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Lipid emulsion rapidly restores contractility in stunned mouse cardiomyocytes: A comparison with therapeutic hypothermia

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Abstract

Objective—Cooling following cardiac arrest can improve survival significantly. However, delays in achieving target temperature may decrease the overall benefits of cooling. Here we test whether lipid emulsion, a clinically approved drug reported to exert cardioprotection, can rescue heart contractility in the setting of delayed cooling in stunned mouse cardiomyocytes.

Design—Cell culture study

Setting—Academic research laboratory

Subjects—Cardiomyocytes isolated from 1–2-day old C57BL6 mice

Interventions—Cardiomyocytes were exposed to 30 minutes of ischemia followed by 90 minutes reperfusion and 10 minutes isoproterenol with nine interventions: 1) no additional treatment; 2) intra-ischemic cooling at 32°C initiated 10 min prior to reperfusion; 3) delayed cooling started 20 minutes after reperfusion; 4) lipid emulsion + delayed cooling; 5) lipid

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CONFLICT OF INTEREST DISCLOSURE

Guy Weinberg holds a US Patent related to lipid resuscitation and is a co-founder of ResQ Pharma, LLC. No other conflicts of interest.

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emulsion (0.25%) administered at reperfusion; 6) lipid emulsion + intra-ischemic cooling; 7) delayed lipid emulsion; 8) lipid emulsion + delayed cooling + Akt inhibitor (API-2, 10 μ M) and 9) lipid emulsion + delayed cooling + Erk inhibitor (U0126, 10 μ M). Inhibitors were given to cells 1 h prior to ischemia.

Measurements and Main Results—Contractility was recorded by real-time phase-contrast imaging and analyzed with pulse image velocimetry in MATLAB. Ischemia diminished cell contraction. The cardioprotective effect of cooling was diminished when delayed but was rescued by lipid emulsion. Further, lipid emulsion on its own improved recovery of the contractility to an equal extent as intra-ischemic cooling. However, co-treatment of lipid emulsion and intra-ischemic cooling did not further improve the recovery compared to either treatment alone. Moreover, Akt and Erk inhibitors blocked lipid emulsion-induced protection.

Conclusion—Lipid emulsion improved contractility and rescued contractility in the context of delayed cooling. This protective effect required Akt and Erk signaling. Lipid emulsion might serve as a treatment or adjunct to cooling in ameliorating myocardial ischemia/reperfusion injury.

Keywords

Stunning; Contractility; Lipid Emulsion; Cooling; Akt; Erk; Intralipid; Stunning

INTRODUCTION

The reversible myocardial stunning suffered during an episode of ischemia is far from clinically benign, complicating the recovery from cardiac arrest (CA) and cardiac surgery alike (1). Despite a lack of tissue death, stunned myocardium takes weeks to regain full contractile function, elevating morbidity and mortality (2) and requiring inotropic support to maintain cardiac output (3, 4). Many drugs have been tested to protect the myocardium from stunning and related injury, but with mixed clinical outcomes (5). Targeted temperature management using active cooling improves long-term neurologically-intact survival following CA (6–8) and has been proposed to aid cardiac recovery as well. In laboratory studies, cooling reduces cell death following myocardial ischemia (9) by activating Akt and Erk1/2, which are both members of the reperfusion injury salvage kinase pathway (10, 11). However, timeliness in reaching target temperatures is difficult even for mild cooling (12), and delays can negate the cardiac benefits of cooling (9).

Infusion of lipid emulsion (ILE) has been reported to have similar protective effect to cooling with activation of Akt and Erk1/2 pathways (13, 14), but has received little clinical attention. Jones et al originally proposed the beneficial effects of the soybean-based ILE as a myocardial energy substrate after ischemic arrest (15) and Van de Velde et al demonstrated the benefit of a systemic-infusion of ILE during recovery in several animal models of cardiac stunning (16, 17). The use of ILE during resuscitation is advantageous from a practical standpoint since ILE are readily available, clinically approved (as a parenteral nutrition supplement), easily infused, and exhibits a favorable safety profiles (18–20). These clinical advantages have led to the use to ILE as an effective treatment for cardiac pharmacotoxicity (21, 22). Therefore, we hypothesized that ILE could be used as adjuvant to cooling, especially when delayed. We also compared the effect of ILE to intra-ischemic

cooling and tested whether the combined treatment of ILE and cooling could further the cardiac protection in a cell model of myocardial stunning. Moreover, we examined the effect of ILE on relevant signaling pathways.

