GAPDH-knockdown reduce rotenone-induced H9C2 cells death via autophagy and anti-oxidative stress pathway

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**HIGHLIGHTS**

- GAPDH pre-knockdown by siRNA reduced rotenone-induced H9C2 cell death.
- That occurred via autophagy and anti-oxidative stress pathway.
- Underexpressing GAPDH was associated with a demonstrable preservation of ATP.

**ABSTRACT**

**Background:** GAPDH, well known for its house-keeping functions, has also been shown to be involved in cell injury, apoptosis and death under conditions of stress such as starvation, chemical injury and oxidative stress. This study examines the effect of GAPDH knockdown on cell injury in response to rotenone.

**Methods:** GAPDH was knocked down in H9C2 cardiomyoblasts using siRNA prior to exposure to rotenone (0 nM, 20 nM, 40 nM and 80 nM). Autophagy was detected by western blot for autophagy proteins (Beclin-1, Atg5, LC-3A/B and p62) and MDC staining for acidic substances. Pro-apoptosis protein and flow cytometry were used to assess cell apoptosis and death and intracellular ATP relative concentration was measured. Oxidant stress was assessed by measuring DCFH-DA, TBARS, GSH and SOD.

**Results:** In this study, GAPDH-knockdown enhanced autophagy in rotenone-induced H9C2 cells, decreased oxidant stress and increased antioxidant pathways; and reduced cell apoptosis and death. Furthermore, GAPDH-knockdown preserved cell energy.

**Conclusion:** siRNA-mediated GAPDH knockdown reduced rotenone-induced H9C2 cell death occurring via autophagy and anti-oxidative stress pathway. This study enriches the understanding of GAPDH pathophysiology role, and provides potential new therapeutic targets for cardiac disease states characterized by oxidative stress.

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1. Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is not just a mono-functional house-keeping protein but a multifunctional protein, involved in cell proliferation, differentiation and apoptosis as data shown by recent studies (Pierce et al., 2008; Sen et al., 2008). In addition to glycolysis, GAPDH has been shown to be involved in numerous physiological pathways such as membrane fusion and transport, microtubule assembly and polymerization, nuclear RNA output and DNA repair, gene post transcriptional modification, mitochondrial damage mediation and autophagy regulation and so on (Baek et al., 2008; Sen et al., 2009; Nakamura and Lipton, 2009; Krishnan et al., 2010). Under baseline conditions, there is less GAPDH expression in mitochondria. But under conditions of cell starvation, oxidative stress, radiation damage and other stresses, GAPDH expression is increased in the mitochondria. Under these conditions of stress, additional GAPDH...
is deposited on the mitochondrial outer membrane in association with voltage-dependent anion channels 1 (VDAC1), resulting in increased mitochondrial membrane permeability (MMP), swelling of the mitochondrial matrix and reduction of transmembrane voltage, finally contributing to caspase-independent cell death (CICD) (Pierce et al., 2008). Furthermore, GAPDH can translocate to the nucleus and up-regulate Atg5-Atg12 expression in CICD, enhancing cytoprotective autophagy, and thereby antagonizing apoptosis (Pierce et al., 2008; Colell et al., 2007; Jacquin et al., 2013).

Autophagy is a catabolic process that degrades long-lived proteins and damaged organelles by sequestering them into double membrane structures termed “auto-phagosomes” and fusing them with lysosomes. It is considered a protection mechanism for cells under most conditions of stress (Mizushima et al., 2008).

Rotenone is a common broad-spectrum insecticide that selectively inhibits mitochondrial complex I, inducing oxidative stress and cell death. It has been used to explore the mechanisms involved in autophagy. Rotenone can induce GAPDH accumulation in cell and promote the formation of intermolecular disulfide bonds between GAPDH molecules. This irreversibility oxidized GAPDH dimer and conjugated proteins of GAPDH associated with other pro-apoptotic proteins have been shown to contribute to cell death (Lee et al., 2012; Tristan et al., 2011). Rotenone’s effect on macroautophagy completion may also contribute to its neurotoxic potential (Tang et al., 2011; Yu et al., 2009; Mader et al., 2012). Some studies have reported that rotenone-induced toxicity can be prevented by enhancers of autophagy and be aggravated by inhibitors of autophagy. (Xiong et al., 2013).

