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Recombinant asialoerythropoetin protects HL-1 cardiomyocytes from injury via suppression of Mst1 activation

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ABSTRACT

Background: Recombinant human erythropoietin (rhuEPO) and asialoerythropoietin (asialo-rhuEPO) are cardioprotective. However, the protective effects of rhuEPO could not be translated into clinical practice because of its hematopoiesis-associated side effects while non-erythropoietic asialo-rhuEPO is unavailable in large quantities for clinical studies. This study was designed to investigate the cardiomyocyte protective potential of plant-produced asialo-rhuEPO (asialo-rhuEPO^{P}) against staurosporine (STS)-induced injury in HL-1 murine cardiomyocytes and identify cellular pathway(s) responsible for its cardioprotection.

Methods: HL-1 cardiomyocytes were simultaneously treated with STS and asialo-rhuEPO^{P}. Cellular injury, apoptosis, and cell viabilities were measured by LDH assay, Hoechst staining and trypan blue exclusion method, respectively while western blotting was used to study its effects on apoptosis and autophagy hallmarks.

Results: Our results showed that 20 IU/ml asialo-rhuEPO^{P} provided 39% protection to cardiomyocytes compared to STS-treated cells, which is 2-fold better than that of mammalian cell-produce rhuEPO (rhuEPO^{M}). Asialo-rhuEPO^{P} was found to suppress activation of proapoptotic kinase Mst1 (mammalian Sterile-20-like kinase 1) and FOXO3, leading to inhibition of apoptotic pathway and restoration of autophagy as indicated by the reduction of fragmented/condensed nuclei, altered ratios of Bax/Bcl2, p-Bad/Bad, cytosol/mitochondrial cyt *c* and caspase-3 activation, and the restored levels of autophagy markers Beclin1, p62 and LC3B-II. Additionally, Akt was found to be activated and FOXO3 was phosphorylated on Ser253, suggesting inhibition of FOXO3 transcriptional function.

Conclusions: Asialo-rhuEPO^{P}-mediated cardioprotection occurs through activation of PI3K/Akt pathway leading to suppression of Mst1 activation and promoting cardiomyocyte survival.

General significance: Asialo-rhuEPO^{P} could be used to modulate Mst1 activity elevated under numerous pathological states.

1. Introduction

Cardiovascular diseases are the leading cause of death in the US with an average of 1 death every 39 s [1,2]. Cardiomyocyte injury and apoptosis resulting from various disease conditions and treatments are a major cause of morbidity and mortality [3]. When myocardial infarction occurs, for example, timely reperfusion is the most effective approach to provide the survival benefit to the ischemic myocardium [2,4]. However, not only ischemia but also reperfusion can cause cardiomyocyte injury and apoptosis because the latter generates free radicals that induce further damage [2,4]. Apoptosis therefore, plays a major role in inflicting damage to the heart following myocardial infarction. To block apoptosis and protect the myocardium from injury, interventions involving pre- or post-conditioning or using pharmacological agents have been proposed [2,4,5]. Although numerous compounds targeting different pathways involved in myocardial injury have been tested, identification of an effective agent that would limit myocardial injury from apoptotic cell death remains a challenge [5,6]. The discovery of new therapeutics, directly targeting the apoptotic process to prevent and impede cardiomyocyte injury and apoptosis, is highly desired for the treatment of myocardial infarction as well as other cardiovascular diseases.

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Mammalian sterile-20-like kinase 1 (Mst1), a ubiquitously expressed serine/threonine kinase and component of the Hippo signaling pathway, has an important role in regulating apoptosis besides controlling cell proliferation and organ size [7–9]. During cardiomyocyte apoptosis, Mst1 is strongly activated to promote cardiac dysfunction, whereas the suppression of Mst1 inhibits apoptosis [10,11]. More recently, Mst1 was proved to act as a switch to regulate apoptosis and autophagy dually during ischemic stress in mouse hearts [12]. Both autophagy and apoptosis are important ancient processes, which regulate cell fate under normal and disease conditions [13]. The former occurs through removing damaged organelles and protein aggregates to maintain organelle function and control protein quality [14] while the latter is activated through programmed cell death to eliminate old, unnecessary, and unhealthy cells to prevent releasing harmful substances into the surrounding area [15]. In the context of the heart, maintaining a certain physiological range of autophagic activity is critical because either excessive or inadequate activity is harmful [16,17]. During myocardial infarction, activation of autophagy during an initial stage of ischemia was found to be protective whereas late or delayed activation of autophagy during reperfusion is harmful [18]. Because of its important role in apoptosis and autophagy, Mst1 was suggested as an important therapeutic target for the discovery of new drugs/therapies to prevent cardiomyocyte death [12]. To date, no therapeutics has been identified to regulate the activity of Mst1 for the purpose of cardiomyocyte survival.

Among the tested cardioprotective compounds, a glycoprotein hormone erythropoietin produced in mammalian cell (rhuEPO^{M}), best known for its regulatory role in red blood cell (RBC) production, has been reported to have cardioprotective function in in vitro and in vivo animal models of ischemia/reperfusion (I/R) injury [19,20]. However, its cardioprotective effects for reducing myocardial infarct size from I/R injury could not be observed consistently in clinical studies [5]. The therapeutic application of rhuEPO^{M} for cytoprotection was also tampered by observed adverse effects, such as thrombosis and hypertension, associated with its hematopoietic activity [21–24]. Moreover, higher doses of EPO are needed for cytoprotective purpose, which can result in massive production of RBCs because of unwanted stimulation of erythropoiesis, causing more damage [25]. Therefore, cytoprotective EPO derivatives lacking hematopoietic activity are desired for cytoprotective purposes.

Several strategies have been employed to develop EPO derivatives that retain cytoprotective function, but lack hematopoietic activity [26–29]. Among them, asialo-rhuEPO prepared by enzymatic removal of sialic acid residues of rhuEPO^{M} was found to be non-erythropoietic but tissue protective in stroke, sciatic nerve injury, spinal cord compression and I/R injury [26,29–31]. However, the cost and limited availability of asialo-rhuEPO through enzymatic desialylation were major hurdle in translating its medical benefits. To overcome this, we took advantage of an inexpensive plant-based expression to produce asialo-rhuEPO by stably co-expressing human *EPO* and β1,4-galactosyltransferase (*GalT*) genes in tobacco plants [32,33]. We discovered that plant-produced asialo-rhuEPO (asialo-rhuEPO^{P}) provides better protection to neuronal-like mouse neuroblastoma cells (N2A) [33] and pancreatic beta-cells [34] than rhuEPO^{M}. In pancreatic beta cells, suppression of Mst1 by asialo-rhuEPO^{P} was observed [34]. In the present study, we intended to investigate the cardioprotective potential of asialo-rhuEPO^{P} as well as to determine whether it can suppress Mst1 activation and benefit cardiomyocyte survival in detail. Asialo-rhuEPO^{P} was found to be not only cytoprotective toward cardiomyocytes but also displaying 2-fold better cytoprotective effects than rhuEPO^{M}. Our studies revealed that asialo-rhuEPO^{P}-mediated protective effects occur via activation of the PI3K/Akt pathway leading to suppression of Mst1 activation and FOXO3 thereby promoting cell survival by inhibiting apoptosis and restoring autophagy.

2. Materials and methods

2.1. Materials

Asialo-rhuEPO^{P} was purified from transgenic tobacco line A56-5 [33] using two-step procedure as described previously [35]. The number of asialo-rhuEPO^{P} units was calculated from protein concentration as described by Erbayraktar et al. [26]. Cell culture grade mammalian cell-produced sialylated EPO (rhuEPO^{M}) was purchased from R & D Systems (Minneapolis, MN, USA). Claycomb medium, FBS, norephinephrine, STS and HOECHST 33342 stain were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Lactate dehydrogenase (LDH) assay kit was purchased from Roche (Indianapolis, IN, USA). M-PER mammalian protein extraction reagent, Halt™ protease and phosphatase inhibitor cocktail were obtained from Thermo Fisher Scientific (Grand Island, NY, USA). Bradford reagent was purchased from Bio-Rad laboratories (Hercules, CA, USA). Antibodies for detection of Bcl-2, Bax, p-Bad/Bad, cytochrome *c* (cyt *c*), caspase-3, Mst1, total FOXO3, p-Ser253-FOXO3, p-JAK2/JAK2, p-Akt/Akt, p62, Beclin1, LC3B, tubulin and VDAC were purchased from Cell Signaling (Boston, MA, USA) except anti-p-Ser207 FOXO3 (Zhenjiang Hope Biotechnology Co, Zhenjiang, China) and anti-β-actin (Sigma-Aldrich). West Pico chemiluminescent substrate for western blotting was purchased from Thermo Fisher Scientific.