MATERIALS AND METHODS

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and these procedure were approved by the Animal Care Committee at the University of Illinois at Chicago

Mouse cardiomyocyte culture

Primary mouse ventricular cardiomyocytes were prepared from hearts of 1- to 2- day-old neonatal C57BL6 mice (Jackson Laboratories, Bar Harbor, ME). The details of the isolation protocol were reported previously (10). Experiments were performed on culture day 6–8.

Ischemia reperfusion (I/R) protocol

Synchronously contracting cells on glass coverslips were placed in a 1.2 ml Sykes-Moore perfusion chamber and subjected to ischemia reperfusion protocol as previously described (23). Nine treatment groups were examined (Figure 1) and included: 1) Stunning ($n = 10$). Cardiomyocytes were equilibrated for 30 min and then subjected to 30 min simulated ischemia, and 90 min reperfusion followed by 10 min isoproterenol (10 μ M). 2) Stunning + Intra-ischemic cooling (IC, $n = 10$). Cooling (32°C) was initiated during the last 10 min ischemia and extended 60 min into reperfusion. 3) Stunning + delayed cooling (DC, $n = 6$): Cooling was started 20 min into reperfusion and lasted for 40 min, and then rewarmed to 37°C for 30 min. 4) Stunning + DC + infusion of lipid emulsion (20% Intralipid, Baxter Pharmaceuticals, Deerfield, IL). 0.25% ILE (diluted with balanced salt solution [BSS], $n = 10$) was administered at the reperfusion for 90 min ($n = 11$). 5) Stunning + ILE ($n = 10$) was administered at the reperfusion for 90 min. 6) Stunning + delayed infusion of lipid emulsion (DLE, $n = 6$) treatment: 0.25% ILE was administered at 20 minutes into reperfusion for 70 min. 7) Stunning + IC + ILE ($n = 5$). 8) Stunning + DC + ILE + API-2 ($n = 5$). Cells were pre-incubated in API-2 (10 μ M, EMD Chemicals, Philadelphia, PA) for 1 h prior to ischemia. 9) Stunning + DC + ILE + U0126 ($n = 5$). Cells were pre-incubated in U0126 (10 μ M, EMD Chemicals, Philadelphia, PA) for 1 h prior to ischemia. All reported time values are given as minutes following their specific treatment and include 30 minutes into equilibrium (Eq30), 5 minutes into ischemia (Is5), 10, 20, 30, 60, 90 minutes into reperfusion (R10, R20, R30, R60, R90 respectively), and 10 minutes into Isoproterenol (Iso10). The Iso10 time point was used to assess the reserve capacity of contractility activated by β -adrenergic stimulation.

Measurement of the contractile velocity via speckle image velocimetry

A Nikon ECLIPSE Ti inverted phase/fluorescent microscope (Nikon Instruments Inc., Melville NY) was used for cell imaging. Movement of cardiomyocytes was recorded with phase-contrast microscopy following the same field of cells over time. One field of cardiomyocytes (~500 cells) was selected for each experiment, and eight-second videos were acquired at predetermined time points using phase-contrast optics (20X magnification,

33 frames per second). Speckle Image Velocimetry (24) was used to quantify contraction velocity with custom written MATLAB software (Mathworks, Natick MA). Speckle image velocimetry method was similar to previously described methods (25) and focused on contractions of the entire collection of neonatal cardiomyocytes in a microscopic field as opposed to velocities within a single adult myocyte (26, 27). In brief: A 19×19 array of 30×30 pixel (24×24 microns) windows to be tracked were spaced 25 pixels (20 microns) apart for reliable tracking of windows across contractions. Time of contraction onset for each video was identified based on peak differences in absolute pixel intensity between frames. Displacement matrices of each window were calculated using a Fast-Fourier-Transform cross-correlation algorithm. Vector displacements were converted to velocities (microns/sec) by dividing displacement by time step between frames (1/33 second). Maximum velocities were averaged across windows ($n = 361$) for each contraction and mean contraction velocity was calculated by averaging all time points across the 8-second video. To ensure reproducibility, each set of experiments consisted of replicates (n between 5–11) as listed in the results generated from multiple batches of cells. To ensure consistency between batches, only cells with spontaneous contractions between 100–150 beats per minute were used.

