Remote Ischemic Preconditioning

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Autophagy is a cellular process by which mammalian cells degrade and assist in recycling damaged organelles and proteins. This study aimed to ascertain the role of autophagy in RIPC-induced cardioprotection. Sprague Dawley rats were subjected to RIPC at the hindlimb followed by 30 min transient blockade of the left coronary artery to simulate I/R injury. Hindlimb muscle and the heart were excised 24 h post reperfusion. RIPC prior to I/R upregulated autophagy in the rat heart at 24 h post reperfusion. *In vitro,* autophagy inhibition or stimulation prior to RIPC respectively, either ameliorated or stimulated the cardioprotective effect, measured as improved cell viability to mimic the preconditioning effect. Recombinant IL-6 treatment prior to I/R increased *in vitro* autophagy in a dose dependent manner activating the JAK-STAT pathway without affecting the other kinase pathways such as p38 MAPK, and GSK-3β pathways. Prior to I/R, *in vitro* inhibition of the JAK-STAT pathway reduced autophagy upregulation despite recombinant IL-6 pre-treatment**.** Autophagy is an essential component of RIPC-induced cardioprotection that may upregulate autophagy through an IL-6/JAK-STAT dependent mechanism, thus identifying a potentially new therapeutic option for the treatment of ischemic heart disease.

Keywords: Autophagy ; Preconditioning ; Remote preconditioning ; JAK-STAT ; Cardioprotection ; Myocardial infarction ; RIPC ; Reperfusion ; I/R ; Ischemia-reperfusion

1. RIPC Prior to I/R Upregulates Autophagic Activity In Vitro and In Vivo

To determine autophagic activity, Atg5-Atg12 and LC3-II autophagic markers were assessed in vitro and in vivo. Stimulating with RHPC prior to H/R (RHPC-H/R) in H9c2 cells increased Atg5-Atg12 expression by 1.70 ± 0.08-fold relative to the normoxic control ($p < 0.05$) (Figure 1A). Similarly, LC3-II was significantly increased in RHPC H/R compared to the H/R group (1.95 ± 0.21 vs. 1.38 ± 0.11-fold relative to normoxic control, $p < 0.05$) (Figure 1B). Consistent with the in vitro results, RIPC stimulation in the hindlimb prior to I/R (RIPC I/R) significantly elevated the Atg5-Atg12 conjugate (2.24 ± 0.36 vs. 1.29 ± 0.0.19-fold relative to sham, $p < 0.05$) (Figure 1C) and LC3-II (2.07 ± 0.28 vs. 1.16 ± 0.12-fold relative to sham, $p < 0.05$) (Figure 1D) compared to I/R injury alone. Induction of autophagy was confirmed by pre-treatment of H9c2 cells with bafilomycin A-1 prior to exposing them to H/R. Increased levels of LC3-II in the presence of bafilomycin A-1 are indicative of autophagy flux. However, to assess if H/R and RHPC alter the autophagic flux through substrate digestion, it is important to compare the treatment plus bafilomycin A-1 with the treatment alone group [1]. An additive effect of LC3-II levels with bafilomycin A-1 is suggestive of autophagy flux due to the treatment/intervention; however, if the treatment plus bafilomycin A-1 does not increase LC3-II levels, then it is likely that the autophagy process is impaired [2][3]. In our study, the treatment plus bafilomycin significantly increased LC3-II levels compared to the treatment alone ($p < 0.001$) in all the study groups, suggesting functioning autophagy flux in the normoxia, H/R, and RHPC H/R groups (Figure 1E).

Figure 1. Effect of RIPC prior to I/R on autophagy protein expression in vitro and in vivo. Western blot analysis of Atg5-Atg12 conjugate in (**A**) H9c2 cells, (**B**) rat heart lysate and LC3 protein levels in (**C**) H9c2 cells, (**D**) rat heart lysate, and (**E**) bafilomycin-A1-treated H9c2 cell extracts, expressed as mean ± SEM, fold relative to control; * $p < 0.05$, ** $p < 0.01$.

