Metformin preconditioning protects against myocardial stunning and preserves mitochondrial dynamics and protein translation in a mouse model of cardiac arrest

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Running Head: Metformin therapy protects against cardiac arrest

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Abstract

Cardiac arrest (CA) causes high mortality due to multi-system organ damage attributable to ischemia-reperfusion injury. Recent work in our group found that among diabetic patients who experienced cardiac arrest, those taking metformin had less evidence of cardiac and renal damage after cardiac arrest when compared to those not taking metformin. To investigate the potential for metformin to impact cardiac arrest outcomes, the current study investigates metformin interventions on cardiac and renal outcomes in a non-diabetic CA mouse model. We found that two weeks of metformin pretreatment protects against reduced ejection fraction and reduces kidney ischemia-reperfusion injury at 24 hours post-arrest. This cardiac and renal protection depends on AMPK signaling, as demonstrated by outcomes in mice pretreated with the AMPK activator AICAR or metformin plus the AMPK inhibitor compound C.

At this 24-hour time point, gene expression analysis showed that metformin pretreatment caused changes supporting autophagy, antioxidant response, and protein translation. Further investigation found associated improvements in mitochondrial structure and markers of autophagy. We also found that markers of protein nitrosylation between sham and arrest were not significantly different and thus unlikely to be a driver of gene expression differences. Notably, Western analysis indicated that protein synthesis was preserved in arrest hearts of animals pretreated with metformin. The AMPK activation-mediated protection of protein synthesis was also demonstrated in a hypoxia/reoxygenation cell culture model. Despite the positive impacts of pretreatment *in vivo* and *in vitro*, metformin did not preserve ejection fraction when deployed at resuscitation. Taken together, we propose that metformin's *in vivo* cardiac preservation occurs through AMPK activation, requires adaptation before arrest, and is associated with preserved protein translation.

New and Noteworthy

We leveraged a mouse model of cardiac arrest to study whether metformin pretreatment could protect cardiac function after arrest. We find that AMPK activity is crucial to this protection and characterize several pathways associated with this response. Signaling nodes with strong association may be precise targets to promote cardiac function in ischemia/reperfusion injury or in cardiac stunning.

52 **Introduction**

53 Cardiac arrest (CA) refers to the abrupt cessation of cardiac function and affects over 600,000 patients
54 annually in the United States (1,2). Patients who return spontaneous circulation (ROSC) after CA
55 experience systemic ischemia-reperfusion injury, typically resulting in multi-system organ damage.
56 Typical findings include cardiogenic shock, acute renal failure, liver damage, and neurologic dysfunction
57 (3–5). Previous observational studies in cardiac arrest patients have shown that low cardiac ejection
58 fraction (EF) (6) and reduced kidney function (7,8) after arrest are predictors of increased mortality.
59 Despite the prevalence of CA, no pharmacologic therapy has been shown to improve overall survival.

60 A recent study by our group found that diabetic patients taking metformin had less evidence of cardiac
61 and renal damage after CA than diabetic patients on other treatments (9), suggesting that metformin
62 pretreatment may be beneficial to CA patients through an undefined mechanism. Metformin is an oral
63 antihyperglycemic agent used as a first-line agent for type 2 diabetes that enhances insulin sensitivity
64 and normalizes glucose and lipid homeostasis (10–13). Beyond its role in controlling diabetes,
65 metformin has demonstrated clinical benefit across a wide variety of pathologies, including decreased
66 mortality in the setting of coronary artery disease (14), congestive heart failure (15), acute kidney injury
67 (16), chronic kidney disease (15), septic shock (17,18), and major surgical procedures (19).
68 Cardiovascular studies suggest that improved outcomes occur independently of the glucose-lowering
69 effects of metformin and may instead be attributable to metformin's pleiotropic effects (20). In a
70 preclinical rat model of CA, metformin pretreatment improved neurologic outcomes and survival (21),
71 and similar protection was recently reported using metformin as a post-arrest therapy (22). However,
72 the mechanisms underlying this protection and the value of metformin as a rescue therapy after CA
73 remain unclear.

74 Several mechanisms beneficial to cardiovascular health have been implicated in metformin's effects,
75 including reduced oxidative stress, anti-apoptotic activities, JNK inhibition, complex I inhibition, and
76 AMP Kinase (AMPK) activation (21,23,24). AMPK activation is prevalent in the metformin literature, and
77 AMPK activity is essential for survival after ischemic stress (25), but it is unclear whether AMPK activity is
78 the mediator of metformin-mediated protection in CA. Furthermore, it is unclear whether the adaptive
79 upregulation of AMPK during cardiac stress is optimal or whether further activation could even more
80 strongly impact recovery in cardiac injury models.

81 In this study, we sought to test metformin's potential benefit on heart and kidney protection after CA
82 and begin to assess the drivers of the effects witnessed clinically in our earlier diabetic patient study. We
83 evaluated cardiac and renal outcomes one day after CA in untreated sham (Sham), untreated arrest
84 (Arrest), metformin-pretreated sham (Sham+Met), and metformin pretreated arrest mice (Arrest+Met)
85 mice. Additionally, we tested the AMPK-dependence of metformin's effects by pretreating with the
86 AMPK activator 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR; Arrest+AICAR) instead
87 of metformin or by inhibition of AMPK via compound C given in the presence of metformin
88 (Arrest+Met+Comp C). We also evaluated a small cohort of mice with intravenous metformin as rescue
89 therapy. From the pretreatment group, we identified transcriptomic changes in metformin-treated
90 sham and arrest mice and tested many of the pathways implicated by Western blot analysis or
91 confirmed changes in a cell model of hypoxia/reoxygenation (H/R).

92

Methods

Sudden Cardiac Arrest Model

Eight-week-old male and female C57BL/6J mice (Jackson Labs, Bar Harbor, ME, #000664) underwent cardiac arrest by delivery of potassium chloride (KCl) directly into the LV by percutaneous, ultrasound-guided needle injection (Figure 1) as previously described (26). Briefly, mice were anesthetized using vaporized isoflurane (Henry Schein, Melville, NY, #1182097) and endotracheally intubated and mechanically ventilated (MiniVent, Harvard Apparatus, Holliston, MA, #73-0043) at 150 bpm and volume of 125 µL for females and 140 µL for males. Body temperature was maintained using a rectal temperature probe and heating pad (Indus Instruments, Webster, TX, #THM150). The chest was cleaned of hair using Nair and sterilized with betadine before the introduction of a 30-gauge needle into the LV under ultrasound guidance (Visual Sonics Vevo 3100 with Vevo LAB v 5.5.1 software, Toronto, Canada), followed by delivery of 40 µL of 0.5M KCl to induce asystole. The ventilator was stopped, and the mice remained in asystole for 8 minutes. 7.5 minutes after KCl dosing, 500 µL of 15 µg/mL epinephrine in saline (37 °C) was injected into the LV over approximately 30 seconds. At 8 minutes, the ventilator was restarted, and CPR was initiated by finger compression at about 300 bpm for 1-minute intervals. An electrocardiogram (ECG) was evaluated for return of sinus rhythm after each 1-minute interval. Animals not achieving ROSC by 3 minutes after CPR initiation were euthanized. Mice remained on ventilator support until spontaneous breathing frequency was greater than 60 times per minute. Sham mice received no KCl, but rather a single injection of epinephrine. After the procedure, all animals were placed in a recovery cage under a heat lamp.

Animal Treatment Groups

Treatment groups in the study were as follows: untreated sham (Sham), untreated cardiac arrest (Arrest), metformin-pretreated sham (Sham+Met), metformin-pretreated cardiac arrest (Arrest+Met), 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) pretreated cardiac arrest (Arrest+AICAR), metformin + compound c pretreated cardiac arrest (Arrest+Met+Comp C), and metformin rescue treatment cardiac arrest (Arrest+Rescue Metformin). Metformin pretreatment consisted of *ad libitum* access to metformin (Major Pharmaceuticals, Livonia, MI, #48152) in drinking water (1 mg/mL) for 14 days before the procedure. AICAR pretreated mice were given IP injections of 500 mg/kg AICAR (Toronto Research Chemicals, Toronto, CA, #A611700) in saline every other day for 14 days before CA. Compound C pretreated mice were given 20 mg/kg IP injections of compound C (Cayman Chemical, Ann Arbor, MI, #11967) in saline daily for 14 days before arrest. Metformin rescue therapy was given as a single direct LV injection (1250 µg/kg) dissolved in saline and 500 µL of 1 mg/mL epinephrine (Par Pharmaceutical, Chestnut Ridge, NY, #10977) at the time of resuscitation. Mice were randomly assigned to treatment groups. Male and female mice were used in all groups. All mouse studies were performed at the University of Pittsburgh in compliance with the National Institutes of Health Guidance for Care and Use of Experimental Animals. This protocol was approved by the University of Pittsburgh Animal Care and Use Committee (Protocol #18032212).

Echocardiography and Ultrasound

Immediately before arrest CA, mice were evaluated by transthoracic echocardiography using Vevo 3100 imaging systems (Visual Sonics) with a 40 MHz probe as previously described (27). Repeat echocardiography was performed one day after arrest under isoflurane anesthesia delivered by nose

134 cone. Heart rate was maintained between 400-500 bpm during imaging by adjusting isoflurane
135 concentration. B-mode images taken from the parasternal long-axis were captured, and LV EF was
136 calculated using modified Simpson's methods (28). A cohort of all groups was assessed for renal
137 perfusion after resuscitation. The ultrasound probe was oriented transversely across the abdomen at
138 the plane of the right kidney and monitored for renal artery blood flow by Doppler imaging. All image
139 analysis was performed by a blinded sonographer (Vevo Lab 5.5.1, Visual Sonics).

140 **Tissue and Serum Collection**

141 After euthanasia with isoflurane and cervical dislocation, mice underwent cardiac puncture to collect
142 blood by heparinized syringe. Blood was separated by centrifugation at 2,000 x g at 4°C for 10 minutes,
143 and the serum was flash frozen. These samples were evaluated for blood urea nitrogen (BUN) and
144 creatinine by the Kansas State Veterinary Diagnostic Laboratories (Manhattan, KS). Hearts from the mice
145 were excised, and LVs were isolated and flash frozen.