In this study, we examine the effect of GAPDH silencing on rotenone-induced cell death and autophagy, with particular focus on ROS pathways.

2. Methods

2.1. Cell culture and chemical treatment

H9C2 cells (rat cardiac muscle cells) were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 1% L-glutamine, 1% antibiotic (penicillin and streptomycin) at 37°C under 5% CO2 in air. After transfection with GAPDH or scrambled siRNA (30 nM for 12 h; Entrez Gene ID, 24383; organism, rat; Refseq No., NM-017008; AM4650, Thermo Fisher Scientific Inc), cells were treated with a range of concentrations of rotenone (0 nM, 20 nM, 40 nM, 80 nM) for 24 h. DMSO (Sigma–Aldrich) was used as a solvent to dissolve rotenone, and as a vehicle control (0 nM rotenone). 1 mM pyruvate was added into transfection medium in both siRNA–GAPDH group and negative control.

2.2. Immunoblot analysis

Equal amounts of protein in each sample were loaded and separated by any-kD SDS-precast gel electrophoresis under reducing or non-reducing conditions after homogenized in a buffer. Then, they
were transferred to PDVF membranes and blocked in 5% non-fat milk at least 2 h. Blots were hybridized with the following antibodies: a monoclonal GAPDH antibody at 1:1000 dilution (sc-365062, Santa Cruz, Inc.); a monoclonal LC-3A/B antibody at 1:1000 dilution (#4108S, Cell Signaling Technology, Inc.); a monoclonal Beclin-1 antibody at 1:1000 dilution (#3738S, Cell Signaling Technology, Inc.); a monoclonal Atg5 antibody at 1:1000 dilution (#2630S, Cell Signaling Technology, Inc.); a monoclonal p62 antibody at 1:1000 dilution (#5114S, Cell Signaling Technology, Inc.); a monoclonal p53 antibody at 1:1000 dilution (sc-99, Santa Cruz, Inc.); and a monoclonal caspase-3 antibody at 1:1000 dilution (sc-271028, Santa Cruz, Inc.); and reacted with the corresponding secondary antibody. α-tubulin and β-actin protein were used as loading and internal control.

Fig. 3. Relative fluorescence density of cytoplasmic MDC. A. Arrow shows cytoplasmic acidophil particle in rotenone group. (×400) B. Rotenone induced an increase in intracellular acidophil particles and relative fluorescence density of MDC in two groups. (×200) p < 0.05 was considered statistically significant, n = 3.
2.3. MDC staining

Cells were stained with monodansylcadaverine (MDC), a fluorescent dye, which interacts with lipids on autophagic vacuole membranes. Cells were examined under fluorescence microscopy (maximum excitation 335 nm, maximum emission 518 nm) (Tiwari et al., 2014). Analysis software was used for relative fluorescence intensity. For each group, 3 regions of interest were selected on

Fig. 4. Representative western blots for Beclin-1, Atg5 and (A–B) LC3A/B expression under different concentrations of rotenone in negative control group and siRNA–GAPDH group for 24 h. p < 0.05 was considered statistically significant, n = 3.
3 independent slides. 0 nM negative control was adjust as standard regulation and nucleus fluorescence was excluded.

2.4. ROS and antioxidants measurement

2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) is de-esterified intracellularly and turns to highly fluorescent 2′,7′-dichlorofluorescein upon oxidation by cellular esterases. Intracellular levels of oxidative stress are reflected by DCF fluorescence intensity (excitation wavelength 485 nm, fluorescence wavelength 530 nm) (Eruslanov and Kusmartsev, 2010; Annangi et al., 2014). We used 2 μM DCFH-DA in 2% bovine serum albumin (BSA) (37°C for 20 min) to measure fluorescent intensity by flow cytometry. 2% BSA without DCFH-DA was used as control in 37°C for 20 min.