2. Autophagy Functions as a Signaling Mechanism for RIPC and Confers Cardioprotection Against I/R Injury in Rats

Consistent with the increase of autophagy in H9c2 cells exposed to RHPC-H/R, RHPC alone significantly increased LC3-II protein by 2.29 ± 0.44-fold relative to the normoxic control ($p < 0.05$) (Figure 2A) in vitro. In order to evaluate the contribution of RIPC alone, without left coronary artery (LCA) occlusion and reperfusion, on myocardial autophagy and the cardioprotective JAK-STAT3 pathway, myocardial tissue was assessed for LC3-II and phosphorylated STAT3 levels immediately post-RIPC (0 min post-RIPC) and 24 h post-RIPC. In rats subjected to RIPC only, LC3-II protein in the myocardial tissue increased 1.37 ± 0.13-fold relative to the control group at 24 h post-RIPC ($p < 0.05$ vs. sham, $p < 0.05$ vs. 0 min post-RIPC) (Figure 2B). However, no effect on LC3-II was observed at 0 min post-RIPC compared to the control

group (1.04 ± 0.08-fold relative to sham). Interestingly, at 0 min post-RIPC, the autophagy regulator STAT3 was increasingly phosphorylated (3.97 ± 1.33-fold relative to the sham ($p < 0.05$) in myocardial tissue (Figure 2C). However, this value decreased to 2.21 ± 0.45-fold relative to the sham ($p = 0.32$ vs. 0 min post-RIPC) at 24 h post-RIPC.

In confirmation of previous studies, infarct size 24 h following I/R injury was reduced to 36.66 ± 3.87% with RIPC prior to I/R injury compared to I/R injury alone (52.96 ± 1.48%) ($p < 0.01$) (Figure 2D). There was no significant difference in the area at risk (AAR) between groups (Figure 2E). This cardioprotection from RIPC was confirmed by the reduction of cleaved poly(ADP-ribose) polymerase (PARP), an apoptotic marker, in RIPC I/R cardiac tissue compared to I/R alone (1.75 a± 0.44 vs. 5.45 ± 1.12-fold relative to sham, $p < 0.01$) (Figure 2F). Cardioprotective kinase signaling pathways, JAK-STAT, GSK-3β, and p38 MAPK, showed varied outcomes with RIPC prior to I/R. In RIPC I/R, STAT3 phosphorylation was significantly increased compared to I/R only (3.81 ± 0.26 vs. 2.26 ± 0.38-fold relative to sham, $p < 0.01$) (Figure 2G). Interestingly, phosphorylated GSK-3β was significantly elevated in I/R (1.78 ± 0.14-fold relative to the sham group ($p < 0.05$) but reversed to almost the sham group level in RIPC I/R (0.80 ± 0.07-fold relative to the sham group, $p < 0.05$) (Figure 2H). In contrast, p38 MAPK phosphorylation was unchanged after RIPC I/R and I/R alone (Figure 2I).