146 **Western Blot**

147 Frozen LV tissue was homogenized in lysis buffer containing a protease/phosphatase cocktail (Sigma-
148 Aldrich, St. Louis, MO, #11697498001) and normalized for protein content using a BCA assay (Life
149 Technologies, Carlsbad, CA, #23235). Samples were separated on NuPage 4-12% gradient SDS-PAGE gels
150 (ThermoFisher, Waltham, MA, #WG1403BOX) and iBlot transferred onto nitrocellulose membranes
151 (Invitrogen, #IB21001). Membranes were blocked in 5% milk (non-phosphorylated antibodies) or 5% BSA
152 (phosphorylated antibodies) for 1 hour and then incubated overnight at 4 °C with primary antibodies,
153 including, p-AMPK (Thr172, 1:1000, Cell Signaling, Danvers, MA, #2535), AMPK (1:1000, Cell Signaling,
154 #2532), GAPDH (1:5000, Millipore, St. Louis, MO, #AB2302), p62 (1:1000, Sigma-Aldrich, #P0067), LC3
155 (Invitrogen, 1:1000, PA1-16931), OxPhos Rodent Antibody Cocktail 1:5000 (ThermoFisher, #458099),
156 OPA1 (Invitrogen, 1:1000, PA5-57875), MFN2 (Invitrogen, 1:1000, #711803), p-DRP1 (Ser616, 1:1000,
157 Thermo, #PA5-64821), DRP1 (1:1000, Cell Signaling, #5391), p-S6 (Ser240/244, 1:1000, Cell Signaling,
158 #2215), S6 (1:1000, Cell Signaling, #2217), p-4EBP1 (Thr37/46, 1:1000, Cell Signaling, #2855), 4EBP1
159 (1:1000, Cell Signaling, #9644), nitrotyrosine (Santa Cruz, 1:1000, #32757) and ATF4 (1:1000, Thermo,
160 #PA5-27576). Following incubation, membranes were washed with TBS-tween and then probed for 1
161 hour at room temperature with anti-mouse or anti-rabbit secondary antibodies conjugated with either
162 horse radish peroxidase (Jackson ImmunoResearch, West Grove, PA, #115-035-003 and #115-035-144)
163 or infrared secondary (IRDye 680 and 800, LICOR, Lincoln, NE, #926-32210 and #926-68071). Images
164 were obtained by developing on a ChemiDoc XRS imaging system (BioRad, Hercules, CA) or Odyssey CLx
165 infrared imaging system (LICOR) and analyzed using ImageJ software (National Institutes of Health,
166 Bethesda, MD).

167 **Microarray Analysis**

168 Microarray analysis was performed on cDNA through the Affymetrix microarray analysis service
169 (ThermoFisher). Sham, Arrest, Sham+Met, and Arrest+Met mice were included in these studies with an
170 even distribution of males and females (total n=6 per group). Differential gene expression analysis was
171 performed using Transcription Analysis Console (Thermofisher). Gene-level p-values less than 0.05 were
172 considered significant for gene inclusion. Subsequent pathway analysis was performed to compare
173 untreated sham and arrest groups through Ingenuity Pathway Analysis (Qiagen). Complete datasets
174 were deposited in GEO (accession no. GSE176494).

175 **Cell Model**

176 AC16 cells, a hybrid cell model derived from the fusion of adult ventricular myocytes with stable,
177 proliferating SV40 fibroblasts (29), were cultured in DMEM with 10% FBS and penicillin/streptomycin
178 (Thermo, #15140122). Cells were incubated overnight at 37° C in DMEM with and without AICAR (1.25
179 mM) before the hypoxia/reoxygenation (H/R) challenge. Immediately prior to challenge, media was
180 replaced with either DMEM (normoxia group) or Esumi buffer containing 137 mM NaCl, 12 mM KCl, 0.5
181 mM $MgCl_2$, 0.9 mM $CaCl_2$, 20 mM HEPES, and 20 mM 2-deoxy-d-glucose at pH 6.2 (H/R group). Cells
182 were challenged with either normoxia (20% oxygen, 5% CO_2) or hypoxia (1% oxygen, 5% CO_2) for 4
183 hours, and then media was exchanged with normoxic DMEM. After 30 min of normoxia, cells were
184 stained with NucBlue Live (Hoechst 33342, Invitrogen) and NucGreen Dead (Sytox green, Invitrogen)
185 according to manufacturer recommendations and visualized on a Cell Insight CX7 HCX Platform
186 (Thermo) at 20x to quantify the ratio of living to dead cells. These cells were pelleted and flash frozen
187 for protein quantification.

188 **Protein Synthesis Assay**

189 The normoxia vs. H/R challenge was repeated for protein synthesis assays using the methionine analog
190 1-azidohomoalanine (AHA; Thermo). Immediately after normoxia/hypoxia, cell media was exchanged
191 with methionine-free media (RPMI, Thermo) containing AHA for 30 min. After treatment, media was
192 removed, and cells were fixed and permeabilized. The AHA reaction cocktail was added to cells to detect
193 AHA signaling as a marker of protein synthesis, and cells were co-labeled with Hoechst stain. Cells were
194 visualized for fluorescent signal and total fluorescent intensity quantified in a ring around the nuclei
195 using the Cell Insight CX7 HCX Platform.

196 **Respirometry and Hydrogen Peroxide Assay**

197 Oroboros high-resolution respirometry was performed on fresh whole-tissue homogenate using an
198 Oroboros Oxygraph-2K (Innsbruck, Austria). Homogenization was performed by adding 50 mg of frozen
199 LV to Mir05 buffer ($MgCl_2$-$6H_2O$ 3 mM, K^+MES 105 mM, taurine 20 mM, KH_2PO_4 10 mM, Hepes 20 mM,
200 d-sucrose 110 mM, and fatty acid-free BSA 1 g/l). After baseline respiration was established, 5 mM
201 pyruvate, 2 mM malate, and 10 mM glutamate (PMG) were added to assess respiratory capacity
202 through complex I, followed by ADP (2 mM) titrations to stimulate respiration. Next, 10 µM cytochrome
203 C was added to evaluate mitochondrial integrity, followed by 10 mM succinate to assess the combined
204 activity of Complex I + II, then 0.5 µM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) to uncouple
205 the mitochondrial membrane and induce maximal respiration. Hydrogen peroxide levels were assessed
206 from these samples using Amplex Red Assay (Thermo, #A22188) according to manufacturer instructions.

207 **Statistical Analysis**

208 Data were expressed as mean ± standard error in all figures. $P \leq 0.05$ was considered significant for all
209 comparisons. One-way ANOVA with Dunnett's multiple comparisons test was used to all compare
210 groups except Figure 8, in which student's t-test was used. All statistical analysis was completed using
211 Graphpad Prism 8 software (San Diego, CA).

212

Results

Metformin pretreatment protects cardiac EF and is dependent on AMPK signaling

To test the cardioprotective effects of metformin in CA, mice were pretreated for 2-weeks with metformin before the CA procedure (Figure 1) and compared to untreated arrest mice. There were no significant differences between groups to baseline animal characteristics, including the ratio of female mice (Table 1), body weight (Table 1), or baseline EF (Figure 2A) in these mice. Twenty-four hours after arrest, untreated arrest mice had significantly lower EF than untreated sham mice (Sham: 59.5±1.7%; arrest: 41.1±2.7%, $p<0.0001$, Figure 2A), as expected based on our previous description of this model (26). Metformin pretreatment significantly protected EF measured 24 hours after CA (Arrest+Met: 51.6±2.6%, $p<0.01$ vs. Arrest, Figure 2A). There were no changes to postoperative body temperature, time to resuscitation, or time to extubation (Table 1). Sham mice pretreated with metformin had no change to baseline characteristics or EF 24 hours after the procedure. These baseline and control measures found no confounding variables.

Metformin has multiple potential modes of action (30), with AMPK activation being dominant in the literature. Because of AMPK's role in cardiac resilience (25), we tested whether direct AMPK activation was necessary and sufficient for the observed enhanced EF. Cohorts of mice were treated for 2-weeks with the AMPK activator AICAR (31) or metformin and compound C, an established AMPK inhibitor (12). Both groups underwent two weeks of intraperitoneal (IP) injections before CA and were evaluated by echocardiography at baseline and twenty-four hours after arrest. No changes were observed in baseline EF (Figure 2A) or baseline characteristics, including age, % female, or body weight compared to the arrest group (Table 1). Like the metformin results, AICAR pretreated arrest mice had significantly improved EF compared to untreated arrest mice at 24 hours post-CA (Arrest+AICAR: 52.0±2.4%, $p<0.05$ vs. arrest, Figure 2A). Compound C not only prevented the beneficial effects of metformin on cardiac EF but also caused significantly reduced EF (Arrest+Met+Comp C: 30.0±2.9%) when compared to the untreated arrest group ($p<0.05$). The AICAR pretreatment phenocopies metformin pretreatment, and compound C blocks the benefit of metformin, suggesting that AMPK activation is necessary and sufficient for the metformin-mediated protection of cardiac function after CA.

To determine AMPK activation status, we assessed phosphorylation of threonine-172 of the AMPKα subunit in the myocardium (32). 24 hours after the CA procedure, LVs from sham and arrest groups (n=6/group) were assessed for p-AMPK, total AMPK, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein expression and were normalized to the arrest group (Figure 2B, Supplemental Figure 1). p-AMPK/AMPK was not significantly changed between Sham (1.44±0.47), Arrest (1.00±0.17), and Sham+Met (1.15±0.16) groups. p-AMPK/AMPK was elevated in the Arrest+Met mice (2.19±0.19, $p<0.01$ vs. Arrest) and Arrest+AICAR mice (1.91±0.31, $p<0.05$ vs. arrest) but unchanged in the Arrest+Met+Comp C mice (1.024±0.12). Total AMPK was unchanged between the treatment groups (Supplemental Figure 1). The strongest activation of AMPK was with metformin or AICAR plus arrest, suggesting a synergistic effect of drug and arrest on activation at this time point.

Metformin pretreatment protects against kidney injury and is dependent on AMPK signaling

The effects of whole-body ischemia/reperfusion injury can be detected in peripheral tissues, particularly in the kidney (26). Untreated arrest mice have significant kidney damage by tubular injury scoring compared to untreated sham mice at one-day post-CA (sham: 0.11±0.04; arrest: 3.33±0.24, $p<0.0001$,

254 Figure 2C). Metformin pretreated sham mice have no evidence of damage by tubular injury scores
255 (0.14±0.04, p<0.0001 vs. arrest). Metformin pretreated arrest mice had significantly lower tubular injury
256 scores (2.26±0.24, p<0.05) than the arrest group. AICAR pretreated arrest mice had lower injury scores
257 (1.45±0.42, p<0.001) than arrest mice, while Metformin + compound C mice had no protection evident
258 by score (2.54±0.47). To test peripheral markers as a proxy for tubular injury scores, serum markers of
259 kidney damage, creatinine and BUN, were also assessed in each group (Supplemental Figure 2). Serum
260 creatinine was significantly elevated in the arrest mice compared to sham (sham: 0.36±0.04 mg/dL;
261 arrest: 1.49±0.14 mg/dL, p<0.0001). There was no significant change to metformin pretreated arrest
262 mice (0.97±0.21 mg/dL), though significant changes were found in the AICAR arrest (0.67±0.25, p<0.01)
263 and Metformin + compound C mice (0.85±0.07, p<0.05) compared to sham. BUN was elevated in arrest
264 mice compared to sham (sham: 26.6±5.2 mg/dL; arrest: 153.9±28.4 mg/dL, p<0.001) but was unchanged
265 between arrest mice and Metformin pretreated arrest (121.2±28.0 mg/dL), AICAR arrest (85.7±8.9), and
266 Metformin + compound C mice (135.8±18.6). No changes were found when comparing the time to
267 kidney reperfusion between the untreated arrest and arrest treatment groups by Doppler flow analysis
268 of the renal artery (Table 1). Taken together, systemic activation of AMPK reduces histological markers
269 of CA-induced kidney damage, but 24-hour serum markers show limited sensitivity to this protection.