Western blot analysis

Protein was processed as previously described (10). In brief, cells were lysed in lysis buffer. Samples were resolved on a 10% SDS-Page gel, transferred to a nitrocellulose membrane and incubated with primary antibodies against p-Akt (T308 and S473), p-Erk1/2, or p-GSK3 β S9 (Cell Signaling, Boston, MA) or α -tubulin (Neomarks Fremont, CA). The membranes were further incubated with secondary antibodies conjugated to horseradish peroxidase, and visualized by SuperSignal (Thermo Scientific, Waltham, MA). Unless otherwise noted, lysates were analyzed at R30.

Statistical analysis

Results are expressed as mean $\pm$ SEM. Data was analyzed in Prism 6.0 (GraphPad, San Diego, CA) Before any individual comparisons; data sets were analyzed for normality using a D'Agostino & Pearson omnibus normality test if possible and using a Kolmogorov-Smirnoff normality test if numbers were too low to perform the D'Agostino & Pearson. Before any group comparisons, groups were analyzed for equal variances using a Bartlett's test for equal variance. When data were normal and exhibited equal variances, treatment groups were analyzed using a one-way ANOVA with post-hoc Bonferroni multiple-comparison tests of selected time sets. If variances were unequal or data were non-normal, treatment groups were analyzed with a Kruskal-Wallis test and post-hoc Dunn's multiple comparison tests. For inter-condition comparisons, data during reperfusion (R10, R30, R60, R90) was analyzed as a group; reserve capacity of contractility at the Iso10 time-point was separated out and analyzed with a Mann-Whitney U-Test due to difference in test conditions relative to reperfusion time-points. Differences from baseline (100% at Eq30) were assessed using a Wilcoxon Rank-sum test.

RESULTS

Stunning after ischemia and reperfusion of mouse cardiomyocytes

The velocity of contractility at Eq30 was used as baseline (100%). Cell contraction stopped by Is5 with a relative contraction velocity of $3.5 \pm 0.8\%$ of baseline. It gradually recovered during reperfusion at R30 ($p^{\#} < 0.05$), R60 ($p^{\$} < 0.0001$) and R90 ($p^{\$} < 0.0001$). The contractility was further improved with isoproterenol treatment to reflect the reserve capacity ($p < 0.0001$). However, it was not fully recovered to baseline level ($p^< < 0.01$) (Figure. 2).

Ischemic cooling

Contractility aided by IC was analyzed by Kruskal-Wallis. IC did not improve contractility during reperfusion in contrast to stunning alone until R90 ($60.6 \pm 3.5\%$ vs. $41.8 \pm 1.9\%$). Isoproterenol improved contractility to $107.9 \pm 6.6\%$ which was indistinguishable from baseline, but was significant relative to that in stunning ($p^{\$} < 0.0001$) (Figure 3A). Delaying the cooling 20 min provided no benefit in contractility improvement compared to stunning alone (Figure 3B).

Lipid treatment

Supplementation of delayed cooling with ILE rescued the recovery of contractility (Figure 4A) from 27.3 ± 2.3 to 40.77 ± 1.7 at R30 ($p^{\$} < 0.0001$), from 35.3 ± 2.0 to 50.69 ± 1.8 at R60 ($p^{\$} < 0.0001$) and from $39.3 \pm 1.8\%$ to $56.7 \pm 3.1\%$ at R90 ($p^{\$} < 0.0001$). DC+ILE also preserved reserve capacity of contractility upon supplementation of isoproterenol ($p < 0.0001$). Further, treatment with 0.25% ILE alone resulted in a rapid and dramatic recovery (Figure 4B). By R10, ILE markedly improved contractility ($40.4 \pm 2.8\%$ vs. $13.2 \pm 1.9\%$ in stunning alone, $p^{\#} < 0.05$) and the contractility remained elevated throughout reperfusion. Moreover, reserve capacity was preserved and improved to $112 \pm 4.2\%$ at Iso10 ($p < 0.0001$). Perfusion with ILE in the absence of stunning for 3 h had no effect on contractility compared to BSS perfused alone (data not shown). Delaying ILE by 20 minutes showed no benefit in recovery of contractility compared to stunning alone (data not shown).