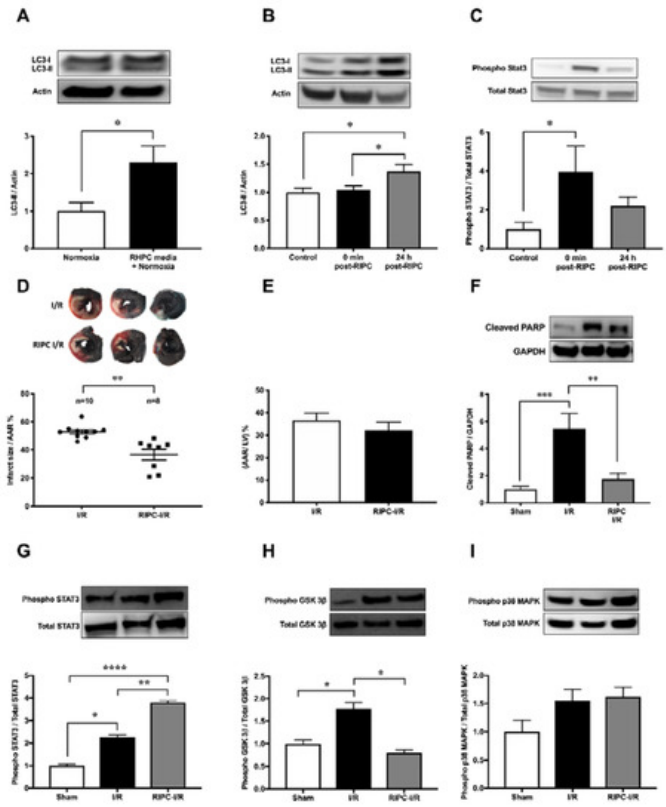

Figure 2. Effect of RIPC on autophagy and the cardioprotective signaling mechanism. Western blot analysis of (**A**) LC3 in H9c2 cells subjected to RHPC (preconditioned) media under normoxic conditions and (**B**) LC3 in the rat heart and (**C**) STAT3 phosphorylation in the rat heart assessed in the control group (without any intervention or sham surgery), at 0 min and 24 h post-RIPC. (**D**–**I**) Rats subjected to I/R with and without prior RIPC. (**D**) MI size expressed as the percentage of infarct size/AAR, (**E**) cumulative data of AAR/LV expressed as the percentage of the mean ± SEM. Western blot analysis of rat heart tissue assessing the (**F**) cleaved PARP ($n = 8$), (**G**) phosphorylated STAT3 ($n = 8$), (**H**) phosphorylated GSK-3β ($n = 8$), and (**I**) phosphorylated p38 MAPK ($n = 8$) levels at 24 h post-I/R with and without prior hindlimb RIPC. Results are expressed as mean ± SEM relative to control; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3. Autophagy is Essential for RHPC-Induced Cardioprotection In Vitro

To assess the importance of autophagy in the RIPC protective effect, H9c2 cells were pre-treated with 3-Methyladenine (3-MA) (inhibits autophagy) or rapamycin (Rapa) (promotes autophagy) prior to stimulation in the H/R injury model (Figure 3A). Pre-treatment with 3-MA significantly reduced cell viability in 3-MA RHPC-H/R cells compared to RHPC-H/R alone (38.89 ± 4.44% vs. 78.43 ± 3.68%, $p < 0.0001$). In contrast, pre-treating the cells with rapamycin significantly reduced cell viability in the RAPA-RHPC-H/R group compared to RHPC-H/R alone (64.93 ± 3.47% vs. 78.43 ± 3.68%, $p < 0.05$) and in Rapa H/R compared to H/R (61.32 ± 2.22 vs. 34.83 ±3.63, $p < 0.05$). Consistent with these results, pro-apoptotic Bax protein expression was significantly increased with 3-MA RHPC-H/R treatment compared to RHPC-H/R alone (7.02 ± 1.60 vs. 1.44 ± 0.11-fold relative to the normoxic control, $p < 0.01$) (Figure 3B). Additionally, 3-MA pre-treatment resulted in a significant increase in H9c2 apoptosis in the RHPC-H/R group compared to the untreated RHPC-H/R group (6.13 ± 0.77% vs. 2.97 ± 0.69%, $p < 0.05$) (Figure 3C). However, treatment with rapamycin prior to H/R resulted in a significant decrease

in apoptosis in the H/R group compared to the untreated H/R group (2.75 ± 0.79% vs. 5.01 ± 0.30%, $p < 0.05$) (Figure 3D). Changes in the mitochondrial membrane potential triggers the cycle of reactive oxygen species (ROS) formation [4], and is linked with cell death [5][6]. We analyzed the mitochondrial membrane potential level in H9c2 cells with and without RHPC prior to H/R. The mitochondrial membrane potential was lost in the H/R group (0.66 ± 0.05%-fold relative to control, $p < 0.01$); however, the mitochondrial membrane potential was maintained in the RHPC-H/R group (1.01 ± 0.08%-fold relative to control, $p < 0.05$ vs. H/R) (Figure 3E).