270 **Metformin causes broad transcriptome changes in sham and arrest mice**

271 To better understand the signaling changes caused by metformin therapy, transcriptomic analysis was
272 performed on LVs from Sham, Arrest, Sham+Met, and Arrest+Met groups (n=6/group) by microarray.
273 Differentially expressed genes were identified for three comparisons: Arrest vs. Sham, Sham+Met vs.
274 Sham, and Arrest+Met vs. Arrest (p<0.05, no fold restriction, Supplemental Figure 3). Pathway analysis
275 was then performed on these transcriptome changes through Ingenuity Pathway Analysis. The top 15
276 most significantly changed pathways are included in Figure 3, along with a z-score to predict the
277 upregulation or downregulation of each pathway. Prominent pathways include changes to autophagy,
278 AMPK signaling, unfolded protein response, nuclear factor-erythroid 2-related factor 2 (NRF2) mediated
279 oxidative stress, and eukaryotic initiation factor 2 (EIF2) signaling. We performed initial confirmation
280 studies for these pathways and focused on changes detectable at 24 hours. When no supporting data
281 were found, we advanced other pathway studies. When support was identified, additional studies were
282 done for confirmation.

283 **Metformin affects mitochondrial morphology and markers of autophagy**

284 Interestingly, the autophagy pathway shows the most significant changes between Arrest and Sham and
285 Sham+Met and Sham, but not between Arrest+Met and Arrest. Because metformin has been reported
286 to increase cardiac mitophagy in cardiomyopathy (33,34) and autophagy was the top hit in two of the
287 sample comparisons, we evaluated protein expression for markers associated with autophagosome
288 formation, including p62/Sequestosome 1, a cargo receptor associated with degradation of
289 ubiquitinated proteins (35), and microtubule-associated protein light chain 3 (LC3) processing (Figure 4).
290 p62 expression (normalized to GAPDH) was not significantly changed between groups. The relative
291 levels of microtubule-associated protein light chain 3 (LC3), specifically levels of uncleaved (LC3-I) and
292 cleaved (LC3-II) forms, were also monitored as an indicator of changes in autophagy initiation (36).
293 Interestingly, the LC3-II to LC3-I ratio was significantly increased in untreated arrest mice (1.91±0.15)
294 compared to sham (1.00±0.18, p<0.01). In contrast, the metformin-pretreated arrest mice had
295 significantly reduced LC3-II/LC3-I (1.17±0.17) when compared to untreated arrest mice (p<0.05). LC3-I

296 and LC3-II expression levels (normalized to GAPDH) were not significantly changed between groups
297 (data not shown). The normalization of LC3-II/LC3-I may be interpreted as metformin restoration of
298 mitophagy; however, ischemia/reperfusion is well known to induce mitochondrial damage and
299 mitophagy (37), and reduction in mitochondrial damage could yield a similar change in LC3 ratios.

300 To assess mitochondrial damage, we used electron microscopy to examine mitochondrial
301 structural changes among hearts from untreated sham, untreated arrest, and metformin-pretreated
302 arrest mice (Figure 5A). The untreated arrest mice showed a decrease in mitochondrial perimeter and
303 area (perimeter: 3.06±0.06 μm; area: 0.60±0.02 μm^2) when compared to sham (perimeter: 3.81±0.09
304 μm; area: 0.87±0.04 μm^2, $p<0.0001$ for both measures) in cardiomyocytes (Figure 5B). Myocardium from
305 metformin-pretreated arrest mice showed a modest but significant increase in mitochondrial perimeter
306 and area (perimeter: 3.33±0.07 μm; area: 0.70±0.03 μm^2) when compared to that of untreated arrest
307 mice ($p<0.05$ for both measures). Both the untreated arrest mice (0.76±0.001 μm, $p<0.0001$) and
308 metformin-pretreated arrest mouse heart (0.75±0.01 μm; $p<0.0001$) had more circular mitochondria
309 than the untreated sham mouse (0.69±0.01 μm). These data demonstrate modest changes in
310 mitochondrial morphology at 24 hours post-CA, with normalization of mitochondrial area and perimeter
311 but not circularity in metformin-pretreated arrest mice compared to untreated arrest mice, which may
312 be consistent with diminished mitochondrial damage or normalization of membrane dynamics.

313 Because of the change in mitochondrial architecture, we also looked at markers of
314 mitochondrial biogenesis and damage in the LVs to explain the protection by metformin. First,
315 representative proteins of mitochondrial respiratory complexes were assessed from LV extracts taken 24
316 hours after arrest from untreated sham, untreated arrest, metformin-pretreated sham, and metformin-
317 pretreated arrest mice (Figure 5C). There was a mild but statically significant elevation in a subunit of
318 complex II in the metformin-pretreated arrest mice (1.29±0.10) compared to untreated sham mice
319 (0.98±0.04, $p<0.01$); but otherwise, relative expression of representative proteins was unchanged across
320 the groups. Consistent with these results, we found no significant differences in Oroboros measurement
321 of mitochondrial oxygen consumption in heart homogenates in sham and arrest cohorts (Supplemental
322 Figure 4A). After sequential stimulation with PMG, ADP, Cytochrome C, succinate, and FCCP, we found
323 no significant differences in oxygen consumption rates (OCR). Prior work shows that mitochondrial
324 dysfunction induced by ischemia-reperfusion injury resolved in less than 24 hours (38,39), so this result
325 only informs that the respiratory defect is not protracted in metformin+arrest heart.

326 **Metformin pretreatment protects against mtDNA damage after arrest**

327 We next examined mtDNA relative abundance and damage in the cardiac tissue from these
328 groups (Figure 5D). Unlike in failed hearts (40), the arrest group did not show a statistically significant
329 decrease in mtDNA levels at 24 hours post-CA, consistent with the evidence above that mitochondria
330 are not being dramatically removed by autophagy. The metformin-pretreated arrest mice had slightly
331 higher relative mitochondrial DNA (mtDNA) levels (1.16±0.06) than untreated arrest mice (0.89±0.05,
332 $p<0.05$) but were not significantly different from the sham group (1.00±0.05). Notably, the metformin-
333 pretreated arrest mice had significantly less mtDNA damage in a long extension assay (0.03±0.1
334 lesions/10kb mtDNA) than untreated arrest mice (0.59±0.16 lesions, $p<0.05$). There was no statistical
335 difference between sham and arrest+met groups. The small but significant increases in mtDNA and
336 complex II in the metformin-pretreated arrest mice could be consistent with an increase in
337 mitochondrial biogenesis, but the combined significant improvement in mitochondrial morphology and

338 mtDNA integrity suggests an improvement in overall mitochondrial quality, decreased damage, and are
339 in line with the changes in autophagy markers.

340 **Metformin pretreatment affects markers of mitochondrial dynamics**

341 To understand the alterations in mitochondrial morphology better, we examined the relative
342 levels of several proteins involved in establishing mitochondrial shape (Figure 5E). Mitofusin 2 (MFN2), a
343 mitochondrial outer membrane GTPase involved in fusion, had reduced expression in metformin-
344 pretreated arrest mice (Arrest+Met: 0.53±0.07) compared to all other groups (Sham: 1.00±0.10, $p<0.01$;
345 Arrest: 1.06±0.07, $p<0.001$; Sham+Met: 0.89±0.04, $p<0.05$). OPA1, also a dynamin-related GTPase that
346 resides in the inner membrane to perform fusogenic functions (41), followed a similar overall trend with
347 significantly reduced levels in metformin-pretreated arrest mice (0.75±0.04) when compared to
348 untreated arrest mice (1.13±0.10, $p<0.05$). In contrast, dynamin-related protein 1 (DRP1), whose activity
349 is regulated by its phosphorylation at Ser-616 (42), showed no significant change in p-DRP1/DRP1
350 between sham and arrest groups. Metformin pretreatment appeared to improve p-DRP1/DRP1 post-
351 arrest modestly, but the effect was not statistically significant, likely because the decline in p-DRP in
352 untreated arrest hearts was not significant. Indeed, while mitochondrial perimeter and area are
353 increasing with metformin-pretreatment in 24-hour post-arrest hearts, markers of fusion and fission are
354 not significantly changed by arrest (relative to sham), suggesting that these markers are not the driver of
355 those morphological changes. In fact, the MFN2 and OPA1 decrease and p-DRP1/DRP1 increase by
356 metformin pretreatment in arrested mice (relative to arrest mice) are moving in the opposite direction
357 from what would be expected to correct mitochondrial morphology. We suggest that other
358 mitochondrial quality control processes contribute to mitochondrial morphological rescue.

359 **Metformin pretreatment protects against changes to markers of protein synthesis**

360 Interestingly, the MFN2 decrease is consistent with the activation of autophagy (43,44), where it
361 is ubiquitinated and targeted for proteolysis. Metformin has been reported to impact heart autophagy
362 through AMPK signaling (33,34). To test engagement of autophagy in the CA model, LVs collected 24
363 hours after arrest from untreated sham, untreated arrest, metformin-pretreated sham, and metformin-
364 pretreated arrest mice were evaluated for markers of autophagy by Western blot analysis (Figure 6).
365 First, we evaluated mTOR activation as a crucial negative regulator of autophagy (45). As a positive
366 downstream marker of mTOR activity, S6 ribosomal protein (S6) phosphorylation at Ser-240/244 (p-S6)
367 was assessed (Figure 6A). Both the p-S6/GAPDH and p-S6/S6 showed that the mTOR pathway activity
368 was highest in the arrest + metformin. We suggest that mTOR is potentially highly activated in
369 arrest+metformin hearts at 24 hours post-arrest because mTOR activity inhibits autophagy, the concept
370 that AMPK activation and subsequent mTOR inhibition to increase autophagy is insufficient to explain
371 the survival benefit of metformin.

372 EIF2 signaling was the most significantly altered pathway between the Arrest+Met and Arrest
373 groups (Figure 3) and is activated by mTOR (46,47). EIF2 signaling is commonly referred to as a marker of
374 the endoplasmic stress downstream of protein kinase RNA-like ER kinase (PERK) activation (48). In that
375 role, EIF2 activation promotes the expression of activating transcription factors (ATFs), including ATF4,
376 an integrated stress response regulator known to be activated by metformin (49,50). However, we did
377 not find significant changes in ATF4 expression between our treatment groups (Supplemental Figure 5).
378 The mRNA changes driving EIF2 signaling are overwhelmingly related to ribosomal proteins
379 (Supplemental Figure 5B), leading us to explore markers of protein synthesis.

