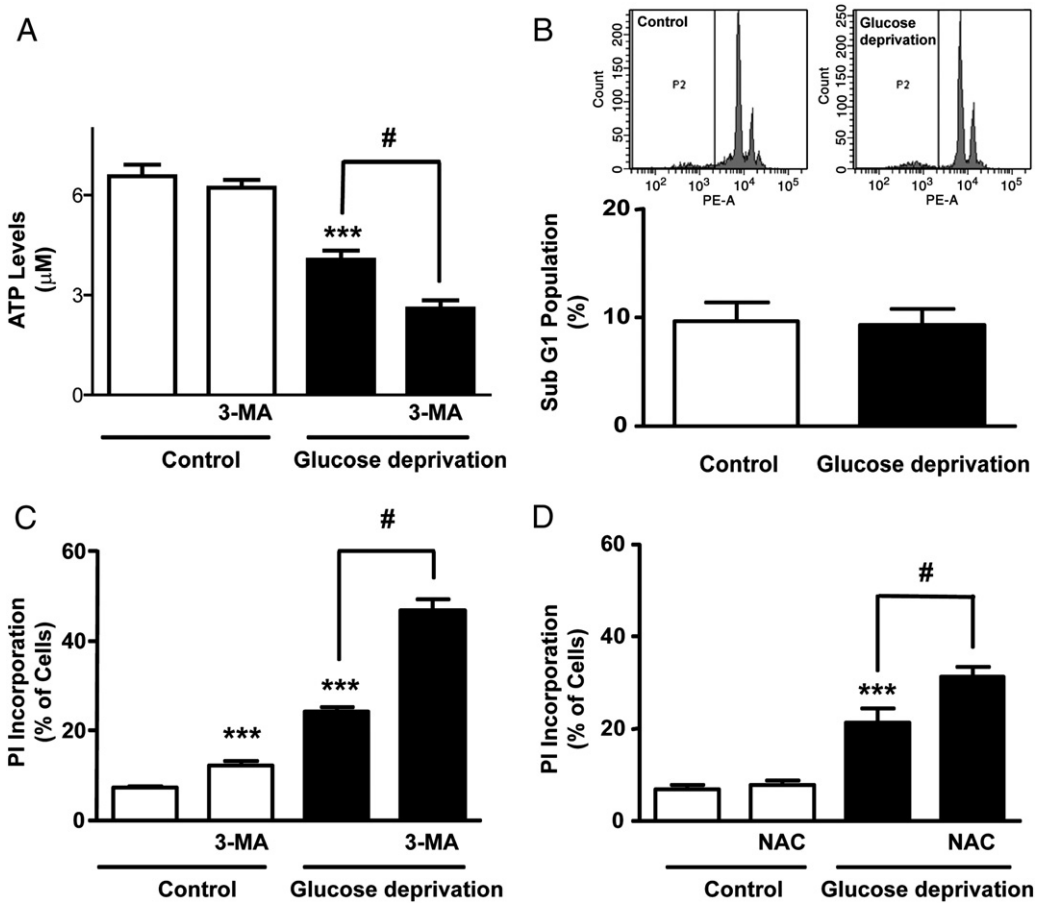

Fig. 5. Effect of NAC and 3-methyladenine (3-MA) on the decrease of intracellular ATP levels and stimulation of cell death induced by glucose deprivation (GD) in cultured cardiac myocytes. (A). Cells were preincubated with or without 3-MA (10 mM) and then exposed to GD for 24 h. Intracellular ATP levels were determined by luminescence using the luciferin/luciferase assay. (B) Cardiac myocytes were exposed to GD for 24 h. DNA laddering was determined by flow cytometry as a sub-G1 subpopulation in methanol permeabilized and PI labeled cells. Upper panel shows a representative sub-G1 population flow cytometry plot. Lower panel shows quantitative data. (C–D) Cells were preincubated with or without 3-MA (panel C) or NAC (panel D) and then exposed to GD for 24 h. After treatment, cells were labeled with PI and analyzed by flow cytometry. $^{***}p<0.001$ vs control; $^{\#}p<0.05$ vs GD.

prevented by catalase, mannitol, and NAC [64]. They also reported that the enhanced level of H_2O_2 in GD astrocytes appeared to be secondary to the depletion of reduced GSH and prevented by an NADPH oxidase inhibitor [64]. As described before, a link between cytosolic Ca^{2+} rise and ROS formation has been proposed [64]. Even though intracellular Ca^{2+} levels were not investigated in our study, an impairment of reticular calcium homeostasis is unlikely because GD does not induce endoplasmic reticulum stress in our model. However further studies should investigate if GD induces ROS in cardiac myocytes by one or several pathways and whether exist a link between Ca^{2+}-ROS.

High levels of ROS can oxidize many cell constituents, including lipids, proteins, and DNA, and thus impose a threat to cell integrity. Cells have evolved various defense mechanisms to cope with oxidative stress, among which autophagy plays a major role [65]. Although autophagy is largely considered nonselective, there is accumulating evidence for specific autophagic processes in response to ROS. These include: a) the selective degradation of mitochondria, termed mitophagy, proposed to decrease the potential oxidative damage caused by defective mitochondria [66]; b) chaperone-mediated autophagy [67,68], suggested to exhibit higher efficiency in degrading oxidized substrate proteins than their unaltered counterparts [65,69]; and c) autophagy in plants, shown to act in the degradation of oxidized proteins following severe oxidative stress [70].

There are several evidences that ROS are important both in autophagy and in protein aggregate formation. Specialized literature has reported that superoxide is responsible for autophagy induction in response to starvation and glucose deprivation [71]. Our data show that NAC restores the decrease in intracellular GSH levels induced by glucose deprivation but does not induce a complete restoration in protein aggregation to control values, suggesting that ROS production could be occurring in parallel to autophagy and aggresome formation. However the adenoviral expression of antioxidant enzymes SOD and catalase attenuates stimulation of autophagy and aggresomes formation induced by glucose deprivation (see supplementary Fig. 1). In our system, GD induces ROS production that is required for aggresome formation, autophagy and cell viability.

The pathophysiological mechanisms involved in protein aggregation in the heart are more complex that it was initially thought because both oxidative stress and reductive stress have similar deleterious effects in protein aggregation. Rajasekaran et al. have shown that a transgenic mice overexpressing cardiac-specific mutations in human αB-crystallin gene recapitulate the cardiomyopathy in humans. Likewise these mice are under reductive stress due to a dysregulation of glucose-6-phosphate dehydrogenase activity [72].

In conclusion, the main results were: a) GD stimulated aggresome formation and stimulated autophagy in cultured cardiac myocytes with UPS impairment. b) GD also decreased intracellular GSH levels and triggered ROS production which could be associated with aggresome formation and the induction of autophagy. c) The inhibition of autophagy with 3-MA decreased ATP levels and increased cell death. d) The inhibition the GD-induced ROS production by NAC or

by the overexpression of antioxidant enzymes decreased aggresome formation, autophagy activation and increased cell death in cultured neonatal rat cardiac myocytes exposed to GD.

These results are important because GD mimics an *in vivo* condition like myocardial ischemia. However, a limitation of the present study is that autophagy and protein aggregation may be probably more important in the adult cardiac myocyte than in the neonatal cardiac myocyte. This point and whether ROS are required for autophagy and protein aggregation in the adult heart under hypertrophy or ischemic conditions remains to be determined.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbadis.2010.02.002.

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