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The effect of remote ischemic conditioning on blood plasma heat capacity profiles
A differential scanning calorimetry study

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OBJECTIVE **To shed light on the effect of remote ischemic conditioning (RIC) on the properties of blood plasma of patients with acute myocardial infarction (AMI), using differential scanning calorimetry (DSC), which has recently emerged as a novel tool in biomedicine.** METHOD **We used DSC to examine the protein denaturation heat capacity profiles of blood plasma samples, particularly of plasma albumin and immunoglobulins, collected from patients with acute myocardial infarction, before and after RIC intervention. DSC is a highly sensitive, non-invasive biophysical technique, which provides fast *in situ* monitoring of changes in the thermodynamic behavior of biological samples.** RESULTS **A major finding of the study was the reduction of the albumin peak in the DSC profiles of the patients with AMI as a result of the RIC intervention, with the heat capacities ratio of the albumin to globulin peaks approaching the control (healthy) values. In contrast, when RIC was applied to healthy individuals, it produced no significant changes in the heat capacity profiles of their plasma proteins.** CONCLUSIONS **RIC intervention had a beneficial effect on patients with AMI, bringing their blood plasma thermograms closer to those characterizing healthy individuals. Previous studies have proposed various mechanisms for the RIC beneficial effects and some reports suggest oxidative stress reduction as a possible mechanism mediating the RIC effects. However, since blood plasma oxidation has been previously reported to result in suppression of the albumin denaturation peak, the findings of the present study appear to disagree with the hypothesis that RIC exerts its beneficial effect through oxidative stress reduction.**

Επίδραση της ισχαιμικής περίσφιξης της βραχιόνιας αρτηρίας στο πλάσμα του αίματος. Μελέτη της μεθόδου της διαφορικής θερμιδομετρίας

Περίληψη στο τέλος του άρθρου

Key words

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Remote ischemic conditioning (RIC) is an experimental medical intervention intended to reduce the ischemic injury to an organ such as the heart, most commonly in the situation of a heart attack (when the heart may suffer temporary ischemia), by triggering the natural protection mechanisms against tissue injury.[1,2] The intervention involves temporary cessation of blood flow to a limb to create ischemia in the tissues. Such conditioning activates protective mechanisms against reperfusion injury caused when tissue oxygen returns from low to normal levels after a period of ischemia (hypoxia). Thus, RIC essentially mimics the cardio-protective effects of exercise.[3]

RIC utilizes intracellular signal transduction, but it also involves the transfer of a cardioprotective signal from the ischemic/reperfused remote tissue or organ to the heart, through both neuronal and humoral pathways.[2] The signal transduction pathways of the conditioning phenomena in the human heart are still largely unknown. Data regarding the benefits of RIC are contradictory. Numerous clinical studies have demonstrated its ability to reduce the size of myocardial infarction and improve prognosis, but clinical trials have failed to demonstrate such benefits.[4]

Differential scanning calorimetry (DSC) is a sensitive, non-invasive thermoanalytical technique that provides fast *in situ* monitoring of changes in the thermodynamic behavior of biological samples such as blood plasma. Basically, it provides a specific heat capacity C_p vs temperature T curve, which is a unique signature for the denaturation of a given protein under specific solution conditions. For blood plasma, the $C_p(T)$ curves (thermograms) obtained by DSC reflect the denaturation of a complex mixture of proteins, with the recorded thermogram representing the sum of the individual protein thermograms weighted according to their mass in the solution.[5] DSC has recently emerged as a novel tool in biomedicine, since its potential for disease diagnostics and monitoring based on body fluid (mainly blood plasma) heat capacity profiles has been demonstrated in a number of diseases.[6] This method has been shown to detect specific aberrations in the thermal behavior of blood plasma proteins in a variety of diseases, including lung cancer,[7] multiple sclerosis,[8] brain tumors,[9] etc.