2.2. HL-1 cell culture

HL-1 murine cardiomyocytes were received as a gift from Prof. William C. Claycomb, Department of Biochemistry & Molecular Biology at Louisiana State University Health Sciences Center, New Orleans, USA. HL-1 cells were routinely cultured in Claycomb medium containing 10% FBS, 100 U/ml penicillin/streptomycin, 0.1 mM norepinephrine and 2 mM L-glutamine, and maintained at 37 °C in 5% CO_2 in a humidified atmosphere [36]. HL-1 cells were used for various experiments only after they reached ~80% confluence. To induce apoptosis and to perform cytoprotection assays, HL-1 cells were cultured in Claycomb medium containing 0.2% FBS, 100 U/ml penicillin/streptomycin, 0.1 mM norepinephrine and 2 mM L-glutamine.

2.3. STS dose-response stimulation curve

To establish the EC_{50} value of STS for induction of cellular damage and apoptosis, HL-1 cells (2.0×10^5) were cultured in 48-well cell culture plates in Claycomb medium containing 0, 175, 350, 525, 700 or 875 nM STS. After 24 h treatment, LDH activity was measured in the spent medium using a LDH assay kit according to the manufacturer's protocol. Each treatment was performed in six wells representing six replicates. The test was repeated three times as three biological replicates. The average percentage of cellular toxicity from three batches was then plotted against STS concentrations to obtain the EC_{50} value using GraphPad Prism software.

2.4. In vitro cytoprotection assay

For EPO-mediated cytoprotection assay, HL-1 cells were seeded at a density of 2.0×10^5 in 48-well cell culture plates. Once cells reached ~80% confluence, they were then subjected to different treatments. For dose-response analysis, they were treated with 175 nM STS alone, or simultaneously with 10, 20, 30, 40 and 50 IU/ml asialo-rhuEPO^{P} for 24 h. For comparison of protective effects of asialo-rhuEPO^{P} with commercial rhuEPO^{M}, cells were treated simultaneously with 20 IU/ml asialo-rhuEPO^{P} or rhuEPO^{M} in PBS containing 0.1% BSA and 175 nM STS, which were directly added to the Claycomb medium (with 0.2% FBS). As a vehicle control, same volume of PBS containing 0.1% BSA was added to the medium. For STS alone treatment, 175 nM STS in PBS containing 0.1% BSA was included in the medium. After 24 h treatment,

cellular damage was assessed with LDH assay kit. Each test was performed using six replicates. Assay was repeated three times as three biological replicates.

2.5. Cell morphological changes and quantitative analysis of apoptotic nuclei

To observe morphological changes and analyze apoptotic nuclei, HL-1 cells were seeded at a density of 2.0×10^5 in 48-well cell culture plates till ~80% confluence before applying treatments. Following 24 h treatments with the vehicle, STS alone, STS + rhuEPO^M or STS + asialo-rhuEPO^P as described above, cell morphological changes were first observed using a Nikon Eclipse TS100 (Melville, NY, USA) inverted microscope. Light microscopy images were captured using NIS Elements imaging software. Cells were also stained with DNA-binding dye HOECHST 33342 to examine for morphological evidence of apoptosis. Briefly, cardiomyocytes grown in 48-well plates that were subjected to above treatments were rinsed with PBS and stained with 5 μg/ml HOECHST 33342 for 15 min. After brief rinse with PBS, stained cells were viewed under an Olympus IX51 fluorescence microscope (Waltham, MA, USA) to examine nuclear morphology. Cells were scored as apoptotic if they exhibited nuclear chromatin condensation/fragmentation. Each treatment was performed in three wells representing three replicates. About 250–300 cells were scored from each well. The experiment was repeated twice as two biological replicates.

2.6. Protein extraction and subcellular fractionation

For western blot analysis to understand EPO-mediated cytoprotective mechanism, HL-1 cells were seeded at a density of 8.0×10^5 in T-25 flasks. Once cells reached ~80% confluence, they were then treated with the vehicle, STS alone, STS + rhuEPO^M or STS + asialo-rhuEPO^P as described above. Cell viability was assessed using a Vi-CELL XR cell viability analyzer from Beckman Coulter (Fullerton, CA, USA) after 24 h treatment. Then cells from different treatments were collected. Total protein from HL-1 cells was obtained by lysis in M-PER buffer containing Halt™ protease and phosphatase inhibitor cocktail. Cellular fractionation to obtain mitochondrial and cytosolic fractions was performed as follows. About 5.0×10^6 cells were lysed in 300 μl of buffer 1 (250 mM sucrose, 137 mM KCl, 70 mM NaCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 200 μg/ml digitonin, 10X protease and phosphatase inhibitor cocktail) by performing five freeze-thaw cycles. The lysate was then centrifuged at 10,000 g for 10 min. The supernatant was saved as S1. The pellet was washed with buffer 1 and centrifuged at 10,000 g for 10 min. The resulting supernatant was saved as S2. The pellet was washed three times with buffer 1, and extracted with buffer 2 (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2% Triton X100, 0.3% NP-40, 10X protease and phosphatase inhibitor cocktail). The extract was centrifuged at 20,000 g for 15 min. The supernatant was used as mitochondrial fraction. For cytosolic fraction, the S1 and S2 supernatants were combined and centrifuged at 100,000 g for 30 min. The resulting clear supernatant was used as cytosolic fraction. Protein concentration was measured using Bradford reagent with BSA as a standard. The microassay format in 96-well flat bottom plate was employed as described by the manufacturer.

2.7. Western blot analysis

For western blot analysis, total protein (10–40 μg) or cytosolic/mitochondrial fractions (7 μg) were applied on 7.5–13.5% SDS-PAGE gels depending on the molecular size of targets. Electrophoresis was performed at 200 V, followed by transfer onto a PVDF membrane using Towbin as transfer buffer. After transfer, the blots were blocked with 5% BSA in 1X PBST for 1 h and then probed with antibodies indicated in material section. All antibodies indicated except anti-β-actin were used at a dilution of 1:1000 in 1X PBST containing 5% BSA. Anti-β-actin antibody was used at a dilution of 1:2000. Following incubation with primary antibodies, blots were washed and incubated with 1:2000 diluted HRP-conjugated secondary antibodies for 1 h at room temperature. SuperSignal West Pico chemiluminescent substrate was used to visualize protein bands. In the case of detection of phosphorylated protein targets, the blots were first probed with phosphoprotein-specific antibodies, followed by stripping and re-probing with antibody specific for their unphosphorylated versions. The blots were then stripped and re-probed with anti-β-actin antibody as a loading control. In the case of mitochondrial fraction, anti-VDAC antibody was used as a loading control. The densities of western blotting bands were analyzed using UVP GelDoc-IT^2 imaging system (Upland, CA, USA).

2.8. Statistical analysis

All results were presented as the mean ± SD. Statistical significance was analyzed using One-way ANOVA and student's *t*-tests for pairwise mean comparison ($P < 0.05$).

3. Results

3.1. STS dose-response simulation curve and determination of EC_{50}

To determine the in vitro cardioprotective effects of asialo-rhuEPO^P, STS was used to induce cellular injury and apoptosis in HL-1 murine cardiomyocytes since it is known to induce oxidative stress and apoptosis in many cell types [37]. A dose-response curve was first established by treating HL-1 cardiomyocytes with 0, 175, 350, 525, 700 and 875 nM STS. After 24 h treatment, LDH released in the medium was measured and converted to cytotoxicity values. From the cytotoxicity data (Fig. 1A), an EC_{50} value of 175 nM was obtained, which is similar to previously reported [38]. Therefore, 175 nM STS was used to induce apoptosis to investigate the cardioprotective effect of asialo-rhuEPO^P and its protective mechanism.

3.2. Asialo-rhuEPO^P treatment protected HL-1 murine cardiomyocytes against STS-induced injury

To evaluate the cardioprotective effect of asialo-rhuEPO^P, its ability to protect HL-1 murine cardiomyocytes against STS-induced cell injury was studied. Cardiomyocytes were treated with 175 nM STS alone, or simultaneously with 10, 20, 30, 40 and 50 IU/ml asialo-rhuEPO^P for 24 h; and LDH assay was used to determine cytotoxicity from the amount of LDH released into culture medium. Results showed that treatment of cardiomyocytes with STS alone resulted in 69% cytotoxicity, whereas cells treated simultaneously with STS and 10–50 IU/ml asialo-rhuEPO^P displayed cytotoxicity ranging from 54% to 30%, corresponding to cytoprotection rates of 22, 40, 51, 53 and 57, respectively (Fig. 1B). The above results indicate that asialo-rhuEPO^P protect cardiomyocytes against STS-induced cell injury. To compare the protective effects of asialo-rhuEPO^P with rhuEPO^M, a dose of 20 IU/ml was chosen because there were smaller increases in cytoprotection rates beyond 20 IU/ml (Fig. 1B), and that asialo-rhuEPO^P and rhuEPO^M at 20 IU/ml dose have been shown to be neuro- [33] and cardio-protective [39], respectively. Treatment of HL-1 cardiomyocytes with STS + 20 IU/ml rhuEPO^M resulted in 55% cytotoxicity corresponding to 19% cytoprotection, whereas STS + 20 IU/ml asialo-rhuEPO^P displayed 42% cytotoxicity (39% cytoprotection) (Fig. 2A). These results indicate that the asialo-rhuEPO^P is cardioprotective and that it even has ~2-fold better cytoprotective effect than rhuEPO^M.