GSH (glutathione), Thiobarbituric acid reactive substances (TBARS) and super oxide dismutase (SOD) was measured in each group according to the introduction of GSH assay kit (Jiancheng, Nanjing, China), TBARS Assay Kit (Cayman Chemical Company) and SOD Assay Kit (Dojindo).

2.5. Detection of cellular energy supply

Relative adenosine triphosphate (ATP) concentration was measured by ATPlite Luminescence Assay System (PerkinElmer, USA).

2.6. Cell apoptosis measurement

1′,1′-Di-n-butyl-3,3′,3′-tetramethylindotricarbocyanine (DiLC5) (excitation wavelength 633 nm) is a cationic cyanine dyes binding with mitochondrial membrane protein to reflect mitochondrial membrane potential and membrane potential change, which can be used as an indicator to differentiate live, apoptosis and death cell (Janko et al., 2013). Propidium iodide (PI) (excitation wavelength 488 nm) is a nuclear DNA-binding dye reagents widely used for apoptosis detection. PI does not enter into live cells, but can bind with DNA when cell membrane permeability is increased (Janko et al., 2013). 2 μL DiLC5 (2μM) and 2 μL PI (10 mg/mL) were added into 500 μL 2% BSA cell suspension medium for 20 min in 37°C prior to assessment by flow cytometry.

2.7. Statistical analysis

All data are expressed as mean ± standard (X±s). p < 0.05 was considered statistically significant. According to the homogeneity of variance, LSD test or Games–Howell test was used. ANOVA was used to examine differences between the two groups in SPSS13.0 statistical analysis software.

3. Results

3.1. Rotenone-induced abnormal GAPDH expression detected by non-reducing western blot

Consistent with previous studies demonstrating stress-induced increases in abnormal GAPDH aggregates, we observed increased aggregates of GAPDH in H9C2 cells induced by different concentrations of rotenone (0 nM, 20 nM, 40 nM and 80 nM) for 24 h (Lee et al., 2012; Tristan et al., 2011; Huang et al., 2009). This is illustrated in Fig. 1.
where the 37 kD blot line represents normal GAPDH, and the 70 kD blot line represents abnormal GAPDH aggregates. Furthermore, the ratio of abnormal aggregates of GAPDH to normal GAPDH was significantly increased in response to rotenone at concentrations of 20–80 nM, peaking at a concentration of 40 nM. The lower level of GAPDH at the highest rotenone concentration of 80 nM may reflect blockade of mitochondrial respiration, inhibition of cellular protein synthesis and cell death (Lee et al., 2012; Tristan et al., 2011; Huang et al., 2009).

Fig. 7. ROS and TBARS measurement in H9C2 cells exposed to different concentrations of rotenone, with and without GAPDH silencing. $p < 0.05$ was considered statistically significant, $n = 3$. 
3.2. Effect of GAPDH silencing

Total GAPDH expression in H9C2 cells was decreased by GAPDH specific siRNA transfection to <10% of the scrambled control, as shown in Fig. 2 (p < 0.05, n = 3).

3.3. GAPDH-knockdown enhanced rotenone-induced autophagy in H9C2 cells

The autofluorescent compound MDC was used for in vitro labeling of autophagic vacuoles. Under baseline conditions, only the nucleus of the H9C2 cells was positively stained (Fig. 3). In contrast, rotenone induced speckled staining in the cytoplasm consistent with autophagy (Fig. 3A). In cells exposed to 20–80 nM rotenone, silencing of GAPDH resulted in significant increases in MDC vacuolar staining, as shown in Fig. 3B (p < 0.05, n = 3). There was no significant increase in MDC staining in cells not exposed to rotenone.