Group analysis

We analyzed overall recovery among all groups by comparing contractile levels of all conditions (Stunning, DC, DLE, IC, ILE, DC + ILE, IC + ILE) at the R90 (Figure 5A) and the reserve contractile capacity at Iso10 (Figure 5B). At R90, ILE elevated contractile velocity the most in contrast to stunning ($p^{\$} < 0.0001$), DC ($p^{\$} < 0.0001$) and DLE ($p^{\$} < 0.0001$), while IC elevated contractile velocity in contrast to stunning ($p = 0.0291$) and DC ($p = 0.0352$). ILE induced a significantly higher contractile velocity than IC ($75.0 \pm 3.9\%$ vs. $60.6 \pm 3.5\%$, $p^{\#} < 0.05$). At Iso10, contractility was increased in IC, ILE, IC + ILE & DC + ILE ($p < 0.001$) for all individual Bonferroni post-tests comparing with stunning, DC and DLE while DC or DLE did not improve contractile reserve capacity. In comparison to baseline, IC and IC + ILE showed no difference at Iso10, but ILE recovered the contractility to above baseline level ($p = 0.003$). Further, co-administration of ILE and IC improved

recovery compared with stunning ($p < 0.001$) but did not improve recovery beyond either treatment alone (Figure 4A,B).

Assessment of Akt & Erk1/2 involvement in recovery

Probing for cellular signaling, we found that ILE increased p-Akt S473, T308, p-Erk1/2, and p-GSK3 β S9 by R10 and this phosphorylation persisted throughout the reperfusion (Figure 6A). The protective effects of ILE were partially inhibited by either the Akt inhibitor API-2 (10 μ M) or Erk inhibitor U0126 (10 μ M) (Figure 6B). Figure 6C demonstrated that IC increased phosphorylation of Akt at T308 and S473, which was suppressed by DC. Co-treatment of ILE and DC reversed the effect of DC on p-Akt and was inhibited by API-2. Similarly, p-Erk1/2 was reduced by DC, increased by ILE and inhibited by U0126 (Figure 6D).

DISCUSSION

We demonstrated in a cultured mouse cardiomyocyte model that a low concentration (0.25% -or- 0.25 g/100mL) of a soy-based lipid emulsion given at reperfusion rescues recovery of contractility from ischemic stunning in the context of delayed cooling via activation of Akt and Erk1/2. This protection was blocked by specific inhibitors of Akt (API-2) or Erk1/2 (U0126). Further, the treatment of ILE improved rapidity and degree of contractile recovery and reserve capacity better than IC. These findings suggest that ILE can activate protective signaling pathways in heart cells similar to those activated by cooling and protect against stunning injury.

Stunning model

We designed the shortened ischemia protocol to mimic the effects of cardiac stunning and assess the ability of different pharmacological interventions to maintain cardiac contractility after ischemia. The current work utilized a shortened protocol (30 minutes of ischemia) that diminished cardiac contractility in isolated cardiomyocytes and isoproterenol administration at the end of 90 minutes of reperfusion to assess the recovery of contractility and reserve capacity following stunning. This protocol demonstrated a consistent stunning effect and a time-sensitivity of cooling protection (i.e. within 20 minutes of reperfusion) that has been reported in our mouse CA model. The 30-minute time period also reflects the 20–30 minutes of arrest seen in most human clinical therapeutic hypothermia studies. Previous work has demonstrated that a shortened protocol produces less cell death and LDH release than longer protocols (23), but the current work confirmed that 30 minutes of ischemia severely reduces contractile capacity. Our work supports previous work demonstrating that cooling can protect cardiac contractility against more prolonged detrimental periods of ischemia (28).

Lipid emulsion rescue of contractility via Akt & Erk

Administration of ILE at the start of reperfusion facilitated the rapid recovery of contractility in the stunned cardiomyocytes and this effect depended on phosphorylation of Akt & Erk1/2. Previous work in isolated rat hearts demonstrated that ILE requires Akt signaling to exert myocardial protection (13, 29) and we have previously demonstrated in both cell and mouse that benefit from cooling also requires Akt signaling (10, 11). We confirmed in this

stunning model that inhibiting Akt or Erk signaling pathways blocked the protective effects of ILE in promoting return of contractions following an ischemic insult. A common protective pathway shared by ILE and cooling might explain the rescue of contractility by ILE in the context of delayed cooling and the lack of synergistic effect with co-administration of IC and ILE. Previous studies have demonstrated that delivery of fatty-acids or ILE *during* ischemia further exacerbates injury (30–35) possibly owing to the increased oxygen requirements for fatty acid oxidation compared with glycolysis. The present study demonstrates that low dose ILE delivered at reperfusion rapidly improves cardiomyocyte contractility and in keeping with previous published findings (13, 29), this protection is attributable to activation of survival signaling pathways. However, other studies suggest that beta-oxidation is required for the beneficial effects of ILE (16).