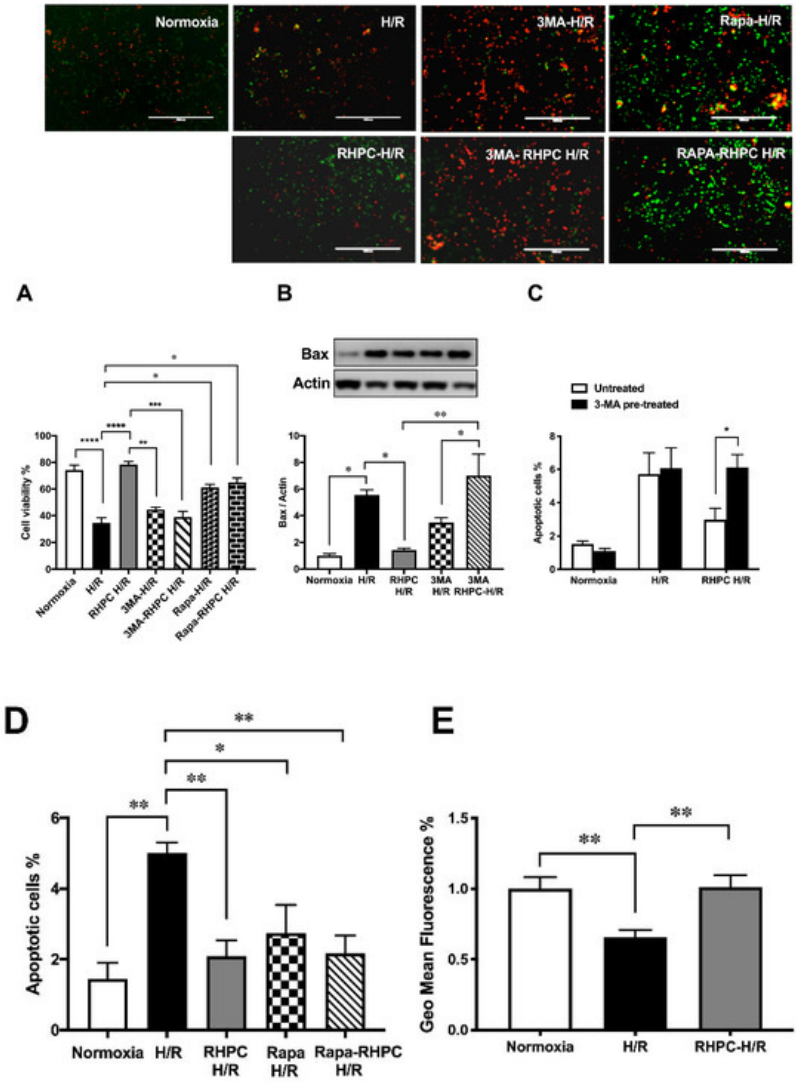

Figure 3. Effect of autophagy modulation on cell viability. (**A**) H9c2 cells were stained with calcein AM (green) and ethidium homodimer-1 (red). Green: live cells, Red: dead cells; the scale bar is 1000 µm. Quantitative analysis of percentage of live/dead cells expressed as mean ± SEM, (**B**) Western blot analysis of pro-apoptotic Bax protein expression are expressed as mean ± SEM, fold relative to the normoxic control, (**C**) and (**D**) Cell apoptosis assessed by fluorescein isothiocyanate (FITC)-labeled annexin V (annexin V-FITC) and propidium iodide (PI) double staining and fluorescence-activated cell sorting (FACS). Quantitative analysis of percentage of apoptotic cells (annexin-V+/PI+) cells are represented as mean ± SEM; (**E**) Mitochondrial membrane potential at 1 h post-reoxygenation assessed by 1,1′,3,3,3′,3′-hexamethylindodicarbocyanine iodide (DiLC5) staining and FACS represented as the percentage geo mean fluorescence ± SEM, fold relative to the normoxia control; n = at least 3 independent experiments, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