In the present study, we used DSC to examine the protein denaturation heat capacity profiles of blood plasma samples obtained before and after RIC from patients with ischemic heart disease-myocardial infarction, to shed light on the mechanism(s) of the beneficial effects of RIC. A major finding of our study was the reduction of the albumin peak in the DSC profiles as a result of the RIC intervention. To our knowledge, this is the first calorimetric study of the effects of RIC on the plasma proteome denaturation profile.

MATERIAL AND METHOD

Subjects

Ten patients (9 men, 1 woman, age 39–75 years, mean age 62±10 years) admitted to the "Attikon" University Hospital, Athens, Greece with acute myocardial infarction (AMI) volunteered to participate in this study, while 6 healthy volunteers [4 men (aged 22, 26, 55 and 79 years) and 2 women (aged 19 and 54 years)] served as control subjects. All the participants gave their written informed consent to participate in the study and all data were handled according to the ethical standards of the Declaration of Helsinki (1975).

Experimental design

The RIC protocol of a single ischemic session was applied in each patient within 48 hours after AMI and primary percutaneous coronary intervention (PCI), and in the healthy control subjects, using bilateral brachial cuff inflation at 200 mmHg for 5 minutes, and a final assessment was performed 25 minutes after the cuff deflation. Blood samples were collected immediately before and 30 min after the RIC intervention. All the AMI patients were being treated with statins, anti-platelet treatment and β-blockers. In addition, all the patients were in sinus rhythm, while exclusion criteria included Killip class >2 during the index event, administration of nitrates, a history of previous known coronary artery or other cardiovascular disease, previous PCI or coronary artery bypass surgery (CABG), and chronic inflammatory and systemic disease. The study protocol was approved by the University General Hospital "Attikon" Institutional Review Board (www.clinicaltrials.gov; unique identifier: NCT3884123).

Differential scanning calorimetry measurements

The DSC measurements were performed using a Nano DSC instrument (TA Instruments). Typically, a sequence of two heating scans was made for each sample at 1 K/min scan rate in the range 1–105 °C. The first scans displayed the denaturation heat capacity profiles of blood plasma, while the second scans displayed heat capacity profiles typical of denatured samples, with no detectable thermal events. The second scans, which originated from the same sample, with the same chemical composition, but displayed no thermal events, were used as a baseline and were subtracted from the first scans. Such an approach is typically used for samples with complex composition, undergoing irreversible thermal denaturational transitions and was rationalized in our previous work.[10–12] The heat capacity profiles were deconvoluted using Gaussian curves.[7,10]

SDS-PAGE electrophoresis

The serum samples were serially diluted with phosphate-buffered saline (PBS) and the resulting dilutions were analyzed for total protein concentration using the bicinchoninic acid (BCA) protein assay (Sigma). From a working solution of 2 mg/mL (final dilution), 30 μg of each serum sample were mixed with loading buffer [62.5 mM Tris pH=6.8, 5% glycerol (v/v), 1% SDS (v/v), 2.5% β-mercaptoethanol (v/v)], denatured at 95 °C for 5 min, and subjected to polyacrylamide gel electrophoresis (SDS-PAGE) [using both a 7% and 17% (w/v) polyacrylamide separating gel, and a 4% (w/v) polyacrylamide stacking gel] and vertically electrophorized at 100 V for approximately 3 hours at room temperature. All the samples of a given subject were always run on the same gel along with a broad-range pre-stained molecular weight marker (Precision Plus Protein™ Dual Color Standards, Bio-Rad). We utilized polyacrylamide separating gels of different concentrations (7% and 17%) to ensure a distinctive and appropriate separation of both the high and low molecular mass proteins contained in the serum samples. After electrophoretic separation of serum proteins, gels were stained with Colloidal Coomassie G-250 and the separated protein molecules were visualized under ultraviolet (UV) light.

