Improved endothelial-dependent and endothelial-independent skin vasodilator responses following remote ischemic preconditioning

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ABSTRACT

Introduction: One week of daily remote ischemic preconditioning (RIPC) improves cutaneous vasodilatory (VD) function. However, the underlying mechanisms and the number of sessions needed to optimize this adaptive response remain unclear. We hypothesized that the responses to localized heating of the skin will be greater after 2 weeks as opposed to 1 week of RIPC. Furthermore, 2 weeks of repeated RIPC will augment cutaneous VD responses to thermal and pharmacological stimuli. **Methods:** Twenty-four participants (24±2 years; 13 males, 11 females) performed repeated RIPC (7 daily sessions over 1 week, n=11; 12 sessions over 2 weeks, n=13) consisting of 4 repetitions of 5 min of arm blood flow occlusion separated by 5 min reperfusion. Laser speckle contrast imaging was used to measure skin blood flow responses, as perfusion units (PU), to local heating (T_{loc}=42 ^{o}C), acetylcholine (ACh), and sodium nitroprusside (SNP) before and after repeated RIPC. Data were expressed as cutaneous vascular conductance (CVC=PU·mmHg^{-1}). **Results:** The VD response to local heating increased after RIPC (ΔCVC from baseline; 1 week: 0.94 ± 0.11 to 1.19 ± 0.15, 2 week: 1.18 ± 0.07 to 1.33 ± 0.10 PU·mmHg^{-1}; $p<0.05$) but the ΔCVC did not differ between weeks. SNP-induced VD increased after 2 weeks of RIPC (ΔCVC; 0.34 ± 0.07 to 0.63 ± 0.11 PU·mmHg^{-1}; $p<0.05$) but ACh-induced VD did not. **Conclusion:** Repeated RIPC improves local heating- and SNP-mediated cutaneous VD. Compared to 1 week of RIPC, 2 weeks of RIPC does not induce further improvements in cutaneous VD function.

NEW & NOTEWORTHY

Repeated RIPC increases the cutaneous vasodilatory response to local heating and to sodium nitroprusside but not to acetylcholine. Thus, endothelial-independent and local heating-mediated cutaneous vasodilation are improved following RIPC. However, two weeks of RIPC sessions are not more effective than one week of RIPC sessions in enhancing local heating-mediated cutaneous vasodilation.

INTRODUCTION

Remote ischemic preconditioning (RIPC), induced by brief, intermittent periods of sublethal ischemia and reperfusion, is a non-invasive means to elicit protection from ischemia-reperfusion (IR) injury (35). IR injury attenuates conduit artery endothelial function, however an acute bout of RIPC prior to inducing IR injury preserved endothelial function (20, 27, 28). However, since the microvasculature comprises about 99% of the vascular tree, evaluating the microvascular responses to RIPC is needed to elucidate the beneficial effects of RIPC. Furthermore, since most studies assessing RIPC have been limited to evaluating the acute responses, the extent to which repeated bouts of RIPC improves microvascular function in humans is unknown.

We recently published work that used the cutaneous circulation to examine the underlying mechanisms of RIPC (24). Because of its accessibility, the cutaneous circulation has been used as a model to investigate *in vivo* human microvascular function noninvasively (13, 19, 30). Our previous work demonstrated that seven days of RIPC improved maximal cutaneous vasodilation elicited by local heating (T_{loc} = 43 ^{o}C) and sodium nitroprusside (SNP) (24). This finding supports other studies that showed angiogenic factors such as vascular endothelial growth factor, hypoxia induced factor, and endothelial progenitor cells are increased following repeated RIPC (2, 16, 21), thereby suggesting that structural adaptations are occurring. Secondly, we found that the nitric oxide (NO) contribution to vasodilation observed at the local heating plateau (T_{loc} =39°C) was not affected by repeated RIPC. However, it is unclear whether other endothelium-derived mediators are altered after repeated sessions of RIPC. Furthermore, the number of ischemic bouts or duration of RIPC may also affect the magnitude of the RIPC response. Consequently, more RIPC sessions may affect microvascular responses differently.

Although repeated RIPC improves cutaneous microvascular function (24), the extent to which endothelium-dependent and endothelium-independent pathways contribute to this response remain unclear. This may be further elucidated by using higher temperature local heating, which evokes greater contributions from non-NO mediators (6), and pharmacological stimuli.