To further investigate the anti-apoptotic effect of asialo-rhuEPO^P, cell morphological changes and nuclear fragmentation were observed in cardiomyocytes subjected to different treatments. STS alone treated cardiomyocytes exhibited severe cytoplasmic shrinkage and were either detached from each other or floated into the medium (Fig. 2B). Their nuclei were extensively fragmented (Fig. 2C). However,

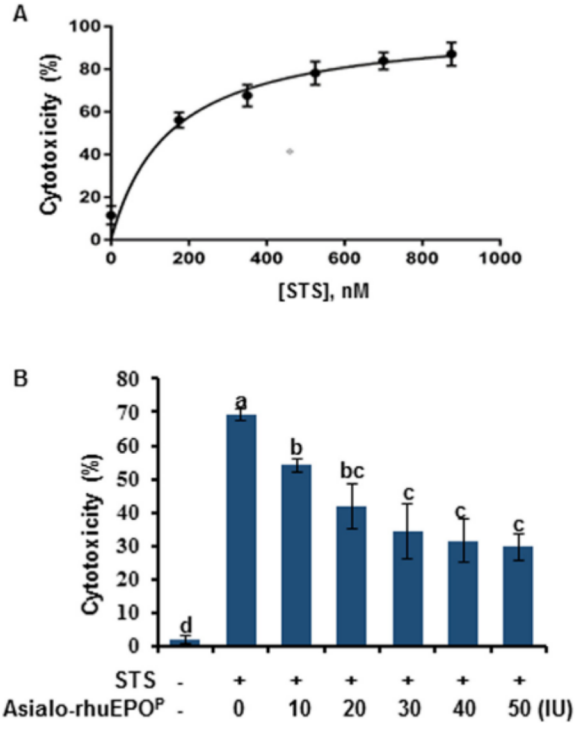

Fig. 1. Dose-response curve of STS treatments in HL1 cardiomyocytes and cytoprotective effects of asialo-rhuEPO^P against STS-induced cell death. A. HL-1 murine cardiomyocytes were treated with 0, 175, 350, 525, 700 or 875 nM STS to establish the EC_{50} value. B. HL-1 murine cardiomyocytes were treated with 175 nM STS alone, or simultaneously with 10, 20, 30, 40 and 50 IU/ml asialo-rhuEPO^P to determine dose-response. After 24 h treatment, LDH released in the medium was measured and converted to cytotoxicity values. All data plotted are the average of three independent experiments ± SD. Different letters labeled represent significant difference at $p < 0.05$ level.

cardiomyocytes treated with STS + asialo-rhuEPO^P exhibited minimal apoptotic cell morphological changes (Fig. 2B), and had lesser fragmented nuclei (Fig. 2C). In the case of STS + rhuEPO^M treated cardiomyocytes, the protective effect was less pronounced compared to STS + asialo-rhuEPO^P treated cells. The above results indicate that asialo-rhuEPO^P treatment and to a lesser extent rhuEPO^M treatment prevented cell death in HL-1 cardiomyocytes compared to STS alone treated cells. This protection appears to be due to inhibition of apoptosis. After treatment with STS + asialo-rhuEPO^P, cells exhibited significant decrease (~44%) in apoptotic nuclear morphological change compared to STS alone (Fig. 2D). In the case of cardiomyocytes subjected to STS + rhuEPO^M treatment, the decrease in apoptotic morphological change was about 37%.

3.3. Asialo-rhuEPO^P treatment inhibited Mst1 activation

Recent studies have shown that Mst1 is strongly activated in cardiomyocytes under toxic or pathologic conditions to promote apoptosis whereas suppressing Mst1 could prevent apoptosis [10–12] and enhance autophagy [12] to benefit cardiomyocyte survival. In our recent study with pancreatic beta-cells we observed that asialo-rhuEPO^P treatment suppresses Mst1 activation [34]. To investigate whether asialo-rhuEPO^P also suppresses Mst1 in cardiomyocytes, we carried out western blot analysis to detect Mst1 activation. Before immunoblotting analysis, cell viability of HL-1 cardiomyocytes subjected to different treatment was measured using Trypan blue dye exclusion method. Results showed that the cell viabilities of vehicle control, STS alone, STS + rhuEPO^M and STS + asialo-rhuEPO^P treatments were 87.5%, 69%, 74.5% and 82.5%, respectively (Fig. 3A). Compared to STS alone, rhuEPO^M and asialo-rhuEPO^P treatments had significantly higher cytoprotective effects; and asialo-rhuEPO^P had more than ~2-fold better cytoprotective effects than rhuEPO^M, which is consistent with observed results based on LDH assay. Therefore, the above treated cells were used to study asialo-rhuEPO^P-mediated cytoprotective mechanism. The results of western blotting showed that STS treatment induced Mst1 activation in cardiomyocytes as evident from a strong 34 kD band representing an active form of Mst1 (Fig. 3B). In STS + asialo-rhuEPO^P treated cardiomyocytes, the intensity of Mst1 active band was however, much lower than the band in STS only treated cardiomyocytes. In rhuEPO^M treated cells, the intensity of 34 kD band was also different from that of STS only treated cells. When Mst1 34/60 kDa (active/inactive form) ratios of STS alone, STS + rhuEPO^M and STS + asialo-rhuEPO^P treatments were calculated and compared to vehicle control (set as 1), they were 3, 2 and 1.6, respectively (Fig. 3B). These results indicated that both rhuEPO^M and asialo-rhuEPO^P could suppress Mst1 activation.

It is known that the proapoptotic function of Mst1 is mediated through transcriptional factor FOXO3 via phosphorylation of its Ser207 [40]. FOXO3 is a Mst1 substrate, and is activated by phosphorylation on Ser207 to trigger its translocation to the nucleus where it initiates transcription of numerous apoptotic genes thereby mediating cell death [40]. Therefore, the endogenous levels of p-Ser207-FOXO3 in cells subjected to different treatments were determined by western blotting to further confirm inhibitory effect of EPO on Mst1. Western blotting results showed that STS alone treated cells had 2.8-fold higher ratio of p-Ser207-FOXO3 to total FOXO3 compared to untreated cells (Fig. 3C), indicating that under stress conditions FOXO3 is activated consistent with activation of Mst1 under the same conditions (Fig. 3B). In the case of rhuEPO^M and asialo-rhuEPO^P treated cells however, the p-Ser207-FOXO3 to total FOXO3 ratios were ~1.5- and 1.3-fold, respectively (Fig. 3C) compared to that of untreated cells, suggesting that rhuEPO^M and asialo-rhuEPO^P treatment prevented FOXO3 activation. The above results indicate that the EPO treatment truly suppresses Mst1 kinase activity as well as FOXO3 activation.

3.4. Asialo-rhuEPO^P treatment inhibited apoptotic hallmarks

Mst1 has been considered as an efficient mediator of apoptosis because it acts both as an activator as well as a target of caspase-3 in the apoptosis signaling pathway [41,42]. Since the suppression of Mst1 in cardiomyocytes was observed following asialo-rhuEPO^P treatment, and that Mst1 activation is both caspase-3 dependent and independent [41], we were interested to see activation status of caspase-3 under different treatments. To investigate this, caspase-3 activation was first measured in HL-1 cardiomyocytes subjected to the above indicated treatments. Western blotting results showed that STS treatment induced caspase-3 activation as evident by release of a 17 kDa active form (Fig. 4A). Compared to STS alone treatment, asialo-rhuEPO^P and rhuEPO^M could suppress caspase-3 activation as shown by the lesser intensity of the 17 kDa active band. The active caspase-3 (17 kDa)/procaspase-3 (35 kDa) ratios for STS alone, STS + rhuEPO^M and STS + asialo-rhuEPO^P treatments were 23, 15 and 7 when compared to that of vehicle control (set as 1). These results indicate that both rhuEPO^M and asialo-rhuEPO^P inhibited STS-induced caspase-3 activation and that the latter one had superior inhibitory effect than the former.