RESULTS

Differential scanning calorimetry

The averaged heat capacity profiles of the blood plasma samples from patients with AMI collected immediately before and 30 min after applying RIC intervention are shown in figure 1. The averaged thermograms were deconvoluted with Gaussian curves and the results are presented in figures 2B, 2C. For comparison purposes, the averaged heat capacity profile of the 6 healthy volunteers is shown in figure 2A. It displays the typical signature DSC pattern of blood plasma proteome, with dominating peaks at about 63 °C and 72 °C, resulting from denaturation of the most abundant albumin and globulins fractions, respectively, a minor fibrinogen peak at approximately 52 °C, and a barely visible hump at about 82 °C, usually attributed to IgG and transferrin components.[5,7] The maximum heat capacities C_p^{max} of the major albumin and globulin peaks are 2.42±0.12 mJ/K and 1.72±0.06 mJ/K, respectively.

As shown in figure 1, the major difference between the heat capacity profiles of the plasma samples collected before and after the RIC intervention is the reduction of the albumin's heat capacity peak, obviously as a result of the RIC. Table 1 presents the ratios of the maximum heat capacities C_p^{max} of the albumin to globulin peaks of the thermograms of plasma samples collected from patients with AMI before and after RIC, as well as from healthy individuals. It appears that the RIC intervention resulted in a peak ratio which approached the control (i.e., healthy) mean value.

In contrast, inspection of the thermograms of plasma samples collected before and after the RIC intervention in healthy individuals revealed no significant differences between the pre- and post-RIC condition (representative thermograms are shown in figure 3).

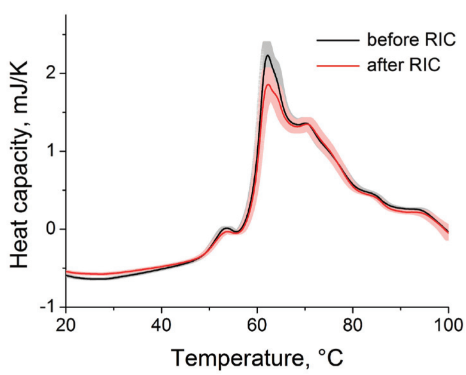

Figure 1. Averaged blood plasma heat capacity profiles of patients with acute myocardial infarction before and after applying remote ischemic conditioning (RIC). The shaded areas show the standard deviations at each temperature.

SDS-PAGE electrophoresis

Inspection of the 17% gel, used for the separation of the low molecular mass proteins (fig. 4), suggests that the samples 5B, 6B, 7B, 9B, 10B, which correspond to the post-RIC condition, exhibited bands of increased density (protein concentration) at the level of approximately 25 kDa, compared to the corresponding samples in the pre-RIC condition, approaching the density of the control sample and implying a possible beneficial effect of RIC on the concentration of those serum proteins. Interestingly,

Table 1. Ratio of the maximum heat capacities C_p^{max} of the albumin and globulin peaks in the heat capacity profiles of blood plasma collected from patients before and after remote ischemic conditioning (RIC), and from healthy control subjects.

Sample	C_p^{max} albumin/globulins
Before RIC	1.75±0.17
After RIC	1.48±0.21
Control	1.41±0.09