Moreover, other studies have used repeated RIPC protocols of varying duration, ranging from 1 – 8 weeks (43), but the most efficacious number of sessions has yet to be determined. Thus, the purpose of this study was to test the contribution of the endothelium to cutaneous vasodilation following repeated RIPC and determine the extent to which longer duration RIPC affects endothelium-mediated responses. We hypothesized that after 2 weeks of RIPC, 1) the cutaneous vasodilatory responses to acetylcholine, SNP, and local heating (T_{loc} = 42 ^{o}C) will be augmented and 2) the hyperemia to local heating will be greater than that observed follwoing only 1 week of RIPC.

METHODS

Subjects

Eleven healthy young subjects (age = 25 ± 4 years old, 6 males, 5 female) received 1 week of daily RIPC while 13 (age=22 ± 2 years old, 7 males, 6 females) underwent 2 weeks of repeated RIPC. All subjects were normotensive (MAP = 87.6 ± 1.5 mmHg), non-smokers, not obese according to body mass index (BMI) (23.2 ± 0.6 kg·m^{-2}), not taking prescription medications or supplements that alter cardiovascular or thermoregulatory control and had no history of chronic skin diseases or skin allergies. On the day of the experiment, subjects arrived at the laboratory fasting (>3 hr), and having refrained from both alcohol/caffeine consumption (>12 h) and participation in novel or strenuous physical activity (>24 h). Verbal and written informed consent was obtained from all participants prior to the study. Experimental procedures conformed to the standards set by the *Declaration of Helsinki* and were approved by the Food and Drug Administration (FDA IND# 138343) as well as the Institutional Review Board at Iowa State University (IRB# 17-608) and Des Moines University (IRB# 08-15-05).

Protocol 1: One Week of Repeated RIPC

As an extension of our previous work (23), this protocol was designed to assess the effects of seven consecutive days of RIPC on the skin microvascular responses to local heating (T_{loc} = 42 ^{o}C). Each daily session of RIPC consisted of 4 cycles of 5 minutes of arm ischemia (i.e., inflating

an upper arm cuff to ~220 mmHg) separated by 5 minutes of no cuff pressure. Skin blood flow responses in the contralateral arm were assessed immediately prior to the first session of RIPC and 1 day after (>24 h but <48 h) the seventh and final session of RIPC.

For the microvascular assessments, participants arrived to the laboratory (T_a = 22 ^{o}C; RH = 36%) between 0600 and 1400 and were positioned in a semi-recumbent position prior to instrumentation. A laser speckle contrast imager (LSCI, moorFLPI-2, Moor instruments, Aximinster, UK),was placed 15-20 cm above the ventral surface of the forearm (wavelength = 785 nm, image exposure time = 4 ms, image acquisition rate = 25 frames/sec). LSCI measures the reflection of a laser light from moving red blood cells in the skin microvasculature, providing a full-field pattern of skin blood flow changes in the forearm with excellent reproducibility between measurements (29, 39). A vacuum cushion was used to stabilize the forearm and minimize movement during imaging. A local heating unit (moor VHS-HEAT, Moor Instruments, Axminster, UK) was attached to the skin surface at least 3 cm away from the antecubital fossa and then set to a thermoneutral temperature (T_{loc} = 33 ^{o}C). Following 15 min of baseline measures, the local heater was increased to 42^{o}C for ~45 min until a stable plateau was achieved (Fig 1). The LSCI continuously measured flux or changes in skin blood flow throughout the experiment. Arterial blood pressure was measured every 10 minutes using an automated system (Suntech Tango M2, Morrisville, NC, USA) that was verified with brachial auscultation. Heart rate was continuously collected using a lead II electrocardiogram (CT-1000 cardiotachometer, CWE inc, Ardmore, PA, USA).

Protocol 2: Two-Weeks of Repeated RIPC

This experimental protocol was designed to test the effects of 2 weeks of repeated RIPC on skin microvascular responses to local heating (T_{loc} = 42 ^{o}C) as well as the iontophoresis of acetylcholine (ACh) and SNP. The RIPC protocol was similar to that described previously except the duration of RIPC was two weeks administered as 3 periods of 4 consecutive days of RIPC sessions separated by a 1 day break (i.e., 12 sessions of RIPC in a 14 day period).