We next examined expression of autophagy proteins Beclin-1, Atg5, LC-3 and p62. (Figs. 4 and 5)

LC-3 is an indispensable component involved in autophagosome assembly and disassembly. The ratio of intracellular LC-3II and LC-3I, and their total expression levels has been shown to reflect autophagy activity and level respectively (Mizushima et al., 2008). As shown in Fig. 4A, silencing of GAPDH resulted in a significant increase in the ratio of LC-3II to LC-3I at all concentrations of rotenone tested (p < 0.05, n = 3). In contrast, when we examined total autophagy, as reflected by total grey density of LC-3I and LC-3II, silencing of GAPDH resulted in significant increases only in cells exposed to 20 nM and 40 nM rotenone in Fig. 4B. In cells exposed to 80 nM rotenone, we observed a reduction in total grey density of LC-3I and LC-3II after GAPDH silencing in Fig. 4B.

Beclin-1 is recognized as a key factor at every step of the autophagy pathway, from the formation to the maturation of the autophagosome. With siRNA–GAPDH preconditioning, Beclin-1 was significant higher than that of negative control group in rotenone-exposed H9C2 cells (p < 0.05, n = 3). In contrast, there was no obvious effect of siRNA–GAPDH on Beclin-1 in cells not exposed to rotenone (p > 0.05, n = 3), as shown in Fig. 4C.

Atg5, which is activated by Atg7, combines with Atg12. This complex is key for LC-3I transformed to LC-3II. In our study, Atg5 expression in the two groups was similar to the pattern of Beclin-1 expression presented in Fig. 4D.
p62 protein, another marker of autophagy signaling, contacts with LC3 and ubiquitin to form a larger aggregate involved in the degradation of autophagosomes. p62 expression was found to be negatively correlated with the level of autophagy in Fig. 5. Silencing GAPDH substantially increased p62 expression compared with scrambled control in cells treated with 20 nM, 40 nM and 80 nM rotenone treatment ($p < 0.05$, $n = 3$).

3.4. GAPDH-knockdown activates antioxidant mechanisms and decreases cellular ROS

We next examined the effect of silencing GAPDH on cellular protective mechanisms against oxidant stress (SOD expression and GSH levels), and markers of cellular oxidative stress (ROS and TBARS). As shown in Fig. 6, knockdown of GAPDH resulted in a significant increase in the expression of SOD, and levels of GSH in cells exposed to 40 nM and 80 nM rotenone treatment ($p < 0.05$, $n = 3$).

3.5. GAPDH-knockdown reduces cell apoptosis and death

p53 and caspase-3 mediate cell apoptosis and death. As shown in Fig. 8, silencing GAPDH decreased p53 expression, and decreased the ratio of active-caspase-3 to pro-caspase-3, signifying a key role of GAPDH in apoptosis. This was further supported flow cytometry of DiCl5 and PI loaded cells. As shown in Fig. 9, siRNA–GAPDH decreased cell apoptosis and death, with a reciprocal increase in viability compared to control.

3.6. GAPDH-knockdown preserves cell energy

We next examined the effect of GAPDH-knockdown on levels of intracellular ATP as an indicator of available cell energy. At low concentrations of rotenone, GAPDH knockdown had no significant effect. However, at 40 nM and 80 nM of rotenone, silencing of GAPDH significantly increased the relative ATP concentration, as shown in Fig. 10.
In the process of physiological aging of cells, detection of protein p62 using autophagosomes was a result of an increase in autophagy activity, with corresponding reduction in cellular apoptosis and necrosis. Interestingly, the increase in autophagy seen in cells silencing GAPDH was associated with a demonstrable preservation of the cells’ key energy source, ATP. That could be due to mitochondrial oxidative phosphorylation and the pyruvate compensate in our study. And autophagy activation can preserve more energy (Ong and Gustafsson, 2012).

In summary, silencing of GAPDH pre-knockdown could reduce the intracellular levels of reactive oxygen species and lipid peroxidation, enhance autophagy activity, and thereby reduce the rotenone-induced myoccardial injury, apoptosis and necrosis. This study provides a new therapeutic target of rotenone-induced myocardial cell injury and enriches the understanding of GAPDH pathophysiology role in vitro.