4. RIPC-Induced Autophagy Regulated by the IL-6-Dependent JAK-STAT Pathway

After RIPC, the secretion of cytokines critical in cardiovascular pathology was assessed. In rats subjected to RIPC, circulating IL-6 protein levels in the plasma were significantly increased to 103.4 ± 1.08 pg/mL compared to 98.4 ± 0.68 pg/mL in the sham group ($p < 0.01$) (Figure 4A). Similarly, in rat hindlimb tissue where RIPC was performed, localized messenger RNA (mRNA) IL-6 expression was upregulated by 1.87 ± 0.22-fold relative to the sham group ($p < 0.05$, Figure 4B). In contrast, RIPC did not modulate the expression of other critical cytokines in cardiovascular pathology, including

cardiotrophin-1 (Card-1) (1.30 ± 0.16-fold relative to the sham group) (Figure 4C), interleukin-11 (IL-11) (0.84 ± 0.33-fold relative to the sham group) (Figure 4D), leukemia inhibitory factor (LIF) (0.89 ± 0.21-fold relative to the sham group) (Figure 4E), and IL-1β (1.53 ± 0.32-fold relative to the sham group) (Figure 4F).

When H9c2 cells were treated with increasing doses of recombinant IL-6, LC3-II protein was upregulated compared to the untreated H/R group ($p < 0.05$) (Figure 4G). However, the LC3-II protein levels in the IL-6-treated H/R groups were similar to the RHPC-H/R group. Similarly, increasing concentrations of IL-6 treatment during hypoxia followed by reoxygenation increased STAT3 phosphorylation in a dose-dependent manner (Figure 4H). However, recombinant IL-6 treatment during hypoxia followed by reoxygenation did not modulate the phosphorylation level of p38 MAPK (Figure 4I) and GSK-3β (Figure 4J).

Pre-treatment of H9c2 cells with the STAT3 inhibitor tyrphostin AG-490 (50μM) prior to RHPC-H/R significantly reduced the RHPC-H/R-induced increase in LC3-II protein levels compared to the untreated RHPC-H/R group (0.41 ± 0.17-fold relative to the untreated RHPC-H/R group, $p < 0.05$) (Figure 4K). Tyrphostin AG-490 pre-treatment prior to H/R failed to upregulate LC3-II protein levels despite pre-treating H9c2 cells with 500 pg/mL of recombinant IL-6 during the hypoxic period prior to exposure to reoxygenation (1.44 ± 0.05 vs. 2.02 ± 0.08-fold relative to the normoxic control, $p < 0.01$) (Figure 4L).