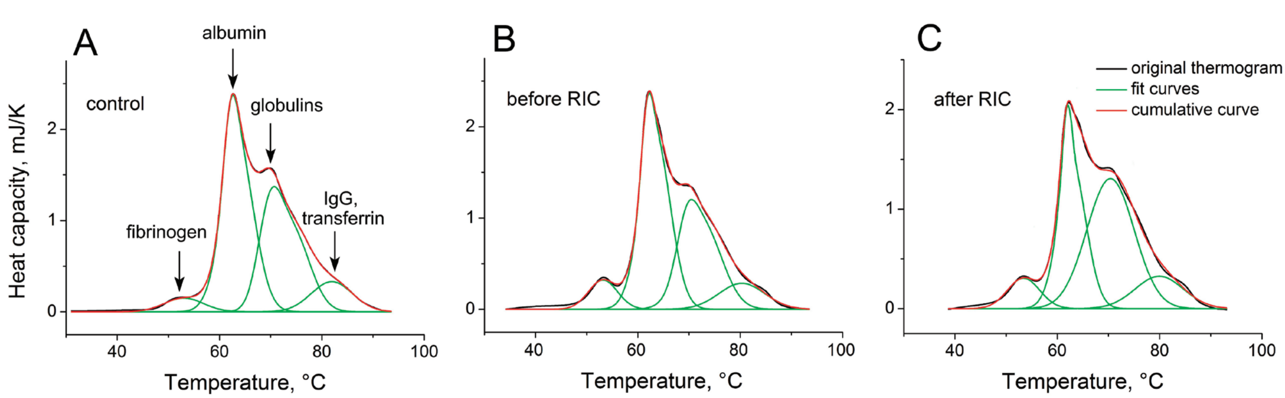

Figure 2. Deconvolutions with Gaussian curves of the averaged heat capacity profiles of healthy control subjects (A) and patients with acute myocardial infarction, before (B) and after remote ischemic conditioning (RIC) (C). The positions of the denaturation peaks of the major blood plasma protein components are indicated.

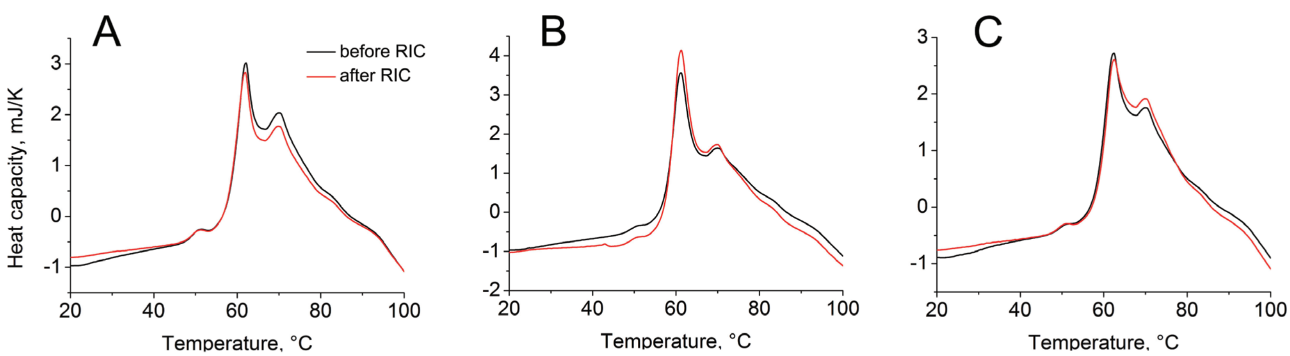

Figure 3. Representative heat capacity profiles of blood plasma derived from healthy individuals before and after the remote ischemic conditioning (RIC) intervention.