When the pro-apoptotic regulators Bax and Bad, and anti-apoptotic proteins Bcl-2 and p-Bad were analyzed, both rhuEPO^M and asialo-rhuEPO^P treatments could lower STS-induced Bax/Bcl-2 ratio to that of vehicle control level, and partially restore p-Bad/Bad ratio suppressed by STS treatment (Fig. 4B and C). However, there were no significant differences in the changes of Bax/Bcl-2 and p-Bad/Bad ratios between rhuEPO^M and asialo-rhuEPO^P treatments. Apart these apoptotic markers, the leakage of cyt *c* from mitochondria into the cytosol is shown to be one of the most important indicators in the apoptotic process [43]. When levels of cyt *c* in mitochondria and cytosol were measured, STS treatment alone resulted in leakage of cyt *c* from mitochondria to

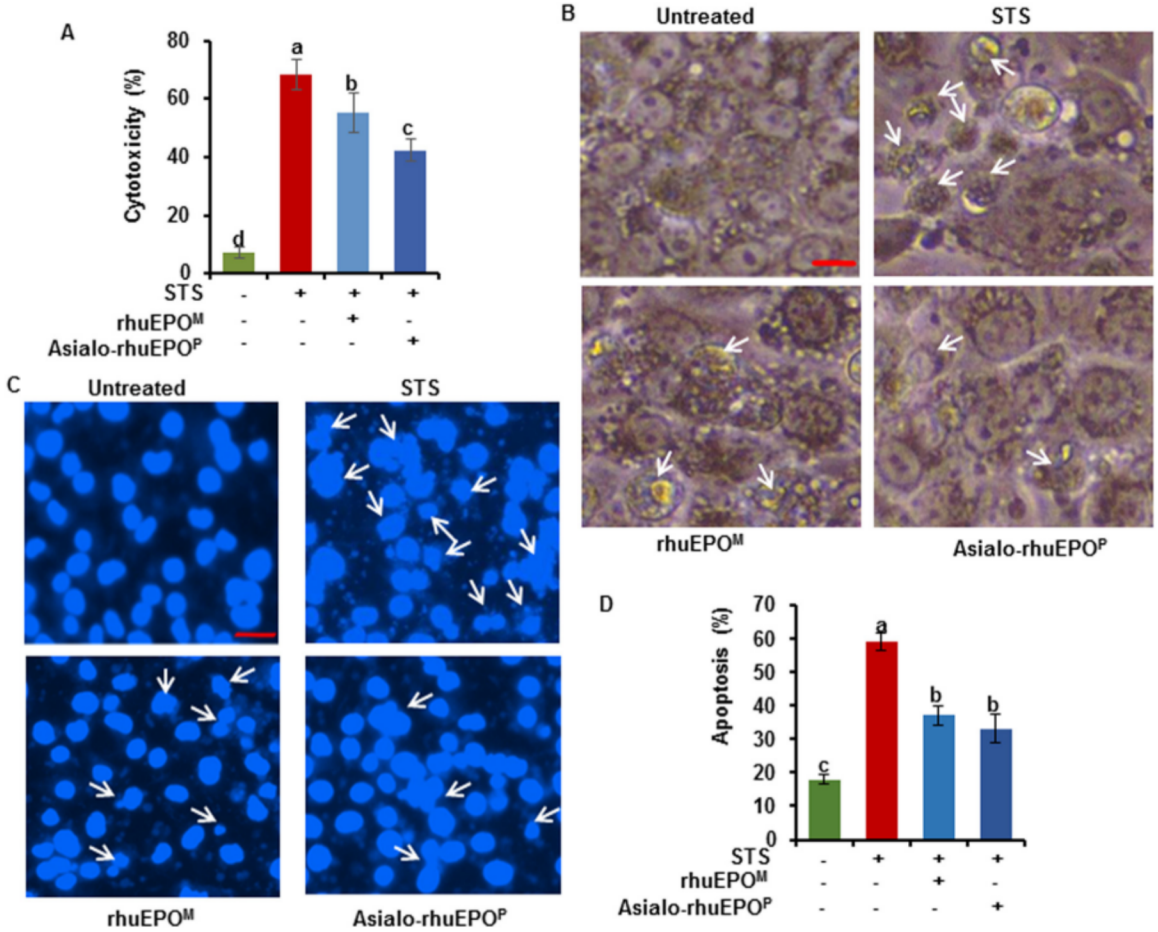

Fig. 2. Protective effects of asialo-rhuEPO^P on HL1 cardiomyocytes. Cells were treated with PBS containing 0.1% BSA (vehicle control), 175 nM STS, 175 nM STS with rhuEPO^M or asialo-rhuEPO^P, respectively. A. Protective effects of rhuEPO^M and asialo-rhuEPO^P against STS-induced cell death. B. Morphological changes after different treatments. Arrows indicate cells rounding and detachment. C. Apoptotic cells displaying fragmented nuclei after different treatments. Arrows indicate fragmented nuclei. D. Apoptosis rate based on fragmented nuclei was calculated as described in METHODS. All data plotted in A and D are the average of three and two independent experiments ± SD, respectively. Different letters labeled represent significant difference at $p < 0.05$ level. Scale bars: 10 μm.

cytosol with cytosol/mitochondria ratio approximately 4-fold higher than the vehicle control (Fig. 4D). When HL-1 cardiomyocytes were treated with either rhuEPO^M or asialo-rhuEPO^P plus STS, both of them could prevent the leakage of cyt *c* from mitochondria into the cytosol, and their cyt *c* cytosol/mitochondria ratios were only 1.7- and 1.3-fold compared to that of the vehicle control. Together, the above western blotting results indicate that both rhuEPO^M and asialo-rhuEPO^P treatments inhibited apoptosis markers to exert their cytoprotective effects in cardiomyocytes. These results are consistent with the decreased apoptotic nuclear morphological changes observed in rhuEPO^M + STS and asialo-rhuEPO^P + STS treated cardiomyocytes (Fig. 2B and C).

3.5. Asialo-rhuEPO^P treatment restored STS-induced changes of autophagy markers

Recently, Mst1 has been proved to act like a switch to dually regulate apoptosis and autophagy during stress [12]. After observing that asialo-rhuEPO^P treatment could suppress Mst1 activation as well as prevent cell death, we set out to know whether suppression of Mst1 activation by asialo-rhuEPO^P affects autophagy since maintaining autophagy within physiological levels is important [44]. Both p62 and LC3 have been widely used autophagy markers [45,46]. Protein p62 is a key substrate of autophagy [47]. The LC3 protein is an ubiquitin-like protein that undergoes conjugation with phosphatidylethanolamine (PE) resulting in formation of LC3-II from unconjugated LC3-I upon induction of autophagy [48]. The latter is then integrated into lipid membranes at the phagophore and autophagosomes [46]. LC3 has multi-isoforms in mammalian cells, but only LC3B correlates with increased levels of autophagic activity and is recommended to be used for autophagy analysis [45,46]. Therefore, the levels of p62 and LC3B along with Beclin1, which play important roles in autophagosome formation and autolysosome fusion, were analyzed in the above four types of treated cells.

Western blotting results showed that STS treatment alone caused a 32% reduction in Beclin1 levels compared to the vehicle control (Fig. 5A). RhuEPO^M and asialo-rhuEPO^P were able to restore 24% and 38% of Beclin1 levels, respectively compared to STS alone treatment. These results are consistent with a previous report that EPO could restore levels of Beclin1 in human neuroblastoma cell line [49]. When protein p62 was quantified, HL-1 cardiomyocytes treated with STS alone had approximately 2-fold higher protein than that of the vehicle control while STS + rhuEPO^M and STS + asialo-rhuEPO^P treated cells had 2.2- and 1.5-fold, respectively (Fig. 5B). These results suggest that STS treatment inhibits autophagy because p62 is typically degraded during autophagy. Asialo-rhuEPO^P had a capacity to significantly restore autophagy, but not rhuEPO^M (Fig. 5B).