Microvascular assessments were conducted similarly to the 1-week protocol. Additionally, iontophoresis active and indifferent electrodes were placed 5 cm to 15 cm apart and between active and indifferent electrodes on the skin surface of the measurement site. Following instrumentation with LSCI and a 15-min baseline, local heating commenced (T_{loc} = 42^{o}C). While heating, iontophoresis of pure saline solution was administered at a separate skin site to ensure that no current-related changes in vasomotor function were occurring. Then, a 2 % ACh solution, diluted in saline, was infused via a 20 μA anodal current for 200 s. After hyperemia fully resolved, the active electrode was relocated to a separate skin site. Skin sites were separated by >2 cm. Pure saline solution was administered at the new site followed by a 1% SNP solution, diluted in saline, administered at 20 μA cathodal current for 400 s. The current applied and duration were based on previous studies that demonstrated a microvascular response without eliciting current-induced vasodilation (24). Throughout the protocol, skin blood flow, heart rate, and blood pressure were measured (HEM-907XL Blood Pressure Monitor, Omron, Japan).

Data analysis

LSCI data were averaged over a 2 min period at baseline, initial peak, and plateau of the local heating response. ACh and SNP peak responses corresponded to the highest flux values averaged over a 10 sec period. Cutaneous vascular conductance (CVC) was calculated as the mean LSCI perfusion units (PU) divided by mean arterial pressure (diastolic pressure + 1/3 pulse pressure) and expressed as an absolute CVC value (PU·mmHg^{-1}) as well as a change from baseline (ΔCVC). The area under the curve (AUC) defined the hyperemic responses to Ach and SNP above the baseline and was calculated as AUC – (baseline CVC x hyperemia time). The total time of hyperemia of ACh and SNP were 6min 20s and 16min 40s, respectively. The duration of the hyperemic responses was determined using the beginning of iontophoresis stimulation as the starting point and the endpoint was chosen based on the time at which most (10 out of 13 subjects) of the hyperemic responses had returned to baseline values.

A two-way mixed model ANOVA [duration (1wk vs. 2wks) x time (pre vs. post)] was used to compare the responses to local heating between 1 week and 2 weeks of RIPC (protocol 1 vs. protocol 2) (SigmaPlot version 14.0, Systat Software, San Jose, CA). A paired Student's t-test (two-tailed) was used to assess Ach and SNP-induced cutaneous vasodilation responses pre- versus post-2 weeks of RIPC (protocol 2). A power analysis was conducted based on our previous work assessing cutaneous microvascular responses to drug treatments (25). This indicated that 11 subjects (power=0.80, α=0.05) would be sufficient to measure meaningful physiological changes. Statistical significance was set at $\alpha = 0.05$. Data are presented as mean ± SEM except for data illustrating individual subject changes with RIPC that include group means and standard deviation.

RESULTS

A representative tracing of the local heating response illustrates an increase in CVC following RIPC (Fig 1). This response is also reflected in the corresponding LSCI image of the local heating plateau before and after RIPC (Fig 2A). The orange and red colors in this image represents skin blood flow augmentation. Group mean skin data, shown in Table 1, indicate that resting MAP (1 week, $p = 0.09$; 2 week, $p = 0.36$) and baseline CVC did not significantly change following RIPC. However, there were significant increases in the initial peak, plateau and in the change in CVC from baseline (ΔCVC) following both 1 week and 2 weeks of RIPC ($p < 0.05$). In comparing the responses consequent to the 2 durations of RIPC (1 week vs. 2 weeks), there were no significant interaction effects (duration of RIPC x pre-post RIPC) for the CVC responses to local heating (initial peak, $p = 0.79$; plateau, $p = 0.34$; ΔCVC, $p = 0.34$). Individual pre-post changes in ΔCVC for 1 week and 2 weeks RIPC are shown in Figure 3.

The group mean Ach- and SNP-induced CVC responses following 2 weeks of RIPC are shown in Table 2. The peak CVC ($p = 0.30$), ΔCVC ($p = 0.34$) and AUC ($p = 0.70$) to Ach iontophoresis were not altered following RIPC. However, each of these variables increased after RIPC in response to SNP iontophoresis ($p < 0.05$). A representative image of the peak SNP response before and

after 2 weeks of RIPC is shown in Figure 2B. Individual pre-post changes in ΔCVC response to SNP, based on responders and non-responders to RIPC are illustrated in Figure 4.