380 As both the EIF2 pathway and p-S6 activation increase mRNA translation, we next performed an
381 additional Western blot analysis of eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1).
382 4EBP1 is a repressor of mRNA translation (51), and when phosphorylated at Thr37/36, 4EBP1 releases
383 eukaryotic translation initiation factor 4E (eIF4E) and is degraded (Figure 6C). eIF4E. proceeds to
384 increase protein synthesis. We found that the ratio of p-4EBP1/4EBP1 was decreased in arrest mice
385 (0.76±0.05) when compared to sham (1.00±0.05, p<0.01; Figure 6B). The Sham+Met (0.89±0.05) group
386 was unchanged from Arrest; however, the Arrest+Met group (0.94±0.03) was significantly higher than
387 Arrest (p<0.05). Sham mice had higher p-4EB1/GAPDH (1.00±0.13) than Arrest mice (0.54±0.05, p<0.01),
388 while there was no change between the Arrest mice and the Sham+Met (0.56±0.04) or the Arrest+Met
389 (0.69±0.05) groups. Similarly, Sham mice had higher 4EBP1/GAPDH (1.00±0.09) vs. Arrest (0.71±0.03,
390 p<0.01), but there was no change between Arrest mice and Sham+Met (0.64±0.02) or Arrest+Met
391 (0.74±0). The elevation of p-S6, p-4EBP1, and activation of the EIF2 pathway strongly implicate mTOR
392 activation in promoting translation that persists at 24 hours post-arrest.

393 **AICAR pretreatment protects against H/R changes to protein synthesis in a cell model**

394 To quantify protein synthesis changes, we replicated the hypoxic insult of CA in cell culture by
395 using AC16 cells (Figure 7A). Cells were pretreated overnight with AICAR to mimic AMPK activation
396 specifically. Cells underwent normoxia or hypoxia (1% oxygen in Esumi buffer) for 4 hours before
397 reoxygenation with and without AICAR pretreatment. Using these conditions, we found that p-S6/S6
398 was decreased in the untreated H/R cells (0.78±0.01) compared to untreated normoxia (1.00±0.06,
399 p<0.01) and unchanged compared to AICAR normoxia (0.91±0.02) but was significantly higher in the
400 AICAR H/R cells (0.93±0.05, p<0.05). Similar changes were found in the p-S6/GAPDH and S6/GAPDH
401 comparisons. Finally, p-4EBP1/4EBP1 was decreased in untreated H/R cells (0.72±0.01) compared to
402 untreated normoxia (1.00±0.08, p<0.05) and unchanged compared to AICAR normoxia (0.83±0.03) and
403 AICAR H/R cells (0.87±0.06). However, p-4EBP1 was significantly lower in the untreated H/R cells
404 (0.56±0.01) compared to all other groups (untreated normoxia: 1.00±0.04, p<0.001, AICAR normoxia;
405 0.74±0.04, p<0.01, AICAR H/R: 0.74±0.04, p<0.01). There was no change in the percent of dead cells
406 after reoxygenation in any cell groups (Figure 7C).

407 Protein synthesis was quantified for each cell group by measuring the fluorescent intensity of
408 labeled AHA given immediately after reoxygenation (n=3000-5000 cells/group). The fluorescent intensity
409 was lower in the untreated H/R cells (1397713±12864) compared to untreated normoxia
410 (2021451±13931, p<0.0001) and AICAR normoxia cells (1969457±16463, p<0.0001; Figure 7D). The
411 AICAR H/R cells had significantly higher intensity (1651628±16842) compared to the untreated H/R cells
412 (p<0.0001). These *in vitro* data suggest that activation of AMPK would restore protein translation
413 disrupted by ischemia/reperfusion injury in heart.

414 **Metformin does not improve outcomes as a rescue therapy**

415 To test metformin's efficacy as rescue therapy in our model, metformin was given directly into the LV in
416 a cohort of mice (n=7) at the time of resuscitation (1,250 µg/kg). Compared to the arrest mice described
417 in Figure 2, there was no change to EF one day after CA (arrest rescue metformin: 42.9±2.4%, Figure 8A).
418 Similarly, there was no change in tubular injury score (2.54±0.423, Figure 8A), creatinine (1.2±0.25,
419 Supplemental Figure 6), or BUN (155.3±14.2) when compared to untreated arrest mice. Baseline EF
420 (61.1±2.1) was not significantly different from the arrest mice. These data are consistent with the notion
421 that metabolic adaptation is required for metformin protection after CA.

422 Hearts from these mice were collected, and Western blots performed comparing untreated
423 arrest vs. arrest with rescue metformin. There was no change in p-AMPK/AMPK, p-AMPK/GAPDH, or
424 AMPK/GAPDH between treatment groups (Figure 8B&C). p-S6/S6 and S6/GAPDH were unchanged
425 between groups, though there was a reduction in p-S6/GAPDH in the arrest + rescue metformin cohort
426 (0.75±0.08) when compared to untreated arrest mice (1.00±0.03, $p<0.05$). There was a mild reduction in
427 p-4EBP1/4EBP1 in the arrest + rescue metformin mice (0.88±0.04) when compared to untreated arrest
428 mice (1.00±0.02, $p<0.05$), but p-4EBP1/GAPDH and 4EBP1/GAPDH were unchanged. These differences in
429 magnitude and duration of activation of these pathways in this model may account for the failure as a
430 rescue therapy.

431

432 **Discussion**

433 Our group recently performed a retrospective analysis of diabetic CA patients with and without
434 metformin therapy before arrest and found that metformin improved markers of cardiac and renal
435 damage acutely after CA (9). This exciting result prompted the search for reports of preclinical models to
436 identify the molecular pathways involved. Of particular relevance are studies of a 9-min rat asphyxial
437 model. Metformin pretreatment has neurologic and 7-day survival benefits but focuses on neuronal
438 markers without measures of cardiac and renal outcomes (21). More recently, a 10-min asphyxial rat
439 model with post-CA treatment demonstrated improved 3-day survival and neurological function,
440 involving normalization of brain activity without activation of AMPK, but also did not examine cardiac
441 and renal function (22). Our current data suggest that metformin-mediated cardiac protection may also
442 promote survival. Indeed, the lack of AMPK activation in the 2-hours post-treatment brain could be
443 consistent with protection arising from other metformin activities. Unfortunately, acute metformin
444 rescue was unsuccessful in our myocardial stunning model, so potential confounders in interpretation
445 among these studies remain.

446 Metformin pretreatment has previously decreased scar size in reversible coronary artery ligation
447 (24,52–56) and *ex vivo* whole-heart ischemia-reperfusion (24,57). However, studies of metformin's
448 effects on cardiac function have primarily utilized ischemia-reperfusion injury models with ischemic
449 periods of 25-30 minutes. The long ischemic duration in these models involves substantial
450 cardiomyocyte necrosis. Much of metformin's beneficial effect has been attributed to reducing mPTP-
451 mediated cell death (58). In contrast, our data demonstrate metformin's protection of *in vivo* EF in a CA
452 model (Figure 1) that features an 8-minute ischemia period without evidence of cardiac cell death (26).
453 We, therefore, set out to identify intracellular processes that adapt the cardiomyocyte to maintain
454 function rather than alter cell survival.

455 Metformin pretreatment has pleiotropic effects involving many molecular pathways, including
456 reducing oxidative stress, inhibiting apoptosis, inhibiting complex I, and activating AMPK signaling
457 (20,24,59,60). In our CA mouse model, we find that AMPK activation is necessary and sufficient to enact
458 metformin's cardioprotective effects. Mice pretreated with AICAR, an AMP-mimetic known to activate
459 AMPK (50), had preserved EF one day after CA compared to the untreated arrest mice (Figure 2A).
460 Conversely, mice pretreated with metformin with the AMPK inhibitor compound C showed no
461 improvement in EF one day after arrest compared to the untreated arrest mice. AICAR conferred similar
462 renal protection as measured by tubular injury score, and the protective effects of metformin on the
463 kidney were negated by compound C (Figure 2C). Interestingly, we did not find evidence of p-
464 AMPK/AMPK changes between the arrest and sham groups by Western blot (Figure 2B), despite
465 indicated AMPK changes in transcriptome pathway analysis (Figure 3). This phosphorylation status of
466 AMPK may have recovered at the time point evaluated (24 hours after arrest), while the continued
467 effects of metformin and AICAR are observed.

468 Pathway analysis was performed on microarray data collected from LVs of Sham, Arrest,
469 Sham+Met, and Arrest+Met mice. A comparison of arrest to sham mice (Figure 3) implicates autophagy
470 and AMPK signaling in our model. Given the evidence of metformin's AMPK-dependent effects (Figure
471 2), it should be expected that metformin therapy causes similar pathway changes in the sham mouse or
472 the untreated arrest model. Several signaling changes were observed when comparing untreated arrest
473 to untreated sham mice (Figure 3, top) when comparing the metformin-treated sham to untreated sham

474 mice (Figure 3, middle). These shared pathways include activation of autophagy, NRF-2 mediated
475 oxidative stress response, NAD signaling, and xenobiotic metabolism signaling. Given this overlap, it is
476 unsurprising that signaling pathways are less profound when comparing the metformin pretreated
477 arrest and untreated arrest mice (Figure 3, bottom). Interestingly, the strongest hit in the Arrest+Met
478 mice compared to the arrest animals was EIF2 signaling, which may suggest an increased ability to
479 support the initiation of translation for protein synthesis after CA.

480 We focused on transcriptional pathways that were altered at the time of cardiac assessment,
481 which showed overt cardiac dysfunction (Figure 1A). Autophagy, AMPK signaling, unfolded protein
482 response, Nrf2-mediated oxidative stress, and EIF2 signaling show differences in transcriptional
483 signatures between Sham, Arrest, and Arrest+Met at 24 hours post-arrest. The absence of changes in
484 gene expression between arrest and metformin arrest may be due to the preservation of cardiac
485 function being upstream of those alterations. In the process, we observed whether given pathway
486 markers being tested showed activation or normalization, i.e., regression to sham levels. For autophagy,
487 the pathway was activated in Arrest vs. Sham and Sham+Met vs. Sham but *not* in Arrest+Met vs.Arrest,
488 suggesting that the pathway wasn't accelerated at the time of analysis. Normalization of mitochondrial
489 function, mitochondrial morphology, LC3-II/I ratios, and hyperphosphorylation of S6, which is inhibitory
490 for autophagy (61), are consistent with the non-activation of autophagy. Although mtDNA lesions are
491 decreased, which could be compatible with accelerated autophagy, the increase in total mtDNA levels
492 and mitochondrial Complex II may indicate some compensatory mitochondrial biogenesis. The
493 significant decreases (i.e., not normalization) of OPA1 and MFN2 and increase in p-DRP1 levels in
494 Arrest+Met relative to Arrest alone (Figure 5E) would suggest a change in mitochondrial dynamics,
495 which has previously been implicated as a major regulator in cardiac arrest outcomes (62). However, we
496 find that mitochondrial area and perimeter are increasing mitochondrial size (Figure 5B) instead of the
497 expected shift to a more fragmented shape due to changes in those proteins (41,63,64). We did not
498 detect oxidative protein modification (Supplemental Figure 4), which is known to be primarily
499 mitochondrial proteins damaged at early timepoints (38,39), suggesting that oxidant activating the Nrf2
500 signaling pathway may have been resolved by 24 hours post-CA. This is in line with the normalization of
501 mitochondrial function by 24 hours post-reperfusion described by others and the lack of differences in
502 respiration described herein (Supplemental Figure 4A).