Clinical Implications

The prevalence of symptomatic coronary artery disease makes protecting the heart against lethal dysfunction after ischemic events a high priority for translational and clinical research. Reperfusion of ischemic myocardium can increase cellular damage and result in considerable morbidity and mortality (36). Cooling has received interest in part because it confers neurological protection and has been recommended for patients who remain unconscious following return of spontaneous circulation (ROSC) after CA (37, 38). In addition, protective cooling following CA requires intra-arrest cooling to 32–34°C induced during the partial reperfusion of cardiopulmonary resuscitation (CPR) (39), a treatment that is currently being clinically studied. However, the practical difficulties of achieving rapid targeted temperature management limits the clinical success of cooling (12). An agent that could be given during or immediately following CPR to mimic cooling protection could potentially be very beneficial.

ILE was identified nearly forty years ago as a potential treatment for cardiac stunning (15–17), but never progressed to the clinical setting. Recently, there has been a renewed interest in the potential for ILE infusion to activate traditional cardio-protective pathways following stunning (13, 14) because of its success in treating cardiac pharmacotoxicity (40) partially through an improvement in contractility (41). Previous experiments have described a benefit of ILE on the recovery from ischemic damage in patients (15), conscious dogs (16), isolated rabbit hearts (17) and isolated rat hearts (13, 14, 29, 42) when delivered at reperfusion. Therefore, we assume that lipid treatment for cardiac dysfunction would most likely be used as an adjuvant in the setting of delayed cooling.

The loss of recovery of contractility when ILE was delayed further supports the concept of an early and critical window of opportunity for rescuing heart function and improving survival after CA. A similar window of protection has been seen in our mouse model of CA both with intra-arrest cooling during CPR (39) and with nitrite infusion (43). These therapies all appear to share common survival signaling pathways in the heart related to Akt and Erk and a common time-sensitivity of treatment within 15–20 minutes after resuscitation. Whether ILE given during or immediately following CPR as an adjunct or to mimic cooling and cooling-related survival signaling activation deserves further study. Whether ILE administration exerts neurological protection also warrants further investigation. Of note,

cooling protection against stroke appears to be mediated in part by Akt (44). In relevant animal CA models, ILE experiments would need to identify appropriate administration parameters, efficacy and safety.

A surprising aspect of the current work was the small concentration of lipid required to produce a substantial benefit in contractility. Previous studies have used larger concentrations (1%) of lipid emulsion to improve cardiac function in drug-induced pharmacotoxicity (45, 46) but here we show that 0.25% lipid emulsion was very effective at increasing recovery from stunning. For instance, a 0.25% final blood triglyceride concentration could be achieved with roughly a 30mL bolus in a 70kg patient – a dose much smaller than typically recommended for reversing local anesthetic toxicity (47).

Limitations

We used an isolated cardiomyocyte model and therefore did not model the entire physiologic context of clinical CA. This cellular model lacks the systemic pharmacokinetic properties associated with lipid emulsion infusion and cannot account for the potential complications of a specific pathologic process like asphyxic arrest. However, this model does allow for careful study of stunning and its treatment at the cellular level apart from the systemic complexity of intact physiological models. Further, these heart cells rely less on fatty acids for metabolism than adult cells which gives them an advantage to study ILE effects apart from metabolic substrate effects. A number of other experimental systems have exhibited a benefit in response to ILE during ischemic stunning including whole animal (16, 17) and whole organ (13, 14, 29) models. Additionally, these models have implicated a number of mechanisms including the requirement for beta-oxidation (16), activation of the Akt & Erk (13, 29), and mitochondrial effects (14, 29). The only study demonstrating a negative effect examined lipid infusion in a rabbit model of hypoxic CA (48) where lipid was infused during asphyxia. Clearly, further pre-clinical studies are required before our results can be considered for translation to the clinical setting.

Conclusions

In a model of cardiac stunning using isolated cardiomyocytes, either intra-ischemic cooling (32°C) or lipid emulsion (0.25%) delivered at reperfusion improved contractile recovery during a 90-minute reperfusion. Additionally, lipid emulsion restored contractility much faster than any other treatment and served as an adjuvant in the case of delayed cooling, demonstrating cooling-like activation of the survival signaling pathways Akt and Erk1/2. The protective effect of intra-ischemic cooling or lipid emulsion was abolished when either was delayed just 20-minutes following reperfusion. This supports the notion of a critical time-sensitivity for the treatment of stunning following CA.

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Figure 1.