DISCUSSION

The purpose of this study was to determine whether repeated RIPC affected the skin hyperemic response to thermal or pharmacological stimuli. We found that the vasodilation response to localized skin heating (42^{o}C), in both the initial peak and plateau, modestly increased by ~10-20% following at least seven daily session of RIPC. However, the duration of RIPC (1 week vs 2 weeks) did not seem to affect the local heating response. In contrast to our hypothesis, RIPC did not appear to affect the ACh-mediated vasodilation response. In support of our previous work (24), SNP-mediated dilation was augmented following repeated RIPC. Cumulatively, these data indicate that local heating-mediated endothelium-dependent and endothelium-independent vasodilation is increased following repeated RIPC. And, it appears that 1 week of RIPC was as effective as 2 weeks of RIPC in eliciting cutaneous microvascular adaptations.

Local heating to 42^{o}C elicits a phasic cutaneous vasodilation response that consists of a transient initial peak, followed by a nadir, and then a sustained plateau (31). The initial peak is mediated in large part by an axon nerve reflex response stimulated by sensory nerves (31), whereas the primary contributors to the plateau phase are endothelium-derived factors such as NO and endothelial derived hyperpolarization factor (EDHF) (7, 8, 17, 30). The contribution of these underlying mechanisms explaining the plateau vary depending on the local heating temperature used (8). Compared to 42^{o}C, local heating to 39^{o}C elicits a plateau that is more dependent upon NO (i.e., ~75% compared to ~40-50% NO contribution) (8). We previously found that one week of RIPC does not increase the plateau response to either 39^{o}C heating or the NO-mediated component of that plateau, which was verified by locally perfusing a nonspecific NOS inhibitor (24). In the present study, local heating to 42^{o}C resulted in a peak vasodilation that was increased by ~10-20% following repeated RIPC. By using a less NO-specific heating protocol, we were able to detect an improved local heating--mediated

response to repeated RIPC. These findings collectively suggest that non-NO mediated endothelial factors, such as EDHF, or altered vascular smooth muscle function are more greatly affected with repeated RIPC.

The observation that local heating induced vasodilation increased after RIPC is in contrast to other reports (14, 15). In the few *in vivo* studies that have examined cutaneous microvascular function following RIPC, the local heating (42^{o}C) plateau was not affected after 1 week of RIPC (14) using a protocol conducted similarly to the current study, nor was it affected after 8 weeks of RIPC (3 bouts/week) (15). These contrasting results may be due to methodology. The previous studies used laser Doppler flowmetry (LDF) whereas the current study used laser speckle contrast imaging (LSCI). LSCI may be superior to LDF with respect to reproducibility (29, 36, 39), thereby limiting day-to-day variability and increasing the precision of detecting changes in a pre-post study design. Additionally, LSCI detects flux or blood flow changes at more shallow depths (i.e., ~0.25 mm from the skin surface) compared to LDF (~i.e., ~0.33 – 1 mm depth depending on the probe). This may explain how our CVC values during the plateau were ~1.5 PU·mmHg^{-1}, which was less than what Jones et al found with LDF at the same local heating temperature, ~2.3 flux · mmHg^{-1} (15). Thus, it is possible that the depth of Doppler assessment may affect the magnitude of the vasodilation detected at the local heating plateau.

The vasodilation to local heating (42^{o}C) was elevated following repeated RIPC in the current study, yet no further increases occurred with the 2 week compared to the 1 week RIPC intervention. The duration of the RIPC intervention has varied between studies, ranging between 1-8 weeks and at different frequencies (i.e., RIPC bouts/week) (40). In studies that assessed conduit vessel endothelial function, it was not apparent that the flow-mediated dilation (FMD) response was greater in the longer duration RIPC interventions (14, 15, 21, 26, 28). Collectively, these findings suggest that the 1 week timeframe is sufficient to fully elucidate any endothelium-mediated changes following repeated RIPC.

In various animal and human models, the underlying mechanisms explaining improvements in vascular function differ between a single bout and repeated bouts of RIPC (14, 21, 26-28, 38, 40). A single bout of RIPC increases endothelial function that lasts for ~24-48 hours; however, the duration of this effect has not been clarified (5, 27). Although there are reports to the contrary (34), previous work in animals has indicated that NO importantly contributes to the increased endothelial response following a single bout of RIPC (1, 23, 37). However, after 1 week of RIPC, we previously found that NO-mediated vasodilation to local skin heating (39^{o}C) was unaffected (24). In that study (10), we assessed NO function ~24 hours after the last RIPC bout. Despite the fact that the response at this time point would reflect both the single bout effect as well as the cumulative repeated bout effect, there was no evident change in NO function. This finding is consistent with the current study demonstrating that ACh-mediated dilation was not different following repeated RIPC.