503 The hyperactivation of p-S6 also showed an accelerated response at 24 hours post-CA in
504 Arrest+Met compared to Arrest alone samples. Initial tests of ATF4 activation downstream of S6K
505 activation did not support the unfolded protein response's involvement at this time. We speculated that
506 p-S6 was not only indicating that AMPK stimulated activity inhibited mTOR, which activated p70S6K, but
507 also the downstream activation of translation (Figure 6C). We also show alterations to the
508 phosphorylation status of the translation repressor protein 4EBP1 in the untreated arrest mice, which is
509 normalized in the arrest mice pretreated with metformin (Figure 6B). Phosphorylated 4EBP1 is
510 dissociated from the translation initiation factor eIF4E (Figure 6C), allowing eIF4E to initiate translation
511 (65).

512 To confirm changes in protein synthesis, we performed H/R studies of AC16 cells. We found that
513 4 hours of ischemia in these cells was enough to insult to drive Western blot markers without causing
514 cell death (Figure 7A, C). Cell homogenates in the untreated H/R groups have elevated p-AMPK/GAPDH
515 compared to normoxia and AICAR-treated normoxia cells (Figure 7B). However, we did not find
516 superactivation of p-AMPK in the AICAR pretreated H/R cells. p-S6/S6 was significantly decreased in the

517 untreated H/R cells compared to normoxia but preserved in the AICAR pretreated H/R group. Similarly,
518 p-4EBP1/4EBP1 was reduced in the H/R mice compared to untreated normoxia cells but preserved in
519 the AICAR pretreated group. The protective effects of AICAR on p-S6/S6 and p-4EBP1/4EBP1 were
520 comparable to those seen by metformin in the animal model.

521 It should be noted that the p-S6/S6 is lower in the untreated H/R cells compared to the
522 untreated normoxia cells, which is discordant with changes seen in the metformin-treated mice;
523 however, the mouse model was complicated by epinephrine injection and surgical handling as well as
524 delayed tissue collection (24 hours in mice, compared to 30 min in cells), which may explain the
525 differences in p-S6/S6 results between the *in vivo* and *in vitro* systems. Importantly, we found that
526 untreated H/R cells reduced protein translation compared to untreated normoxia cells, partially
527 protected by AICAR pretreatment (Figure 7D). While these data suggest that AMPK activation can
528 preserve translation in ischemia/reperfusion stressed cardiomyocytes, additional studies will be needed
529 to establish to what extent such preservation contributes to cardiomyocyte resilience *in vivo*.

530 Finally, we performed a direct injection of metformin into the LV at the time of resuscitation to
531 test the effects of metformin as rescue therapy. To minimize animal injury, we compared the rescue
532 metformin cohort to the large arrest cohort described in Figure 2. However, we completed the analysis
533 of EF and kidney changes in a blinded fashion. This small cohort of mice had no evidence of improved EF
534 or kidney injury compared to the arrest group (Figure 8, Supplemental Figure 7). There has been some
535 controversy concerning the use of metformin after cardiac insult. While several preclinical studies have
536 found beneficial effects of metformin therapy when given at the reperfusion (24,56,59,66), this
537 protection was not evident in a pig model of myocardial infarction (67). Our data suggest that while
538 metformin as a pretreatment protects both the heart and the kidneys against ischemia-reperfusion
539 injury, this protection depends on adaptive mechanisms before the insult and the nature of the insult.
540 When used as rescue therapy, metformin did increase p-S6/S6 as seen in the metformin pretreatment
541 group, but p-4EBP1/4EBP1 was significantly lower in the rescue metformin mice. This supports the
542 notion that metformin's support for mRNA translation after CA requires preconditioning rather than
543 rescue therapy.

544 There are limitations to the results observed in this study. First, while metformin provided clear
545 evidence of renal protection as measured by tubular injury (Figure 2C), these results are less clear when
546 comparing serum markers of kidney injury, including creatinine and BUN. Further, we found unexpected
547 protection against renal damage in the Arrest+Met+Comp C cohort compared to Arrest, which may
548 suggest an additional signaling component in the kidney or varying degrees of AMPK activation between
549 the heart and the kidney. However, these serum measurements depend on other physiologic factors,
550 such as animal fluid status or peripheral tissue breakdown, which may complicate their interpretation.
551 For this reason, we consider tubular injury score a superior marker of damage (68). This renal damage
552 does not appear to depend on kidney reperfusion time (Table 1). However, this reperfusion time was
553 measured by doppler tracing of the renal artery, which may not have the sensitivity to fully detect
554 ischemic duration without contrast imaging (69). Regarding the rescue therapy, intravenous metformin
555 was calculated based on pharmacologic rodent studies comparing oral to intravenous metformin
556 bioavailability (70). The concentration was 10-times larger than an intravenous dose of metformin used
557 as rescue therapy in a mouse infarction model (56), so it is unlikely that this concentration did not exert
558 a significant effect.

559 In summary, we demonstrate that pretreatment with metformin protects against myocardial
560 stunning and renal injury in a mouse model of CA. Our CA mouse involves cardiac injury without cardiac
561 cell death, making it a more focused system to study metformin's mechanism of action. Direct AMPK
562 activation and inhibition studies confirmed that AMPK activation is necessary and sufficient for the
563 cardiac and renal benefits observed with metformin treatment. While multiple molecular mechanisms
564 downstream of AMPK activation may affect these outcomes, microarray data and Western blot changes
565 suggest that preservation of mRNA translation may be a major mechanism underlying metformin's
566 effects. Preserved mRNA translation and protein synthesis were also found in a cell model of H/R when
567 pretreated with AICAR. Unfortunately, these findings did not translate into use as rescue therapy,
568 suggesting that metformin's effects require a degree of adaptation to confer tissue protection.

569

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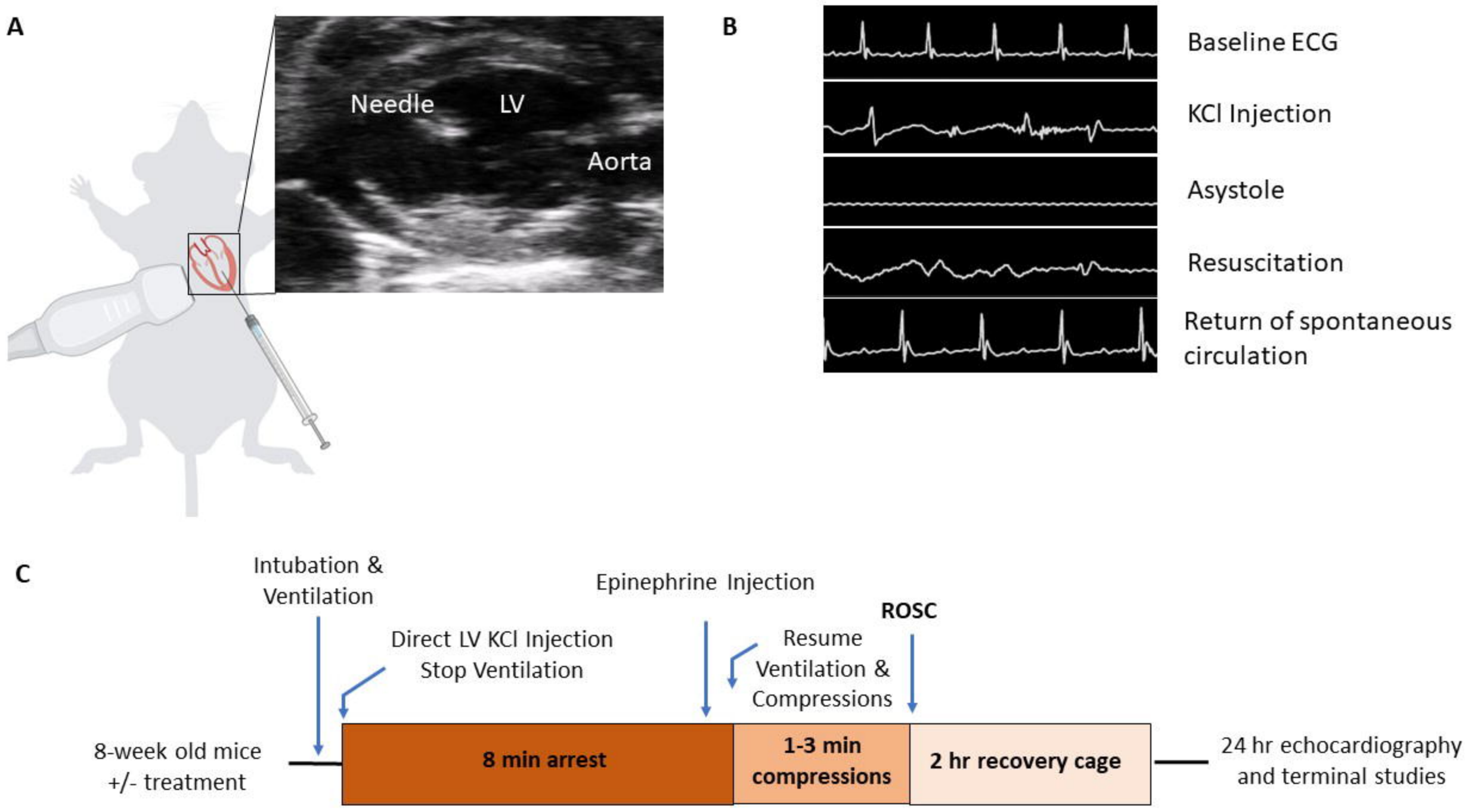

Figure 1. Mouse model of cardiac arrest (CA). A) Cartoon representation of direct left ventricular injection of potassium chloride (KCl) to cause asystole with representative ultrasound image of needle guidance. B) Representative electrocardioagrm (ECG) traces throughout time course of cardiac arrest procedure C) Time course of CA protocol. LV, left ventricle; ROSC, return of spontaneous circulation.