Protocol scheme for Stunning with additions of intra-ischemic cooling (Stunning + IC), infusion of 0.25% lipid emulsion at reperfusion (Stunning + ILE), Delayed cooling (Stunning + DC), Delayed infusion of 0.25% lipid emulsion (Stunning + DLE), Intra-ischemic cooling + infusion of 0.25% lipid emulsion at reperfusion (Stunning + IC + ILE) and delayed cooling + infusion of 0.25% lipid emulsion at reperfusion (Stunning + DC + ILE). Total time of protocol is depicted above timeline with individual timepoints and labels for 30-minute equilibration period (Eq30), 30 minutes ischemia (contractility at Ischemia 5min, Is5, was used as a representative and for proceeding experiments), 10 minutes of reperfusion (R10), 30 minutes of reperfusion (R30), 60 minutes of reperfusion (R60), 90 minutes of reperfusion (R90), and 10 minutes of isoproterenol given following 90 min reperfusion (Iso10).

Figure 2.

Cardiomyocyte contractility following stunning. All time groups are statistically below the Eq30 period ($p^< < 0.001$). Following reperfusion, contractility begins to recover by 30 minutes and is elevated from the total lack of contractility at Is5 ($p^{\#} < 0.05$) and continues to recover during reperfusion ($p^{\S} < 0.0001$).

Figure 3.

A: Effect of intra-ischemic cooling on cardiac contractility following stunning. Comparison of stunning alone ($n = 10$) and stunning + IC ($n = 10$; mean $\pm$ SE). IC enhanced contractility at R90 ($p^{\#} < 0.05$) and Iso10 ($p^{\$} < 0.0001$). **B:** Effect of delayed cooling on cardiac contractility following stunning. Comparison of stunning ($n=10$) and stunning + DC ($n=6$). DC resulted in a loss of cooling protection.

Figure 4.

A: Co-administration of ILE and delayed cooling improved cardiac contractility following stunning. Comparison of DC (n=6) and ILE + DC (DC + ILE; n=11). ILE given at reperfusion in the setting of delayed cooling improved contractility as early as R30 during reperfusion ($p^{\S} < 0.0001$) and at Iso10 ($p^{\S} < 0.0001$). **B:** Effect of ILE improved cardiac contractility following stunning. Comparison of stunning (n=10) and ILE treatment (n=10). ILE improved contractility as early as R10 ($p^{\#} < 0.05$, $p^{\parallel} < 0.01$) and at Iso10 ($p^{\S} < 0.0001$).

Figure 5.

A: Comparison of difference treatment conditions at 90 min reperfusion following stunning. Compared to stunning, IC improved contractility ($n = 10$, $p^{\#} < 0.05$) in contrast to no protection with DC; ILE enhanced contractility while DLE has no improvement ($n = 10$, $p^{\$} < 0.0001$); Co-administration of IC and ILE ($n = 5$) showed no further improvement on contractility compared to either treatment alone. **B:** Comparison of difference treatment conditions at Iso10. IC, ILE, IC + ILE and DC + ILE were all elevated from ($p^{\$} < 0.001$), DC ($p^{\$} < 0.001$), and DLE ($p^{\$} < 0.001$). IC and IC + ILE showed no difference from baseline ($p = 0.096$, $p = 0.43$ respectively). ILE was elevated above baseline ($p^{\>} < 0.0039$) while stunning, DC, DLE and DC + ILE were still ($p^{\#} < 0.05$).

Figure 6.

Role of Akt and Erk in cardiac contractility following stunning. **A**: ILE increased the phosphorylation of Akt S473 and T308, Erk1/2 and GSK-3β-S9 during reperfusion analyzed by Western blot ($n = 3$). α -tubulin was used as a loading control. **B**: ILE-induced improvement on contractility in the context of delayed cooling was mediated by Akt and Erk activation. Akt inhibitor API-2 and Erk inhibitor U0126 blocked the enhancement of contractility in cells treated with ILE + DC compared to DC alone ($n = 5$, $p^{\dagger} < 0.01$ and $p^{\#} < 0.05$ respectively). **C**: Effect of different treatments on Akt phosphorylation. Compared to stunning, IC increased p-Akt S473 and p-Akt T308 which were deactivated by DC, but reversed by ILE ($n = 3$). API-2 + BSS: cells were treated with API-2, but not subjected to stunning. **D**: Effect of different treatments on Erk phosphorylation. A similar effect was observed on Erk as on Akt ($n = 3$).