Acetylcholine-mediated vasodilation is commonly used as a test of endothelial microvascular function in skin (10-12, 18, 30). Although the mechanisms contributing to the dilation response vary depending on dose and method of administration (i.e., iontophoresis or microdialysis) , a moderate bolus dose of ACh is primarily mediated by prostanoids and NO (4, 6, 9-12, 18, 32, 33). Incidentally, the studies indicating that prostanoids have no effect commonly use iontophoresis as a method of ACh administration (4, 22, 32). As such, these results may be affected by artifact dilation due to the administration of electrical current (4, 9, 32, 33). The current used in the present study was ~10 fold less and there was no evident dilation in the iontophoresis of the saline vehicle. At higher doses of ACh (≥10 mM), there may be a greater indirect role of EDHF, which may be reflected in the interaction, or 'cross-talk', between EDHF and NOS and COX pathways (6). Thus, the lower concentrations of ACh delivered in the current study, evidenced by the reduced magnitude and duration of hyperemia, may not have been strong enough to detect an EDHF contribution. Nevertheless, both the direct and indirect contribution of EDHF is greater with local heating as opposed to ACh-mediated vasodilation(6-8). Thus, the observation in the current study of greater local heating but not ACh-mediated vasodilation may indicate that EDHF is a primary endothelial mechanism affected by RIPC.

An increase in endothelium-independent vasodilation occurs after repeated RIPC. This was supported by our previous study indicating that 1 week of RIPC improved the maximal cutaneous vasodilation elicited by local heating combined with SNP, a NO donor (24). However, this maximal dilation response reflects both endothelium-dependent and independent contributions. The present study verifies the endothelium-independent contribution in that the vasodilation response to SNP alone is augmented after 2 weeks of RIPC. Nevertheless, improved endothelial independent vasodilation may be explained by increased vascular smooth muscle sensitivity or angiogenesis. Vascular smooth muscle contractility is regulated by myosin light chain kinase (MLCK) phosphorylation and dephosphorylation. Vascular smooth muscle sensitivity to NO can influence vascular smooth muscle contractility by readily inducing MLCK dephosphorylation(39). However, we are not aware of any study that assessed the effects of RIPC on MLCK. With respect to the latter, there is evidence in humans indicating that vascular endothelial growth factor and endothelial progenitor cells are increased after 4 weeks of RIPC (21, 26). Thus, it is plausible that more extended periods of RIPC may allow structural adaptations to occur. Although our data indicated that local heating-mediated endothelium-dependent dilation is not further increased with longer duration RIPC, it remains unclear whether RIPC duration affects endothelium-independent adaptations.

Practical Implications. The general premise of conducting repeated bouts of RIPC is to examine whether longer lasting vascular adaptations occur. This is supported by studies that have detected improved vascular function 1 week after the last RIPC bout (16, 26, 29). It is clear that the underlying mechanisms affected by repeated RIPC is different than the response to only a single bout of RIPC (43). As such, repeated RIPC may be a more effective intervention to elicit longer lasting adaptations that improve cardiovascular function and minimize the effect of IR injury subsequent to an MI or stroke. Repeated RIPC may be of most benefit to people who are unable to embark on more conventional methods of improving microvascular function, such as a traditional exercise program.

Limitations. The time course of cardioprotection following a single bout of RIPC is based upon animal models examining infarct damage. According to this timeline, a 'delayed window' of cardioprotection lasts up to 72 hours after the RIPC bout (3). Although there is a study supporting this timeline with FMD (27), we are not aware of any similar study assessing the microvasculature. However, because our microvascular measures were collected 24 hours after the last RIPC bout, our findings not only reflect the 'repeated bout' effect of multiple RIPC sessions but also, in part, the 'single bout' effect from the last RIPC session. Further studies are needed to determine whether single bout microvascular responses are occurring and the latency of those effects. A second limitation was that the participants and the microvascular assessments, with the exception of local heating, differed between protocol 1 and protocol 2. Thus, the conclusion that 2 weeks of RIPC does not elicit further benefits than 1 week RIPC is limited to the comparison of the local heating response. Further study is needed to determine whether this is also observed with endothelium-independent function.