4. Discussion

In this study, we have demonstrated a critical role of GAPDH in cellular oxidative stress, energy supply, and cell death, with silencing of GAPDH resulting in a protective effect. These findings suggest the potential value of therapies targeting GAPDH in disease states characterized by elevated oxidative stress.

Rotenone has been shown to inhibit mitochondrial complex I, blocking respiration and resulting in cell apoptosis, a process which can drive GAPDH protein misfolding and aggregation (Huang et al., 2009). Further research has demonstrated GAPDH can bind with pro-apoptosis proteins to promote cell apoptosis under conditions of oxidative stress (You et al., 2013; Nakajima et al., 2009). We have confirmed the effect of rotenone to increase abnormal GAPDH aggregation, and proceeded to examine the role of GAPDH in rotenone-induced injury. We demonstrate the novel finding that silencing of GAPDH is protective against rotenone-induced oxidative stress, apoptosis and cell death. And as data shown by recent studies and our study, GAPDH knockdown could survive cell in a short time without effect on its primary role as housekeeping gene (You et al., 2013; Nakajima et al., 2009; Zhai et al., 2013).

Autophagy is considered a self-protective mechanism for the cell (Matsui et al., 2007; Gurusamy et al., 2009). In this study, silencing GAPDH increased the rate of autophagy, increased the expression of autophagy-associated proteins Beclin-1, Atg5 and LC-3, and increased the number of autophagosomes. Furthermore, this increase in protein markers of autophagy, and number of autophagosomes was a result of an increase in autophagy activity, not dysfunction of autophagy metabolism, as evidence by detection of protein p62 using flow cytometry.

Oxidative stress plays an important role in cell apoptosis and autophagy. In the process of physiological aging of cells, autophagy activation assists in degradation of oxidative material in the cell (Donati et al., 2001; Martin and Barrett, 2002). However, in radiation injury, chemical damage, atherosclerosis and ischemia-reperfusion or other pathological conditions, reactive oxygen species (ROS) interacts with the key molecules (lipids, proteins and nucleic acids, etc.) in cells resulted to cell structural integrity destroy, toxic injury, mutant change and cell necrosis. Elevated ROS in the mitochondria drives lipid peroxidation, mitochondrial DNA mutation, mitochondrial membrane permeability and opening of mitochondrial channels which can further increase intracellular ROS level and promote some of the pro-apoptotic substances (p53 and caspase-3) to be released from the mitochondria (Zorov et al., 2000). Previous research has indicated that accumulation of abnormal aggregations of GAPDH may increase the permeability of the mitochondrial membrane, and activate the mitochondrial voltage-dependent ion channels, inducing an increase in mitochondrial membrane potential and induction of apoptosis (Tarze et al., 2007). In this study, we demonstrate that ROS levels in GAPDH knockdown cells do not change significantly under baseline conditions. However when cells are exposed to rotenone, silencing of GAPDH results in less accumulation of abnormal GAPDH aggregates in cells, lower intracellular ROS levels, reduced lipid peroxidation, and enhanced autophagy activity, with corresponding reduction in cellular apoptosis and necrosis. Interestingly, the increase in autophagy seen in cells silencing GAPDH was associated with a demonstrable preservation of the cells’ key energy source, ATP. That could be due to mitochondrial oxidative phosphorylation and the pyruvate compensate in our study. And autophagy activation can preserve more energy (Ong and Gustafsson, 2012).

In summary, silencing of GAPDH pre-knockdown could reduce the intracellular levels of reactive oxygen species and lipid peroxidation, enhance autophagy activity, and thereby reduce the rotenone-induced myocardial injury, apoptosis and necrosis. This study provides a new therapeutic target of rotenone-induced myocardial cell injury and enriches the understanding of GAPDH pathophysiology role in vitro.

Conflicting of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Transparency document

The Transparency document associated with this article can be found in the online version.

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