Unlike p62, which is rapidly accumulated in cytosol during inhibition of autophagy [47], using LC3 to assess the autophagy activity is not straight forward because the conversion of LC3-II/LC3-I is cell type- and treatment-specific and the accumulation of LC3-II can result from either enhanced autophagosome synthesis or reduced autophagosome turnover to the lysosome [45,46]. Therefore, total LC3B (LC3B-II + LC3B-I),

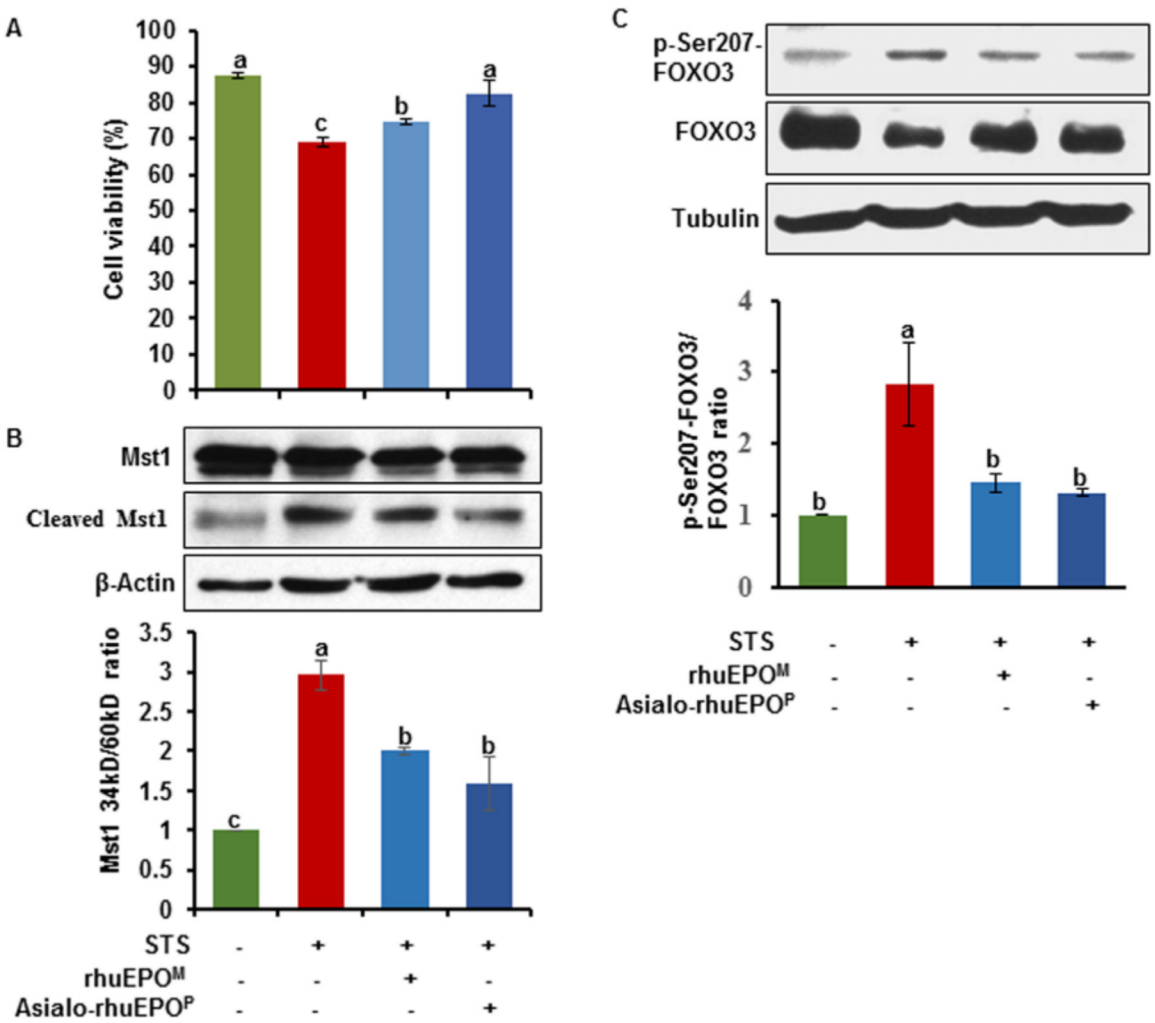

Fig. 3. Cell viability of HL-1 cardiomyocytes subjected to different treatments and western blots showing asialo-rhuEPO^P-mediated suppression of Mst1 and phosphorylation of FOXO3 at Ser207. A. Cells were treated with PBS containing 0.1% BSA (vehicle control), 175 nM STS, 175 nM STS with 20 IU/ml rhuEPO^M or asialo-rhuEPO^P, respectively. Cell viability was assessed after 24 h treatment. B. The levels of Mst1 were measured in cell lysates prepared from above treated cells. Active Mst1 was detected using an anti-Mst1 antibody, which also cross-reacted with proMst1. β-Actin was used as an internal control. C. To obtain the ratio of p-Ser207-FOXO3/FOXO3, the blot was probed with anti-p-Ser207-FOXO antibody first followed by stripping the blot and re-probing with anti-total FOXO3 antibody. Tubulin was used as an internal control. The experiment was repeated twice. All data plotted are the average of two independent experiments ± SD. Different letters labeled represent significant difference at $p < 0.05$ level.

LC3B-II/LC3B-I ratio, and LC3B-II only were compared among different treatments. Approximately 25% increase of total LC3B proteins was observed in STS only treated cells compared to the vehicle control while STS + rhuEPO^M and STS + asialo-rhuEPO^P treated cells had similar levels of total LC3B as the vehicle control (Fig. 5C). When we compared the LC3B-II/LC3B-I ratio among different treatments, it was found to be 45% higher in STS treated cells than the vehicle control while STS + rhuEPO^M and STS + asialo-rhuEPO^P treated cells had a ratio of 19% and 35% higher than the vehicle control, respectively (Fig. 5C). A comparison of LC3B-II only among different treatments showed that STS alone treated cells had 44% higher LC3B-II than vehicle control whereas STS + rhuEPO^M and STS + asialo-rhuEPO^P had about 18% and 4% higher, respectively (Fig. 5C). These results indicate that the higher level of total LC3B observed in STS treated cells resulted from accumulation of LC3B-II, suggesting that the autophagy seems to be impaired at autophagosome-lysosome fusion step [46]. Increased levels of LC3B in STS treated cells is consistent with the higher levels of p-Ser207-FOXO3 levels (Fig. 3C), which is known to induce LC3B gene expression [50]. Both asialo-rhuEPO^P and rhuEPO^M treatment reduced total LC3B levels, which could be due to reduced activation of FOXO3 (Fig. 3C). These results indicate that both asialo-rhuEPO^P and rhuEPO^M appear to have capacity to restore autophagy possibly via alleviating STS-induced autophagosome turnover problem.

3.6. *Asialo-rhuEPO^P treatment restored STS-mediated changes in phosphorylated JAK2 and Akt levels*

Based on previous studies, it is believed that EPO-mediated cell/tissue protection, including cardioprotection is associated with its binding to either homodimeric EPO receptor $(EPOR)_2$ [20,51] or heterodimeric receptor comprising of EPOR and the beta common receptor (β-CR) (EPOR-β-CR) [25]. After binding, JAK2 is activated by phosphorylation (p-JAK2), which then switches on downstream cell signaling pathways, such as PI3K/Akt, STAT5 and MAPK depending on the cell-type and cellular insult [25]. Activation of these pathways then induces regeneration and inhibits apoptosis and inflammation [25]. Since Akt is known to play a central role in promoting the survival of wide range of cell types, and previous studies suggest that anti-apoptotic effects of EPO are mediated through phosphorylation of JAK2 and Akt (p-Akt), the phosphorylated levels of JAK2 and Akt were analyzed by western blotting. The results showed that STS alone significantly reduced p-JAK2/JAK2 ratio by 26% compared to the vehicle control,