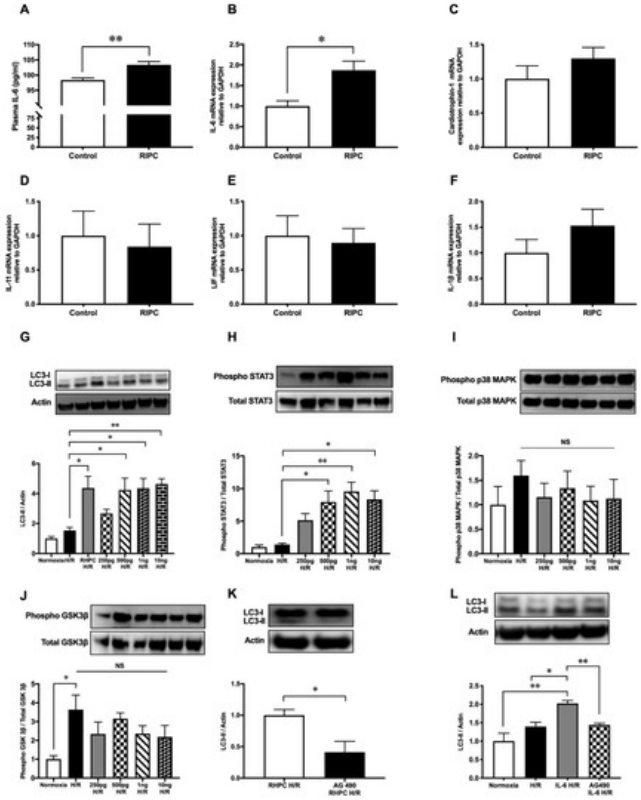

Figure 4. RIPC modulates cytokines' expression and recombinant IL-6 treatment induces autophagy through the JAK-STAT pathway. (**A**) Plasma IL-6 protein levels at 0 min post-RIPC expressed as mean ± SEM, $n = 5$, $p < 0.01$. mRNA expression of (**B**) IL-6, (**C**) cardiotrophin-1, (**D**) IL-11, (**E**) LIF, and (**F**) IL-1β in rat hind limb muscle at 0 min post-RIPC assessed by qPCR and expressed as mean ± SEM; $n = 9$, * $p < 0.05$. Western blot analysis of (**G**) LC3, (**H**) phosphorylated STAT3, (**I**) phosphorylated GSK-3β, and (**J**) phosphorylated p38 MAPK in H9c2 cells pre-treated with different concentrations of IL-6 (250 pg/mL, 500 pg/mL, 1 ng/mL, 10 ng/mL) during hypoxia followed by reoxygenation. The expression levels of phosphorylated proteins were normalized to total STAT3, GSK-3β, and p38 MAPK, respectively. Results are expressed as mean ± SEM; * $p < 0.05$, ** $p < 0.01$. H9c2 cells exposed to RHPC-H/R with and without the JAK-STAT pathway inhibitor tyrphostin AG-490. Representative immunoblots and statistical data of (**K**) LC3 protein are expressed as mean ± SEM, fold relative to the RHPC-H/R group. (**L**) Representative immunoblot of the LC3 protein level in H9c2 cells with IL-6 treatment during hypoxia with and without tyrphostin AG-490 pre-treatment followed by reoxygenation are expressed as mean ± SEM, fold relative to the normoxic control; n = at least 3 independent experiments, * $p < 0.05$, ** $p < 0.01$.

References

1. Klionsky, D.J.; Abdelmohsen, K.; Abe, A.; Abedin, M.J.; Abeliovich, H.; Acevedo Arozena, A.; Adachi, H.; Adams, C.M.; Adams, P.D.; Adeli, K.; et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). Autophagy 2016, 12, 1–222.
2. David C. Rubinsztein; Ana Maria Cuervo; Brinda Ravikumar; Sovan Sarkar; Viktor I. Korolchuk; Susmita Kaushik; Daniel J. Klionsky; In search of an “autophagomometer”. *Autophagy* **2009**, *5*, 585-589, 10.4161/auto.5.5.8823.
3. Sovan Sarkar; Brinda Ravikumar; David C. Rubinsztein; Chapter 5 Autophagic Clearance of Aggregate-Prone Proteins Associated with Neurodegeneration. *Methods in Enzymology* **2009**, *453*, 83-110, 10.1016/s0076-6879(08)04005-6.
4. Guido Kroemer; Bruno Dallaporta; Michèle Resche-Rigon; THE MITOCHONDRIAL DEATH/LIFE REGULATOR IN APOPTOSIS AND NECROSIS. *Annual Review of Physiology* **1998**, *60*, 619-642, 10.1146/annurev.physiol.60.1.619.
5. Derek J Hausenloy; Derek M. Yellon; The mitochondrial permeability transition pore: its fundamental role in mediating cell death during ischaemia and reperfusion.. *Journal of Molecular and Cellular Cardiology* **2003**, *35*, 339-41, 10.1016/s0022-2828(03)00043-9.
6. Guido Kroemer; John C. Reed; Mitochondrial control of cell death. *Nature Medicine* **2000**, *6*, 513-519, 10.1038/74994.