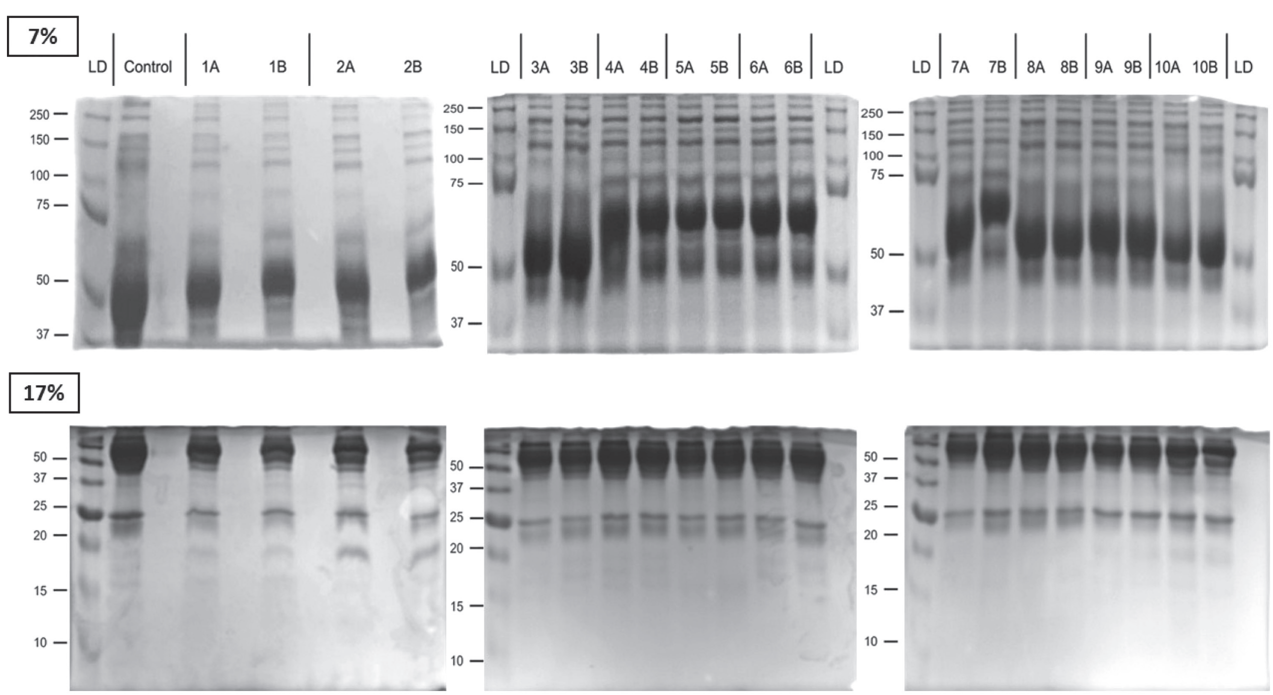

Figure 4. SDS-PAGE electrophoresis gels of samples collected from 10 patients with acute myocardial infarction before and after applying remote ischemic conditioning (RIC) and from a healthy individual (control). Samples marked with "A" indicate pre-RIC condition while samples marked with "B" indicate post-RIC condition. LD: Molecular weight Ladder.

sample 2B (post-RIC) showed a decreased density of the band(s) at 25 kDa compared to its pre-RIC condition (2A), indicating an opposite effect of RIC on this patient.

In the 7% gel, used to separate the higher molecular mass proteins, the patients generally exhibited bands of similar density (protein concentration) in the pre-RIC and post-RIC conditions. Nevertheless, the post-RIC samples 2B and 3B showed bands of decreased and increased concentration, respectively, at approximately 50 kDa, while patient 7 exhibited decreased densities at 50 and 100 kDa post RIC (7B), along with a band of remarkable density at approximately 65 kDa occurred only post RIC.

DISCUSSION

RIC has been believed to reduce myocardial damage in patients with AMI, but its biochemical mechanisms have not yet been defined, and various mechanisms have been deemed responsible for its assumed beneficial effect. The RIC intervention has previously been shown to improve aortic elastic properties and endothelial glycocalyx thickness in patients with AMI.[13] RIC may also prevent adverse myocardial remodeling after the ischemic event, possibly by upregulating the expression of cardio-protective microRNAs.[14] In addition, it has been suggested that RIC by a single 5-min brachial cuff inflation confers acute short-term improvement of vascular function, possibly through oxidative stress reduction,[15] while oxidative stress reduction has come up as a suggested mechanism of RIC protection in other studies.[16–18]

In the present study, the major difference between the heat capacity profiles of the blood plasma samples of patients with AMI, collected before and after the RIC, is the reduction of the albumin's heat capacity peak as a result of the RIC, thus causing the thermograms to evolve towards the healthy configuration. In our previous studies, we have found that plasma oxidation with H_2O_2 leads to a strong suppression of the albumin peak.[8] Thus, once RIC was found to cause similar suppression of the albumin peak in the heat capacity profile (fig. 1), it might be taken as a contradictory to the hypothesis that RIC exerts its beneficial effect through oxidative stress reduction. In contrast to the patients with AMI, RIC did not result in significant differences in the heat capacity profiles of samples collected before and after the intervention in healthy individuals.