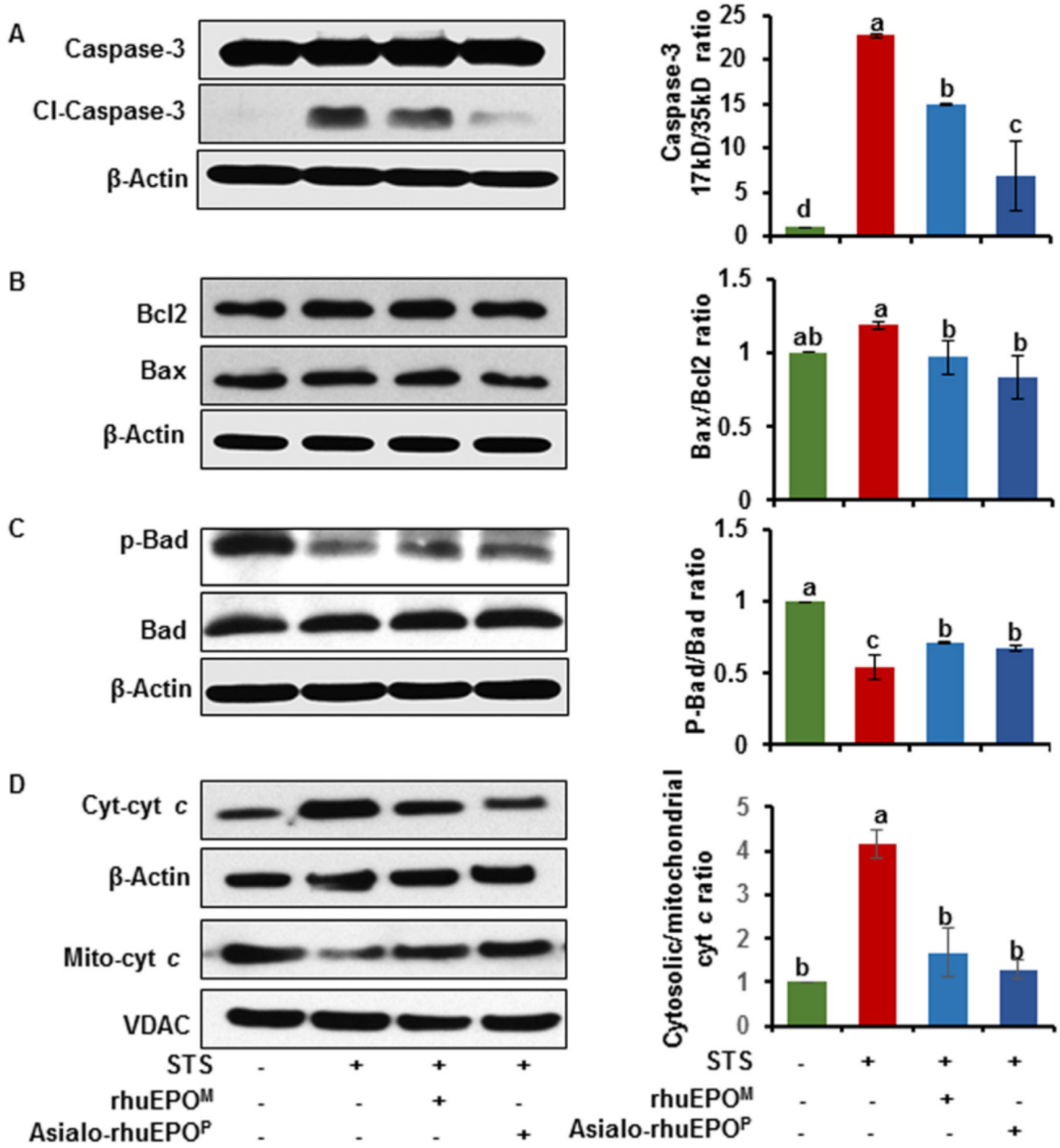

Fig. 4. Asialo-rhuEPO^{P}-mediated inhibition of proapoptotic markers. A-D. caspase-3, Bcl2 and Bax, p-Bad, Bad, and cytochrome *c* (cyt *c*). The levels of these proteins were measured in cell lysates prepared from cells treated with PBS containing 0.1% BSA (vehicle control), 175 nM STS, 175 nM STS with 20 IU/ml rhuEPO ^{M} or asialo-rhuEPO^{P}. Active caspase-3 was detected using an anti-caspase-3 antibody, which also cross-reacted with procaspase-3. Bcl-2 and Bax specific antibodies were used to detect these proteins. To obtain the ratio of p-Bad/Bad, the blot was probed with anti-p-Bad antibody first followed by stripping the blot and re-probing with anti-total Bad antibody. For cyt *c*, cytosolic (Cyt) and mitochondrial (Mito) proteins were isolated separately and then were probed with cyt *c* specific antibody. VDAC was used as an internal control for mitochondrial fraction while β-actin was used as an internal control for total protein extracts or cytosolic fraction. The experiment was repeated twice. All data plotted are the average of two independent experiments ± SD. Different letters labeled represent significant difference at $p < 0.05$ level.

whereas rhuEPO^{M} and asialo-rhuEPO^{P} could partially restore the p-JAK2/JAK2 ratio back to vehicle control (Fig. 6A). However, restoring capacity of asialo-rhuEPO^{P} was not as strong as rhuEPO^{M}. As for p-Akt/Akt ratio (Fig. 6B), STS treatment significantly reduced this ratio by 22% compared to the vehicle control, but both rhuEPO^{M} and asialo-rhuEPO^{P} had similar capacity to restore the above ratio by maintaining the phosphorylated Akt level close to that of the vehicle control (Fig. 6B). These results indicate that both rhuEPO^{M} and asialo-rhuEPO^{P} can activate JAK2 and maintain the active status of Akt.

Since Akt has been shown to promote cell survival by inhibiting FOXO3 transcriptional activity via phosphorylation on Ser253 residue and excluding it from the nucleus [52], we investigated whether activated Akt in rhuEPO^{M} or asialo-rhuEPO^{P}-treated HL-1 cells favor phosphorylation of FOXO3 on Ser253. Western blotting results showed decrease in the ratio of p-Ser253-FOXO3/total FOXO3 in STS alone treated HL-1 cells compared to untreated control cells (Fig. 6C). In the case of rhuEPO^{M} or asialo-rhuEPO^{P}-treated HL-1 cells, the ratio was closer to untreated control cells (Fig. 6C). These results indicate that activation of Akt by rhuEPO^{M} or asialo-rhuEPO^{P} suppresses FOXO3 activation (likely through suppression of Mst1 activation) and possibly excludes it from nucleus favoring cell survival. This is consistent with higher cell viabilities observed in rhuEPO^{M} or asialo-rhuEPO^{P}-treated HL-1 cells compared with STS alone treated cells (Fig. 2A).

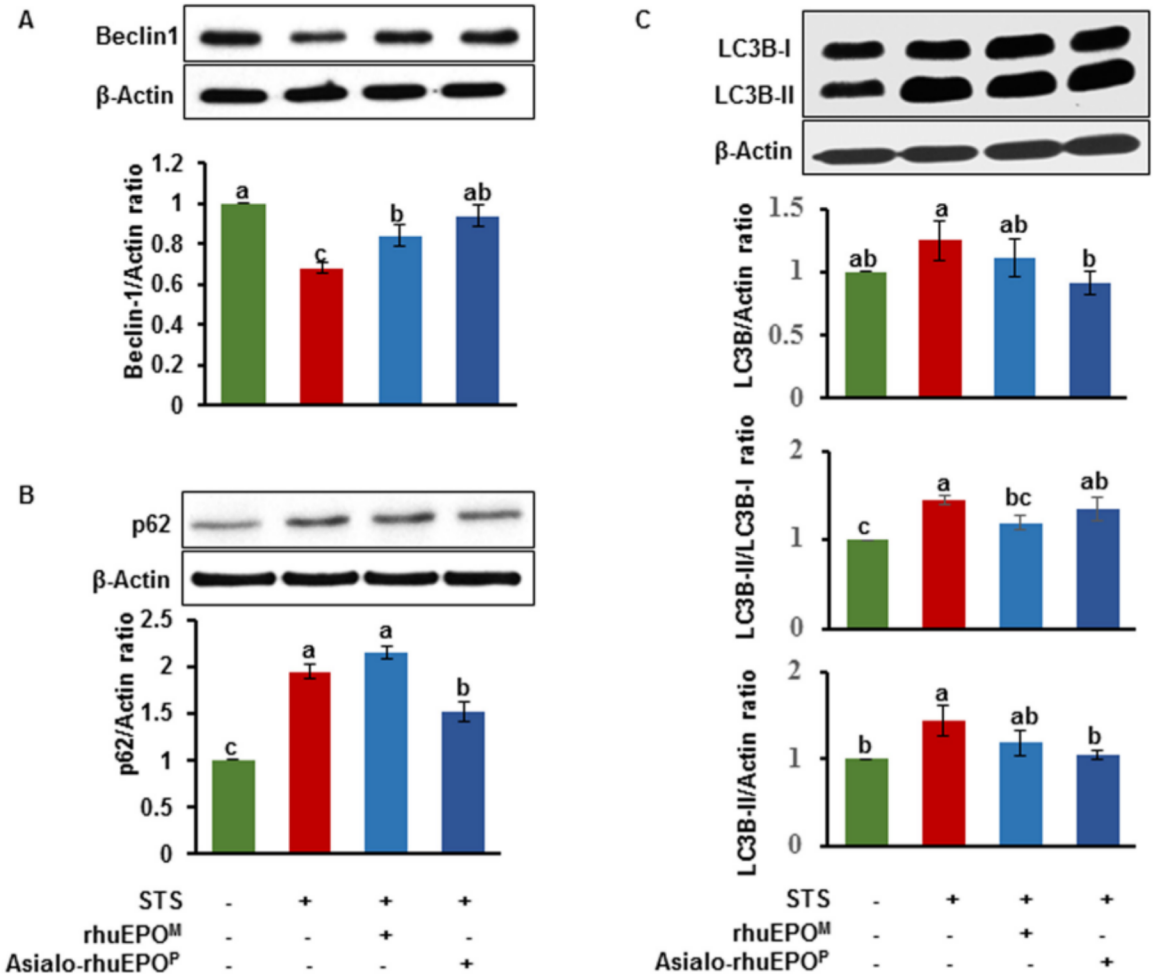

Fig. 5. Asialo-rhuEPO^{P}-mediated alteration in the levels of autophagy markers. A-C. Western blots of Beclin1, p62 and LC3B. The levels of Beclin1, p62 and LC3B were measured in cell lysates prepared from cells treated with PBS containing 0.1% BSA (vehicle control), 175 nM STS, 175 nM STS with 20 IU/ml rhuEPO^{M} or asialo-rhuEPO^{P}. Beclin1, p62 and LC3B specific antibodies were used to detect these proteins. β-Actin was used as an internal control. The experiment was repeated twice. All data plotted are the average of two independent experiments ± SD. Different letters labeled represent significant difference at $p < 0.05$ level.