Conclusion. In conclusion, repeated RIPC improves the skin vasodilation response to the application of 42^{o}C localized heat but not to ACh. This indicates that RIPC improves cutaneous endothelial function by mechanisms that are more specifically activated by heating, such as EDHF. Furthermore, the duration of repeated RIPC (1-week vs. 2-weeks) did not influence the magnitude of local heating induced cutaneous vasodilation improvement. Thus, 1 week may be sufficient to elicit improvements in endothelial function with RIPC. Lastly, SNP-mediated vasodilation was improved following 2 weeks of RIPC, which confirms that endothelium-independent vasodilation is also improved following repeated RIPC.

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DISCLOSURES

The authors report no competing interests.

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FIGURE CAPTIONS

Figure 1: Representative cutaneous vasodilation response to local heating (T_{loc} = 42^{o}C). Both the initial peak and plateau were increased after 2 weeks of RIPC

Figure 2: Representative laser speckle contrast images illustrating the increase in the vasodilation responses to local heating (A) and SNP (B) following 2 weeks of RIPC. LSCI measures the reflection of a beam of the laser by moving red blood cells in the skin microvasculature. The color palette indicates flux value changes. The more red the image, the more red blood cell movement that is detected.

Figure 3: Individual changes in CVC (PU · mmHg^{-1}) from baseline during local heating are shown before and after 1 and 2 weeks of RIPC. Group means were significantly higher after 1 and 2 weeks of RIPC.

Figure 4: Individual changes in CVC (PU · mmHg^{-1}) from baseline during SNP iontophoresis are shown before and after 2 weeks of RIPC. The red hashed lines represent participants that did not exhibit an increase in ΔCVC after 2 weeks of RIPC; however, the group mean was significantly higher after RIPC. * $p < 0.05$ vs pre

489 **Table 1**: Local heating induced changes to CVC (PU·mmHg^{-1}) before and after 1 or 2 weeks of

490 RIPC.

491

	1 Week		2 Weeks	
	Pre	**Post**	**Pre**	**Post**
MAP (mmHg)	89.1 ± 2.9	83.9 ± 2.3‡	85.4 ± 2.0	83.2 ± 2.2
Baseline CVC	0.29 ± 0.04	0.31 ± 0.04	0.34 ± 0.02	0.36 ± 0.03
Initial Peak	1.08 ± 0.09	1.23 ± 0.11*	1.43 ± 0.08	1.55 ± 0.10†
Heating plateau	1.23 ± 0.13	1.50 ± 0.16*	1.52 ± 0.08	1.69 ± 0.11*
Δ CVC	0.94 ± 0.11	1.19 ± 0.15 *	1.18 ± 0.07	1.33 ± 0.10 *

492 * $p < 0.05$ vs Pre, † $p=0.065$ vs Pre, and ‡=0.09 vs Pre

493

494 **Table 2:** Acetylcholine- and sodium nitroprusside- induced changes to CVC ($PU \cdot mmHg^{-1}$) before and

495 after 2 weeks of RIPC.

496

	Ach		SNP	
	Pre	**Post**	**Pre**	**Post**
Baseline	0.39 ± 0.03	0.42 ± 0.04	0.52 ± 0.04	0.52 ± 0.04
Peak	0.96 ± 0.08	1.13 ± 0.13	0.88 ± 0.07	1.16 ± 0.12*
Δ CVC	0.57 ± 0.08	0.71 ± 0.11	0.34 ± 0.07	0.63 ± 0.11*
AUC	140.5 ± 27.3	155.3 ± 31.0	244.9 ± 71.8	395.3 ± 96.4†