In conclusion, in this study, RIC intervention was shown to have a beneficial effect on patients with AMI, bringing the blood plasma thermograms closer to those characterizing healthy individuals. Previous studies have suggested various mechanisms for the beneficial effect of RIC and some reports proposed oxidative stress reduction as a possible mechanism that may mediate the RIC effects. However, since the blood plasma oxidation has been previously reported to result in suppression of the albumin denaturation peak, the findings of the present study appear to disagree with the hypothesis that RIC exerts its beneficial effect through oxidative stress reduction.

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ΠΕΡΙΛΗΨΗ

Επίδραση της ισχαιμικής περίσφιξης της βραχιόνιας αρτηρίας στο πλάσμα του αίματος. Μελέτη της μεθόδου της διαφορικής θερμιδομετρίας

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ΣΚΟΠΟΣ Επίδραση της ισχαιμικής περίσφιξης της βραχιόνιας αρτηρίας (remote ischemic conditioning, RIC) σε συγκεκριμένα χαρακτηριστικά του πλάσματος του αίματος ασθενών με οξύ έμφραγμα του μυοκαρδίου (ΟΕΜ), χρησιμοποιώντας τη μέθοδο της διαφορικής θερμιδομετρίας (differential scanning calorimetry, DSC). **ΜΕΘΟΔΟΣ** Αξιοποιήθηκε η μέθοδος DSC για να εξεταστεί η ενδεχόμενη τροποποίηση των πρωτεϊνών του πλάσματος σε δείγματα αίματος ασθενών με ΟΕΜ που ελήφθησαν πριν και μετά από την παρέμβαση RIC. Η μέθοδος DSC αποτελεί ένα νέο μέσο έρευνας στον τομέα της Βιοϊατρικής, παρέχοντας υψηλής ακρίβειας ειδικές μετρήσεις μέσω μιας μη επεμβατικής διαδικασίας εξέτασης των θερμοδυναμικών τροποποιήσεων των πρωτεϊνών. **ΑΠΟΤΕΛΕΣΜΑΤΑ** Τα κύρια ευρήματα της μελέτης ήταν η μείωση της κορυφαίας τιμής θερμικής αποδιάταξης της λευκωματίνης στο θερμοδυναμικό προφίλ των ασθενών με ΟΕΜ, ως αποτέλεσμα της RIC. Αντίθετα, στους υγιείς εθελοντές δεν βρέθηκαν σημαντικές θερμοδυναμικές αλλαγές στις πρωτεΐνες του πλάσματος μετά την παρέμβαση RIC. **ΣΥΜΠΕΡΑΣΜΑΤΑ** Η RIC φαίνεται να επέφερε ευμενείς προσαρμογές στους ασθενείς με ΟΕΜ, καθώς τροποποίησε τα θερμικά διαγράμματα των πρωτεϊνών του πλάσματος, ώστε να προσομοιάζουν με εκείνα των υγιών εθελοντών. Προηγούμενες μελέτες έχουν προτείνει τη μείωση του οξειδωτικού stress ως μηχανισμό μέσω του οποίου η RIC επιφέρει τα ευεργετικά της αποτελέσματα. Ωστόσο, η μείωση της κορυφαίας τιμής αποδιάταξης της λευκωματίνης μετά από τη RIC υποδηλώνει την αυξημένη οξείδωση του πλάσματος, μη συνηγορώντας υπέρ του ανωτέρου προστατευτικού μηχανισμού.

Λέξεις ευρετηρίου: Διαφορική θερμιδομετρία, Έμφραγμα του μυοκαρδίου, Ισχαιμική προετοιμασία/προπόνηση του μυοκαρδίου, Λευκωματίνη, Πλάσμα αίματος

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