4. Discussion

New therapeutics to prevent cardiomyocyte injury and death resulting from various disease conditions and the adverse side effects of treatments would be helpful to reduce the morbidity and mortality in cardiovascular diseases [3–5,53]. An effective agent that would limit myocardial injury and prevent cardiomyocyte cell death has yet to be identified even though numerous compounds have been tested during the past several decades [4–6,54]. Although rhuEPO^{M} was found to have cardioprotective effects in animal models of I/R injury as well as non-ischemic cardiac dysfunction [55,56], its cardioprotective effects could not be observed consistently in clinical studies [5]. The adverse effects (such as thrombosis and hypertension) associated with its hematopoietic activity might have been contributing factors for its failure in clinical trials [51]. This led to discovery of non-erythropoietic EPO derivatives such as asialo-rhuEPO [26]. It was found that asialo-rhuEPO obtained by enzymatic removal of sialic acids from rhuEPO^{M} has cardioprotective functions in different in vitro and in vivo models of heart diseases [30,31,57]. Nonetheless, it did not find its way into clinical test and practice because of limited and costly production from rhuEPO^{M} and lack of suitable expression system for direct expression. Therefore, our finding that plant-produced asialo-rhuEPO^{P} not only protected cardiomyocytes against STS-induced injury but also had better cytoprotective effects than rhuEPO^{M} (Fig. 2A) is significant. Considering the many potential advantages of plant-based expression, such as the low cost of production, human pathogen free, and ease of scale-up, asialo-rhuEPO^{P} could be potentially developed as a potent cardioprotective drug that many patients in both developed and developing world can afford.

The most important finding in the present study is the suppression of Mst1 by asialo-rhuEPO^{P} leading to inhibition of apoptosis and restoration of autophagy in HL-1 cardiomyocytes (Fig. 7). This discovery adds a new dimension to our understanding of how EPO mediates its cardioprotective effects. So far, there is no report on rhuEPO- or asialo-rhuEPO-mediated suppression of Mst1 activation in cardiomyocytes even though Mst1 is known to be a major player of apoptosis [58] and acts both as an activator as well as a target of caspase-3 [41,58–60]. It is generally believed that the cardioprotective effects of rhuEPO is through anti-apoptotic activity, which involves a number of signaling pathways, such as JAK2/STAT5, PI3K/Akt, MAPK (mitogen-activated protein kinase), and K_{ATP} (mitochondrial potassium channel) depending on the cell-type and type of injury [61–65]. In the present study, we found that asialo-rhuEPO^{P} treatment activated Akt (Fig. 6B), consistent with previous reports that protective effects of EPO are partly mediated by phosphorylation of Akt [57,63,66]. Akt is a key regulator of cellular survival, and is known to inhibit apoptosis by directly phosphorylating key players of the apoptotic pathway, such as Bad, caspase-9 and Bax [67]. Recently, Mst1 was identified as a physiological interacting partner of Akt and its target [68]. Akt has been shown to inhibit proteolytic activation of Mst1 by phosphorylating Thr^{187}

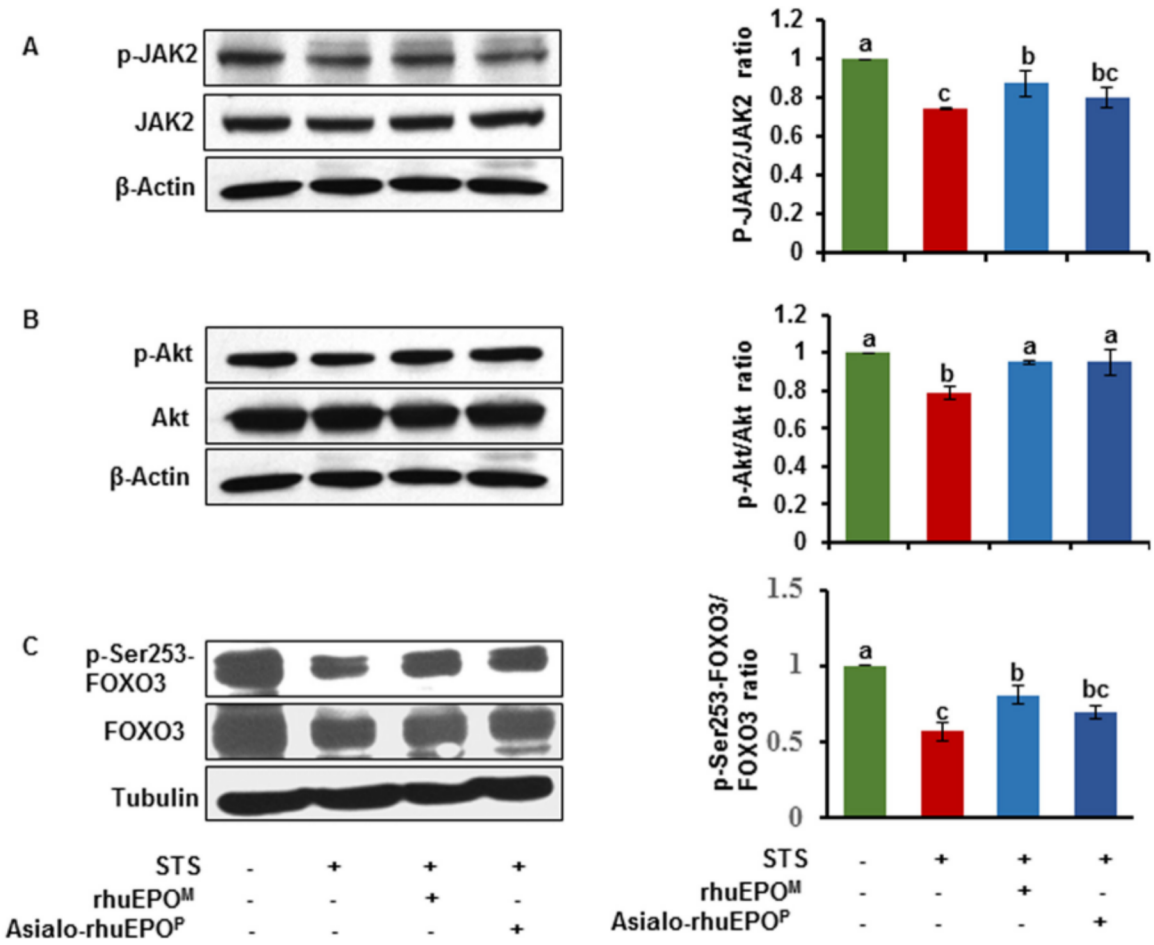

Fig. 6. Asialo-rhuEPO^{P}-mediated activation of cell survival pathway. A-C. Western blots of JAK2, Akt and FOXO3. The ratios of p-JAK2/JAK2 and p-Akt/Akt and p-Ser253-FOXO3/FOXO3 were measured in cell lysates prepared from cells treated with PBS containing 0.1% BSA (vehicle control), 175 nM STS, 175 nM STS with 20 IU/ml rhuEPO^{M} or asialo-rhuEPO^{P}. For their detections, the blots were first probed with antibodies for phosphorylated forms of JAK2, Akt and FOXO3 followed by stripping the blots and re-probing with anti-total JAK2, anti-total Akt antibodies and anti-total FOXO3, respectively. β-Actin and tubulin were used as an internal loading control, respectively. The experiment was repeated twice. All data plotted are the average of two independent experiments ± SD. Different letters labeled represent significant difference at $p < 0.05$ level.

residue, blocking FOXO3 phosphorylation (Ser207) and nuclear translocation thereby benefiting cell survival [69]. Therefore, there is a good reason to believe that activated Akt following asialo-rhuEPO^{P} or rhuEPO^{M} treatment suppressed Mst1 activation and very likely inhibited FOXO3a transcriptional activity. Among the apoptotic hallmarks investigated besides Mst1, we observed that Bax/Bcl2, p-Bad/Bad, caspase-3 activation and cyt *c* cytosol/mitochondria ratios (Fig. 4A–D) were dramatically altered in asialo-rhuEPO^{P} or rhuEPO^{M} treated cardiomyocytes than STS-treated cells. These results further indicate that asialo-rhuEPO^{P} inhibited apoptotic pathway and cell death, which is consistent with the higher cell viability observed in asialo-rhuEPO^{P} treated cardiomyocytes compared to STS alone treated cells (Fig. 3A).