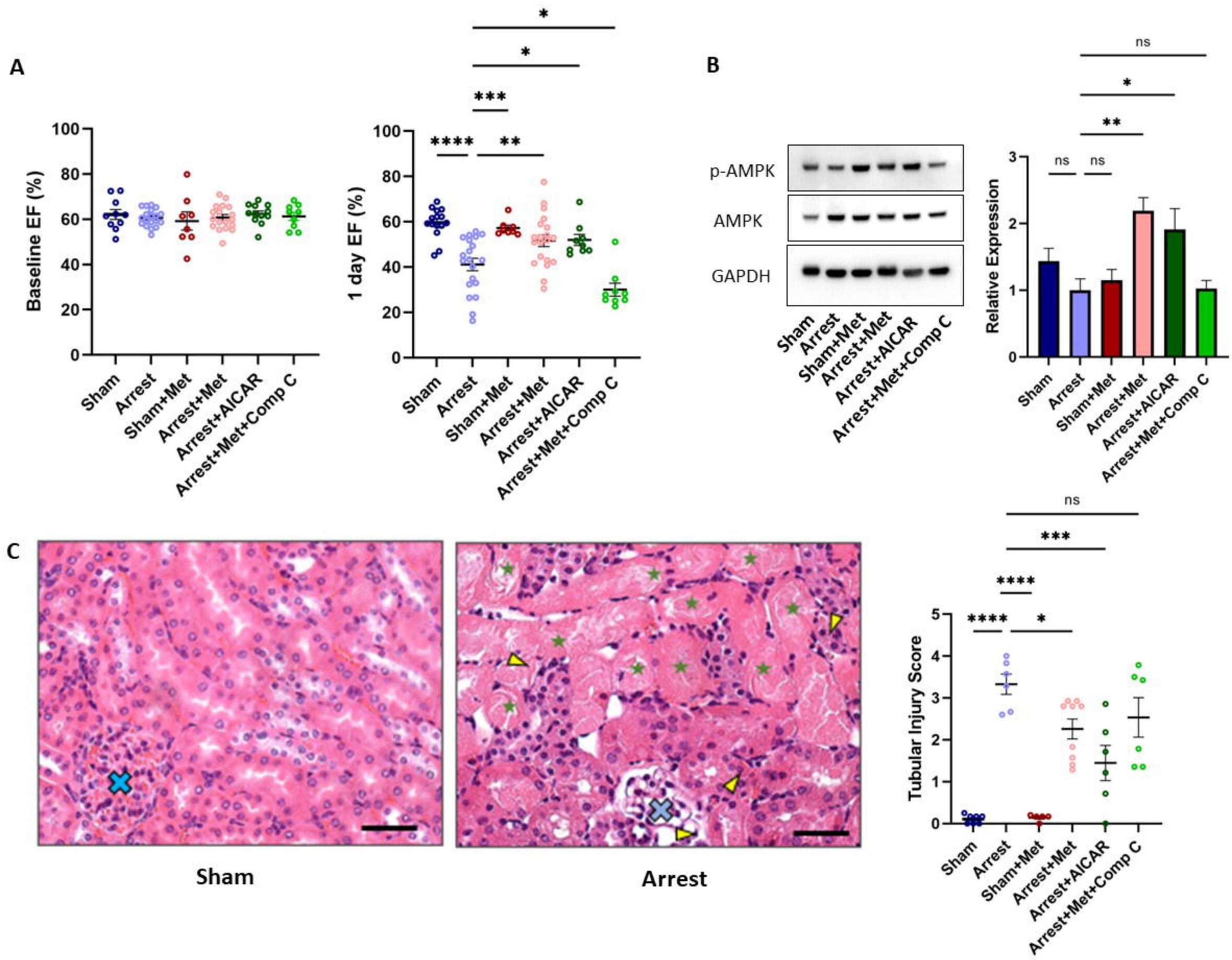

Figure 2. Metformin and AICAR treated mice have improved ejection fraction (EF) , elevated cardiac p-AMPK/AMPK, and less kidney damage than untreated mice one day after cardiac arrest (CA). A) Baseline (left) and one day EF (right) among treatment groups. Circles represent individual mice. B) Representative Western blot analysis of p-AMPK (Thr172), AMPK, and GAPDH for each treatment group and quantification of Western comparing p-AMPK to total AMPK (n=6/group). Full blots are provided in Supplemental Figure 1. C) Representative histologic sections from untreated sham and untreated arrest mice demonstrating proteinaceous casts in renal tubules (green stars) and infiltrates (yellow arrowheads) with glomeruli marked (blue X's). Scale bar = 50 µm) as well as quantification of tubular injury score between groups. Data are expressed as mean ± SEM. P-values: *< 0.05, **< 0.01, ***< 0.001, ****< 0.0001 by one-way ANOVA with Dunnett's multiple comparison test. EF, ejection fraction.

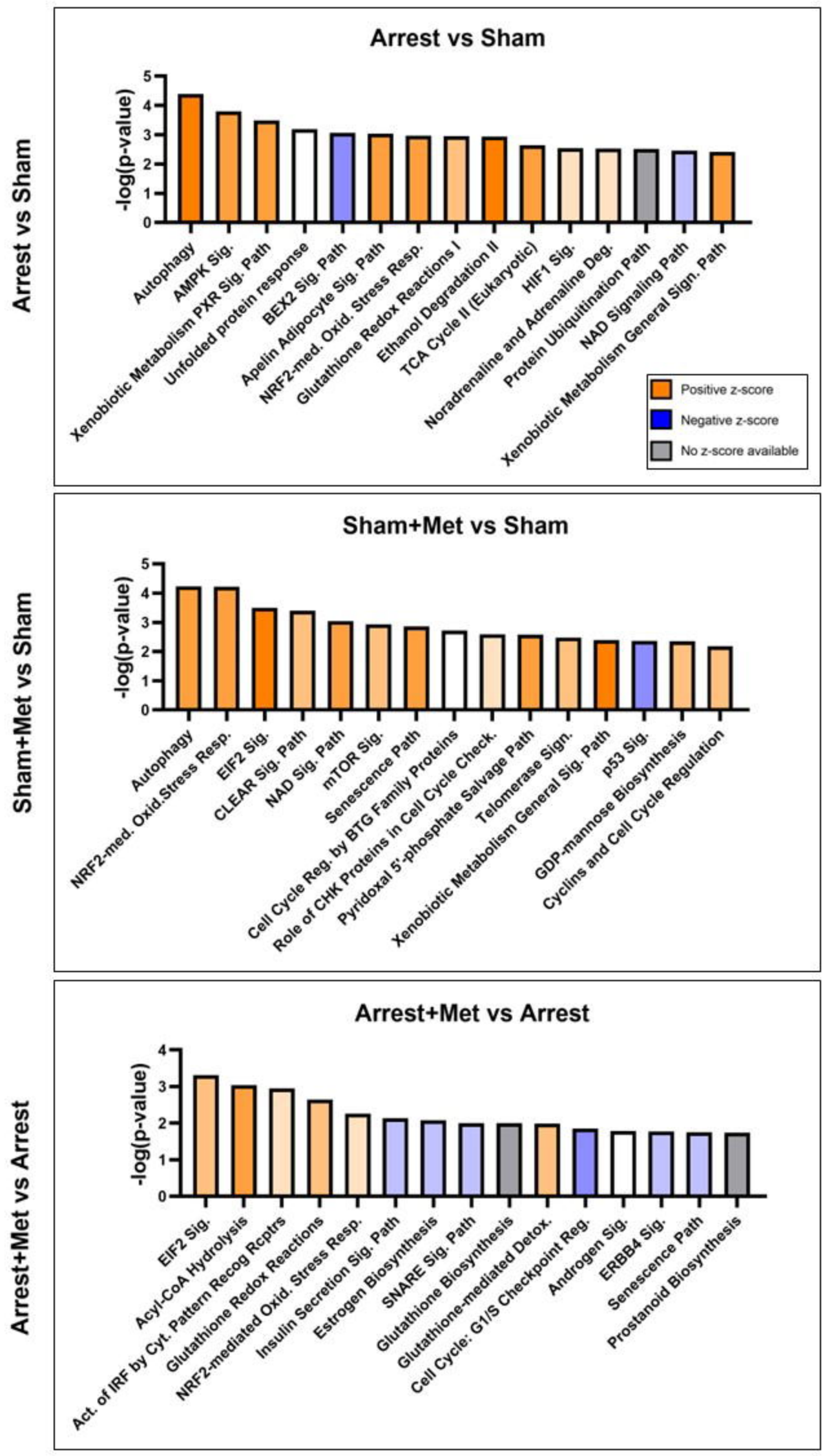

Figure 3. Pathway Analysis Comparing Treatment Groups. Ingenuity Pathway Analysis was used to predict signaling pathways between Arrest and Sham mice (top), Sham+Metformin (Sham+Met) and Sham mice (middle), and Arrest+Metformin (Arrest+Met) to Arrest mice (bottom) based on microarray data collected from left ventricles one day after arrest (n=6/group). Bar-graphs include significance values for pathway changes (-log(p-value)) and are colored according to z-score to predict directionality of changes. Differentially expressed genes were included in the analysis when $p<0.05$ based on microarray data. Principal component analysis and volcano plots are included in Supplemental Figure 2.

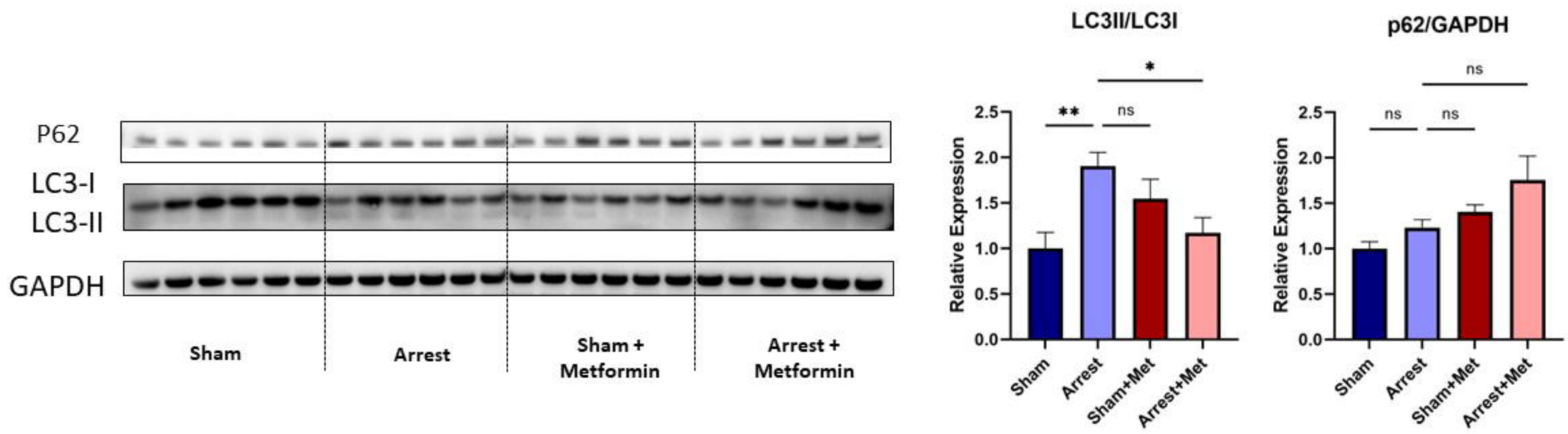

Figure 4. Western Blot Analysis of Autophagy Markers. Western blot images of autophagy-related proteins downstream of AMPK including p62, LC3 (I/II), and housekeeping protein (GAPDH) expression in Sham, Arrest, Sham+Metformin (Sham+Met), and Arrest+Metformin (Arrest+Met) mice (n=6/group). Data are expressed as mean ± SEM. P-values: *< 0.05, **< 0.01 by one-way ANOVA with with Dunnett's multiple comparison.