497 AUC, area under the curve; * $p < 0.05$, † $p = 0.09$ vs Pre

498

499

Figure 1

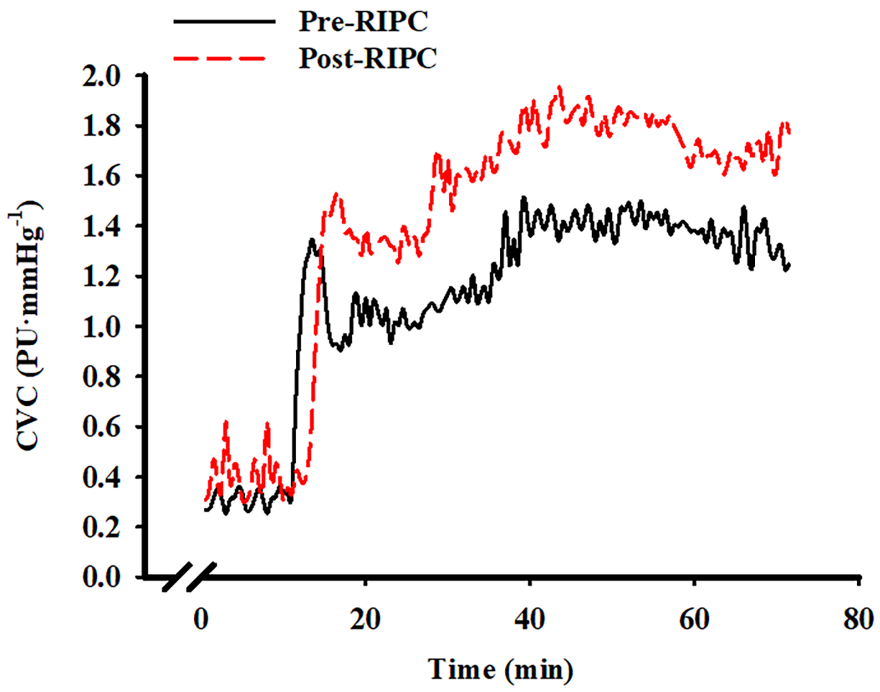

Figure 2

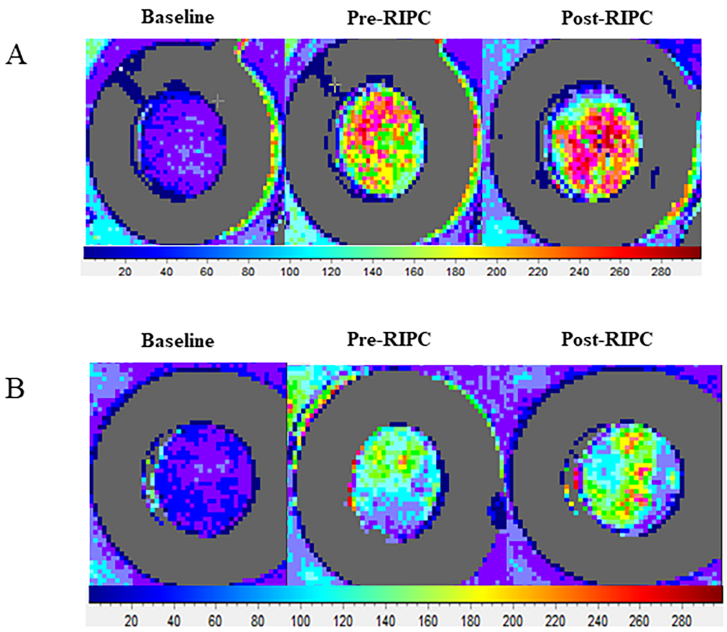

Figure 3

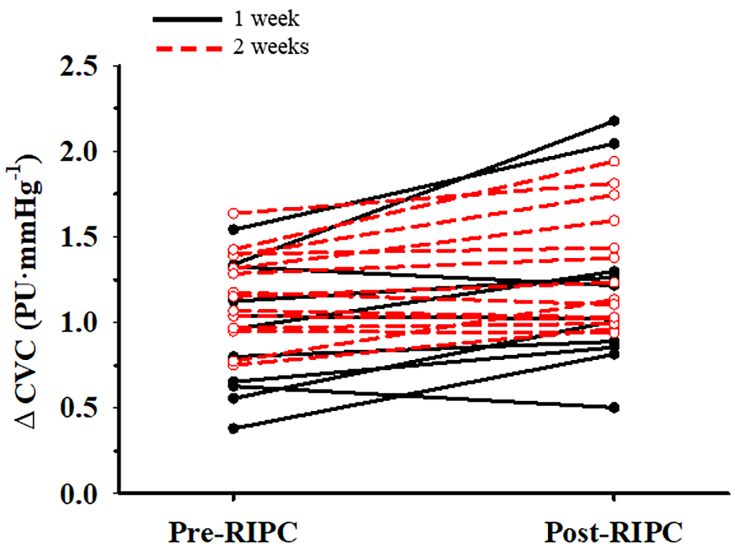

Figure 4