In addition, the observed suppressing effects of asialo-rhuEPO^{P} on Mst1 activation led to the observation that asialo-rhuEPO^{P}-mediated cardioprotection also occurs via partially restoring autophagy activity. Under cellular stress conditions (Fig. 7), Mst1 is activated to phosphorylate Beclin1, which is a component of a type III PI3-kinase complex involved in the nucleation of the autophagic vesicle and a marker of autophagy [12]. The phosphorylated Beclin1 then dissociates from the autophagy complex Atg14L-Beclin1-Vps34 to replace Bax from apoptosis suppressing Bax-Bcl-2 complexes to form autophagy suppressing complex Bcl-2-Beclin1 (Fig. 7) [12,13]. The released Bax from Bax-Bcl-2 complex then promotes apoptosis while breakdown of autophagy complex inhibits autophagy, both of which promote cell death. Our findings that asialo-rhuEPO^{P} treatment inhibited Mst1 activation (Fig. 3B) and restored Beclin1 and p62 levels close to vehicle control (Fig. 5A and B), indicate that autophagy was partially restored. Additionally, Akt which is known to suppress autophagy was found to be activated following asialo-rhuEPO^{P} treatment (Fig. 6B). FOXO3 was also found to be predominantly phosphorylated at Ser253 (Fig. 6C), a form that excludes it from the nucleus and inhibit its transcriptional activity [52]. These results together with the fact that Akt directly inhibits Mst1 activation [69] suggest that activation of PI3k/Akt pathway plays an important role in regulating autophagy via modulation of Mst1 activity. Consequently, the asialo-rhuEPO^{P} mediated modulation of Mst1 activity seems not only to prevent apoptosis but also restore autophagy, thereby promoting cardiomyocyte survival. Apoptotic, necrotic and autophagic cell deaths have been observed in cardiomyocytes during myocardial infarction, I/R injury and heart failure, therefore, intervention based on asialo-rhuEPO^{P} could be used to reduce the damage and improve the cardiac function in patients with the above indicated cardiovascular disease conditions.

Since prevention of Mst1 activation is now recognized to be critical for cardiomyocytes and pancreatic beta-cell survival [12,42], it can be considered as a good target to develop therapeutics for treating cardiovascular diseases and diabetes. However, no drug or approach has been identified yet. Only recently, Fan et al. [70] showed that blocking Mst1/2 activities by a newly identified compound, 4-((5,10-dimethyl-6-oxo-6,10-dihydro-5H-pyrimido[5,4-b]thieno[3,2-*e*][1,4]diazepin-2-l)

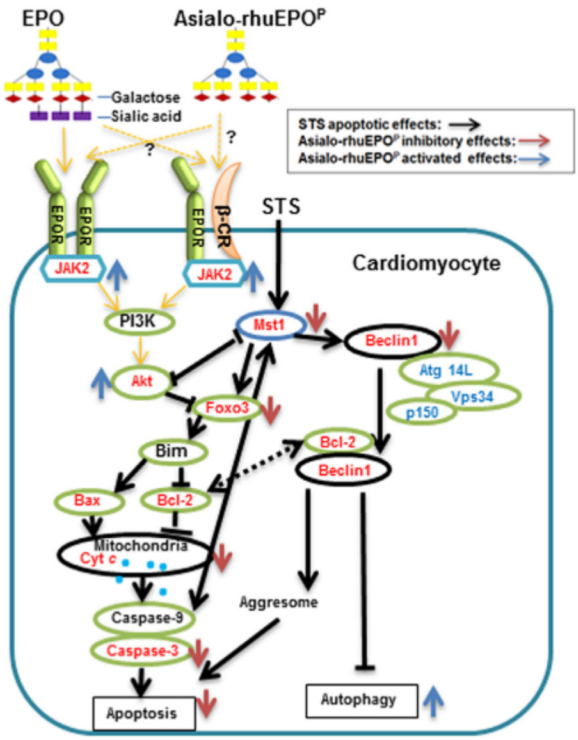

Fig. 7. Possible action model of cardioprotection by asialo-rhuEPO^P. This proposed model is based on the previously described relationship of Mst1 and apoptosis and autophagy in cardiomyocytes [12,13] together with the interaction of EPO and asialo-EPO interact with homodimeric EPOR receptor or heterodimeric EPOR-β-CR (β-common receptor) receptor as summarized by Brines and Cerami [25,74]. Based on findings in the current study, the cytoprotective effects of asialo-rhuEPO^P seem to be mediated through Akt, which is a direct inhibitor of Mst1 and FOXO3. Suppression of Mst1 activation might maintain the autophagy complex (Beclin1/Atg14L/Vps34/p150) with less disturbance of the antiapoptotic Bax-Bcl-2 complex. In the absence of asialo-rhuEPO^P, STS-treatment activates Mst1 leading to disruption of autophagy complex, thereby inhibiting autophagy, whereas the Bax released after sequestration of Bcl-2 by Beclin1 and inhibited by Bim (induced by FOXO3) causes the release of cyt *c* from mitochondria, resulting in initiation of apoptosis. The black arrows and T symbols indicate promoting and inhibitory effects caused by STS treatment, respectively. The blue arrow close each protein pointing upward indicates activated effects of asialo-rhuEPO^P treatment while the red arrow close to each protein pointing downward indicates inhibitory effects of asialo-rhuEPO^P treatment as revealed by western blotting analysis.

amino)benzenesulfonamide (XMU-MP-1), benefits intestinal and liver repair and regeneration in mice. The asialo-rhuEPO^P-mediated suppression of Mst1 activation observed in the current cardiomyocyte protection study as well as in our previous pancreatic beta-cell study [34] suggest that asialo-rhuEPO^P could potentially be developed not only as a cardioprotective drug but also as a broad cytoprotective agent against different types of cell/tissue injuries.

The observed 2-fold better cytoprotective effects of asialo-rhuEPO^P in cardiomyocytes than rhuEPO^M (Fig. 2A) is supported by our previous neuroprotection study, in which it was also found to have 2-fold better neuroprotective effects than rhuEPO^M [33]. Even though the detailed cytoprotective mechanisms remain to be elucidated, the differences in protective effects could result from their variation in *N*-glycan chain structures and their interaction with the receptor. As shown in Fig. 7, rhuEPO bears terminal sialic acid residues whereas these are absent in asialo-rhuEPO. Sialic acid has been shown to have negative effect on receptor binding in which rhuEPO exhibits much less affinity for EPOR than asialo-rhuEPO [71–73]. Moreover, for cytoprotective function, EPO has been suggested to bind to a heterodimeric receptor consisting of EPOR and β-CR [25,74]. EPOR is expressed in cardiomyocytes [75], but whether asialo-rhuEPO^P binds to homodimeric EPOR or heterodimeric EPOR-β-CR is still not clear at the moment. Nevertheless, future studies are warranted to answer which receptor asialo-rhuEPO^P binds to and what are affinities of rhuEPO^M and asialo-rhuEPO^P for the above receptor.

Another possibility that can explain better cytoprotective effects of asialo-rhuEPO^P in cardiomyocytes than rhuEPO^M is their capacities to regulate autophagy. Maintaining autophagy within physiological levels is critical for cardiac homeostasis [44]. The autophagy substrate p62 is a good indicator of autophagy flux and accumulation of cellular p62 levels indicates the inhibition of autophagy [47]. Based on Beclin1, p62 and LC3B-II levels in HL-1 cardiomyocytes subjected to different treatments (Fig. 5), STS could inhibit autophagy whereas asialo-rhuEPO^P had the capacity to relieve STS-induced autophagy inhibition. Why rhuEPO^M failed to relieve autophagy inhibition as much as asialo-rhuEPO^P did is not clear at this time, and warrants further studies.

5. Conclusions

In summary, our findings that asialo-rhuEPO^P not only protected cardiomyocytes against STS-induced apoptosis but also had better cytoprotective effects than rhuEPO^M, and that asialo-rhuEPO^P-mediated cardioprotection involves restoration of autophagy correlating with the suppression of Mst1 activation are significant. These findings set the stage for future in vivo cardioprotective studies in animal models of cardiac injury to determine whether asialo-rhuEPO^P could be developed as a novel agent to protect cardiomyocytes.

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2019.01.004.

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