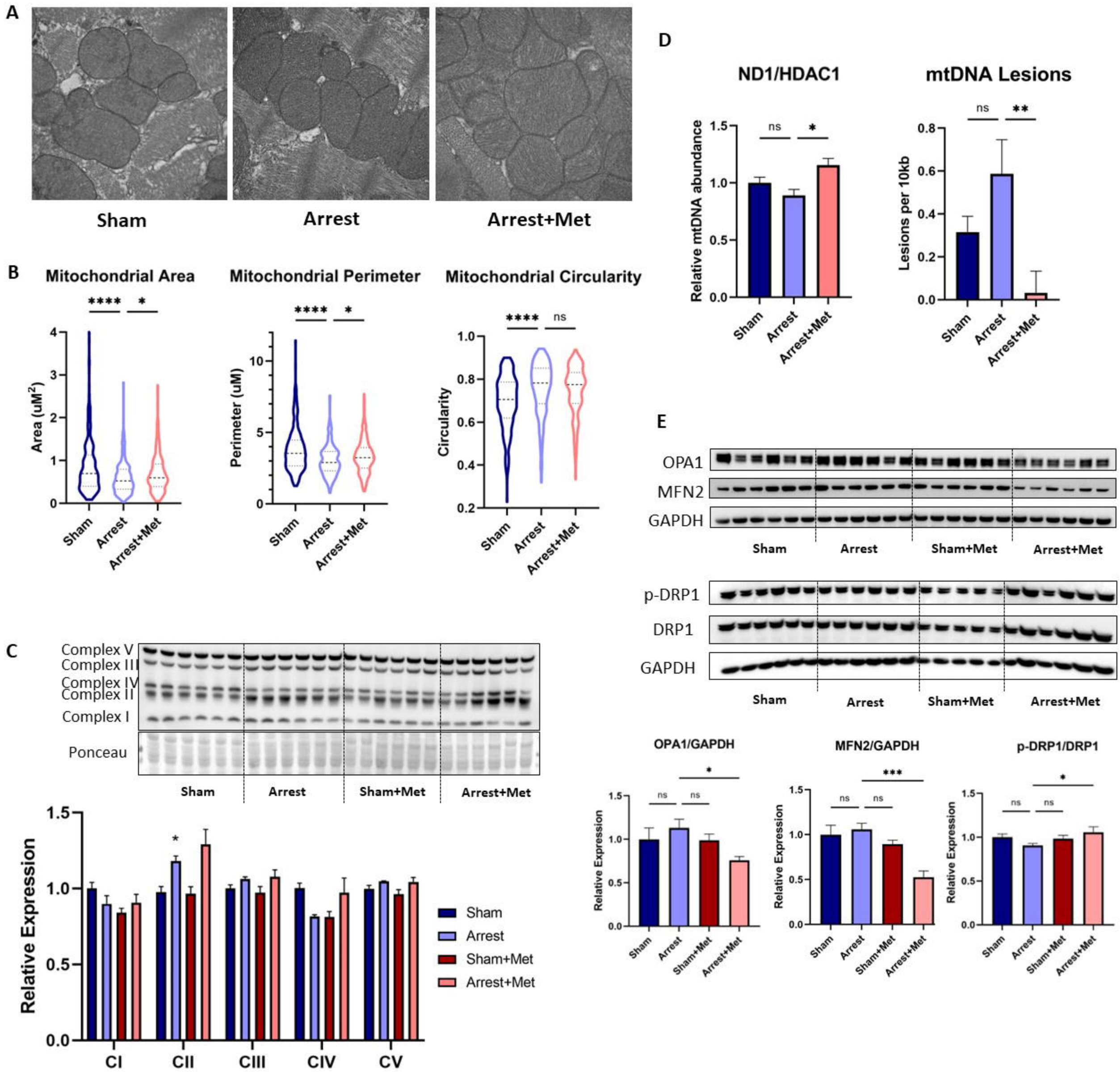

Figure 5. Metformin pretreatment affects mitochondrial size, mtDNA damage, and mitochondrial fission/fusion proteins after cardiac arrest (CA). A) Representative electron microscope images of intrafibrillar mitochondria in Sham, Arrest, and Arrest+Metformin (Arrest+Met) mice one day after surgery (40,000x magnification) B) Violin plot showing mitochondrial perimeter, area, and circularity (n=50 per group). C) Western blot of representative oxidative phosphorylation complex subunits (CI-CV) and bar graph of densitometry (n=6/group, normalized to ponceau stain). Quantitation shown in bar graphs with indicated normalization. D) Bar graph of mtDNA copy number assessment by qPCR quantitation of total DNA preparations of sham (n=5), arrest (n=4), and arrest metformin (n=7) mice. E) Western blot images of markers of mitochondrial fission and fusion (MFN2, OPA1, p-DRP1 (ser616), DRP1) and housekeeping protein (GAPDH) in Sham (n=6), Arrest (n=6), Sham+Met (n=5-6), and Arrest+Met (n=6) mice. B) C). E) Data are expressed as mean ± SEM. P-values: *< 0.05, **< 0.01, ***< 0.001, ***<0.0001 by one-way ANOVA with with Dunnett's multiple comparison. DRP, dynamin-related protein1; MFN2, mitofusin 2; OPA1, dynamin-like 120 kDa protein, mitochondrial.

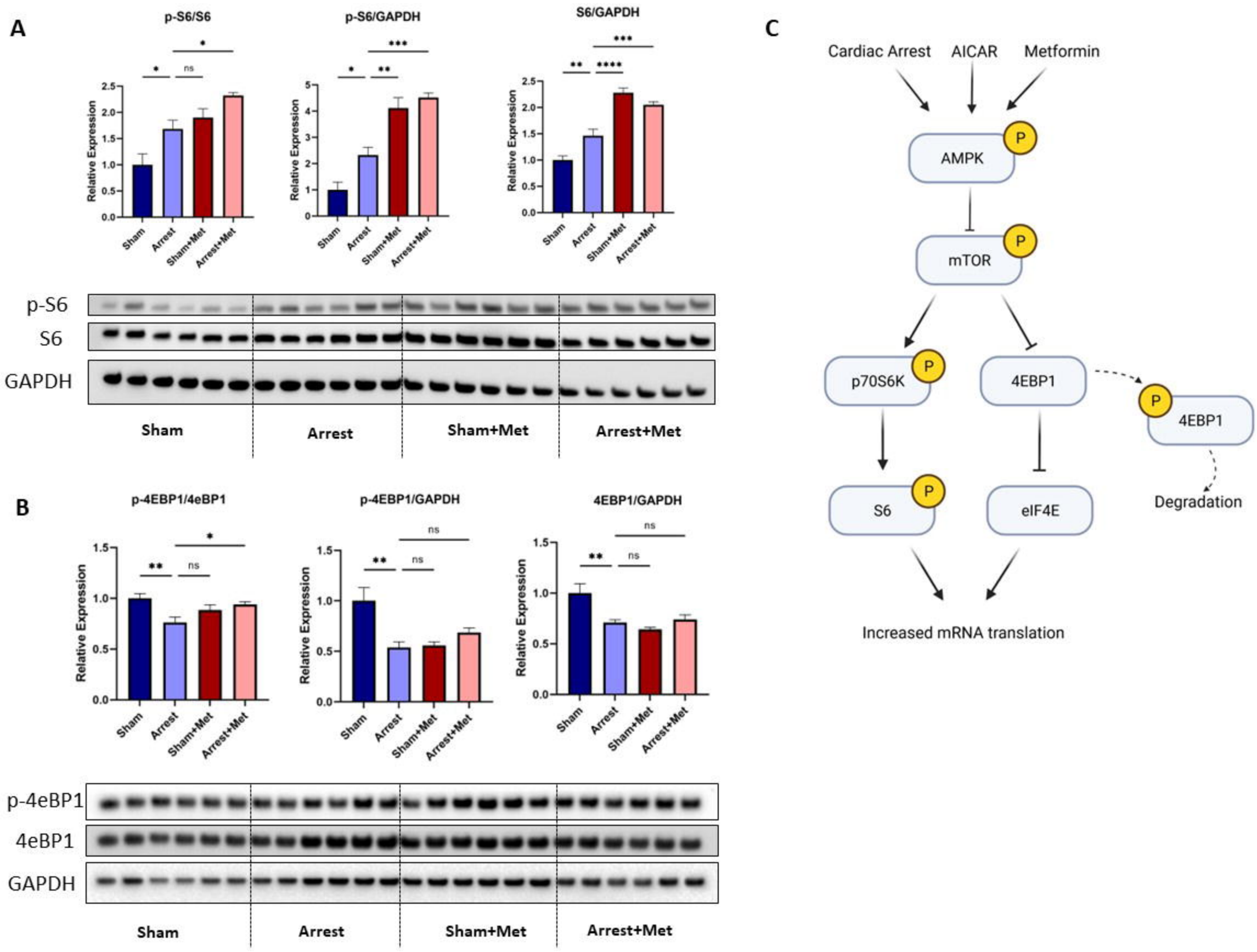

Figure 6. Western Blot Analysis of Protein Synthesis Markers. A) Western blot images of S6 and its phosphorylated form at Ser240/244 and housekeeping protein (GAPDH) expression in Sham, Arrest, Sham+Metformin (Sham+Met), and Arrest+Metformin (Arrest+Met) mice (n=6/group). B) Western blot images of 4eBP1 and its phosphorylated form at Thr37/46 as well as GAPDH. C) Cartoon representation of p-S6 and p-4EBP1 as regulators of mRNA translation downstream of AMPK and mTOR signaling. Data are expressed as mean ± SEM. P-values: *< 0.05, **< 0.01, ***< 0.001 by one-way ANOVA with with Dunnett's multiple comparison test. 4eBP1, eukaryotic translation initiation factor 4e-binding protein 1; mTOR, mechanistic target of rapamycin; S6, ribosomal protein S6.

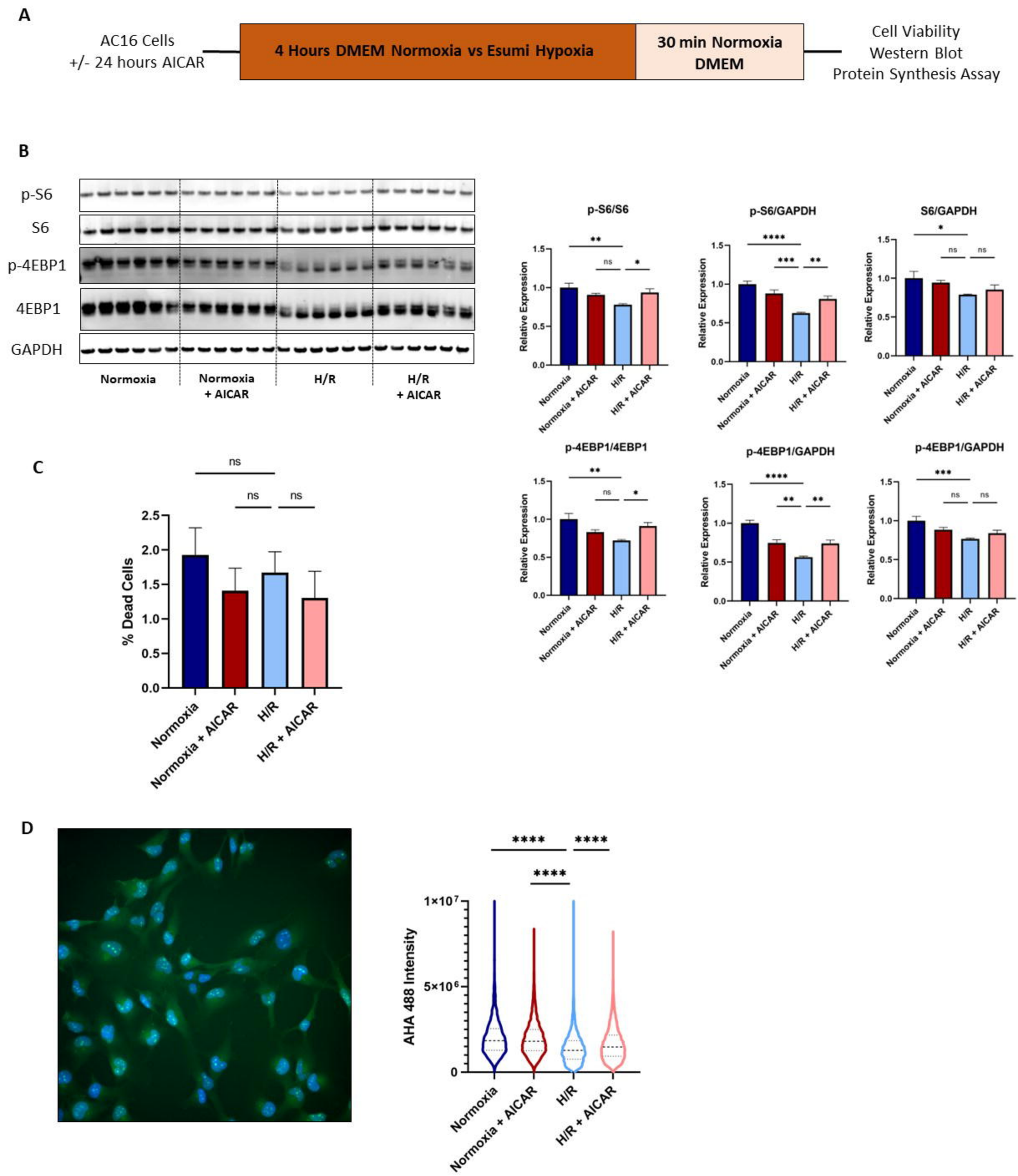

Figure 7. AICAR pretreatment protects against protein synthesis changes in AC16 cells after hypoxia/reoxygenation (H/R). A) Time course of AC16 cells with and without 24 hours of AICAR treatment (1.25 mM). B) Western Blot images of of p-S6 (Ser240/244), total S6, p-4EBP1 (Thr37/46), total 4EBP1, and GAPDH expression from cells 30 min after H/R (n=6/group). C) Quantification of cell death 30-min after H/R. D) Representative image of AHA 488 Protein Synthesis assay demonstrating AHA 488 signal (green) and Hoechst nuclear stain (blue) in AC16 cells 30 min after H/R and quantification of AHA 488 signal intensity surrounding the nuclei after H/R. P-values: *< 0.05, **< 0.01, ***< 0.001 by one-way ANOVA with with Dunnett's multiple comparison test.

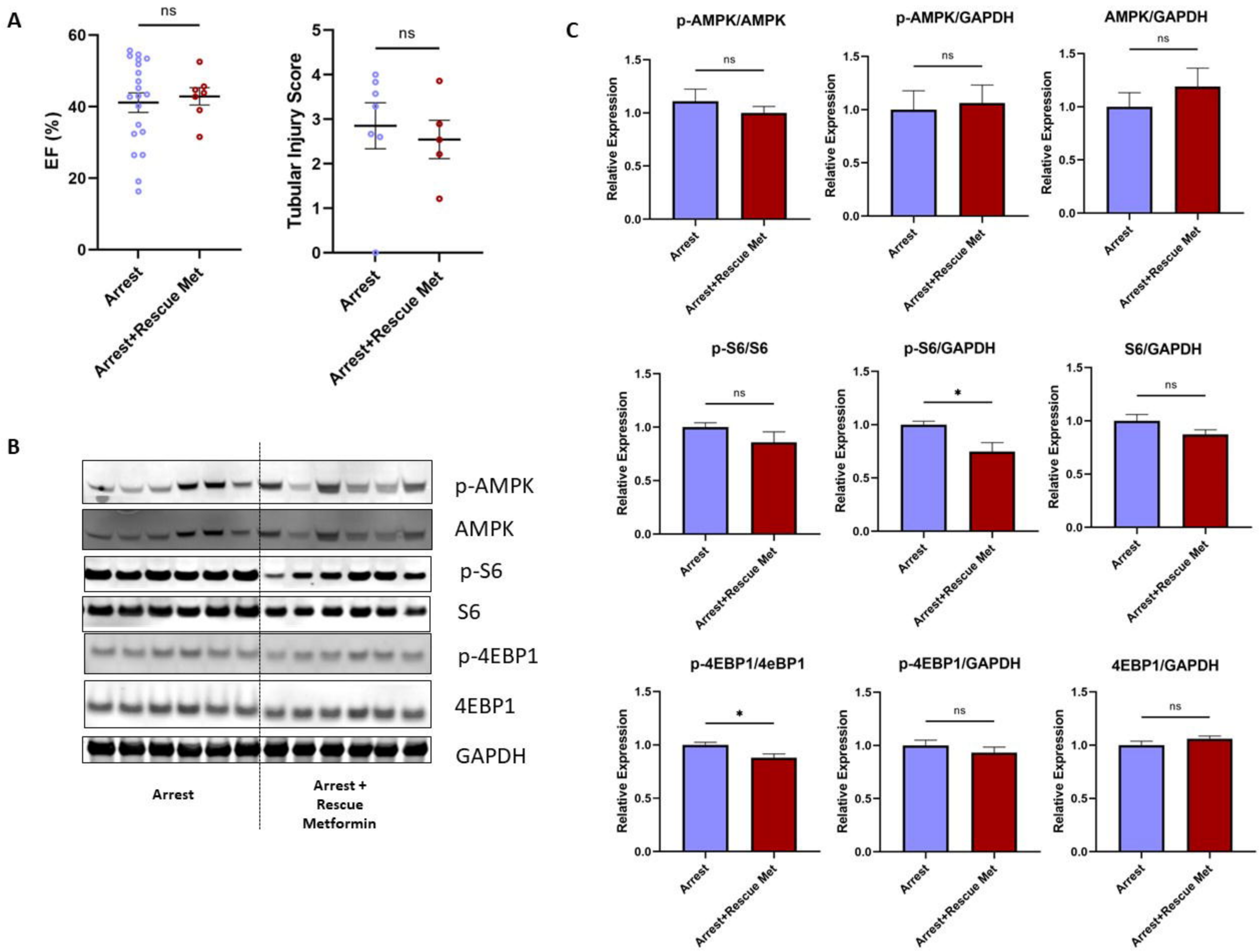

Figure 8. Metformin does not protect cardiac or renal function as a rescue therapy following arrest. A) When given concomitantly with epinephrine, intravenous metformin (rescue met, n=7) did not demonstrate any change to ejection fraction (EF) when compared to untreated arrest mice (n=20; arrest data also presented in Figure 2) or to kidney damage as measured by tubular injury score B) Western Blot images of of p-AMPK (Thr172), total AMPK, p-S6 (Ser240/244), total S6, p-4EBP1 (Thr37/46), total 4EBP1, and GAPDH expression between arrest and arrest + rescue metformin groups (n=6/group) C) Quantification of Western blot changes between groups. *p<0.05 by student's t-test.

Table 1. Surgical data for arrest mice. There are no significant changes to body weight, body temperature at time of extubation, time to return of spontaneous circulation (ROSC), time to extubation, or time to kidney reperfusion between groups when compared to the arrest group. Data are presented as mean ± SEM unless otherwise noted. Statistical comparisons by one-way ANOVA with Dunnett's multiple comparison test.

	Sham (n=20)	Arrest (n=26)	Sham Metformin (n=8)	Arrest Metformin (n=21)	Arrest AICAR (n=9)	Arrest Metformin+ compound C (n=8)
Age (d)	57.55±0.68	57.60±0.59	58.75±0.40	58.80±0.48	58.44±0.50	58.75±0.37
# Female / Total Mice (percentage)	11/20 (55%)	12/26 (46%)	5/8 (63%)	12/21 (57%)	4/9 (44%)	4/8 (50%)
8-wk Body Wt (g)	23.19±0.83	22.97±0.62	21.05±0.88	21.77±0.78	22.59±1.1	22.47±1.23
Temp at Extubation (°C)	35.86±0.15	35.53±0.20	35.96±0.18	35.95±0.12	35.79±0.19	36.21±0.11
ROSC (min)	-	1.31±0.09	-	1.43±0.15	1.44±0.24	1.23±0.12
Time to Extubation (min)	-	22.31±0.66	-	23.64±0.32	22.06±0.90	24.06±0.52
Time to Kidney Reperfusion (min)	-	21.3±0.90	-	21.79±0.57	20.43±1.01	24.70±0.64