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GC/MS-based metabolomic analysis of alleviated renal ischemia-reperfusion injury induced by remote ischemic preconditioning

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Abstract. – OBJECTIVE: Dysfunctional metabolisms have contributed towards ischemia-reperfusion (I/R) injury. However, the role of remote ischemic preconditioning (RIP) in I/R injury is not well known. The present study showed alleviated I/R injury in kidneys treated with RIP.

MATERIALS AND METHODS: We utilized GC/MS-based metabolomics to characterize the variation of metabolomes.

RESULTS: Metabolic category using differential metabolites showed the lower percentage of amino acids in I/R group in comparison to RIP+I/R group, confirming the importance of amino acid metabolism in RIP-treated rat kidney. Further, pathway enrichment analysis showed alanine, aspartate and glutamate metabolism to be involved in the beneficial effects of RIP during renal I/R injury. Furthermore, another crucial enrichment pathway is biosynthesis of unsaturated fatty acids. Other vital metabolites detected in independent component analysis (ICA) analysis were d-glucose, lactic acid and cholesterol. The variation tendency of above-mentioned metabolites was overall consistent with the protective nature of RIP.

CONCLUSIONS: These findings elicited a viewpoint that metabolic strategy affected by RIP are linked to underlying mechanisms of RIP and highlighted the importance of metabolic strategy against I/R injury.

Key Words:

Remote ischemic preconditioning, Metabolomics, Ischemia-reperfusion, Amino acid metabolism, Biosynthesis of unsaturated fatty acids.

Introduction

Acute kidney injury (AKI) is interpreted as an abrupt failure of kidney function and is often related to surgeries like renal transplantation, sepsis, etc., AKI has a high morbidity and poor prognosis due to its vague pathogenesis[1]. Ischemia/reperfusion (I/R) is one of the most common reasons responsible for AKI. Since 1993, the first investigation of the protective phenomenon of remote ischemic preconditioning in myocardial I/R injury[2], many similar adaptive phenomenon have also been reported, which were induced by ischemia/reperfusion of other organs in addition to kidneys[3-5]. Although the hopeful results of the preliminary studies revealed clinical applications of this procedure[6-8], the detailed mechanisms underlying this protective phenomenon are still not well understood.

Metabolomics characterizes small-molecule metabolite profiles in a biological cell, tissue, organ or organism. It is concerned with the quantitative understanding of integrated living systems and their dynamic responses to the physiological as well as pathological changes[9]. It has been exploited in various diseases like diabetes mellitus[10], heart disease[11], cancer[12], as well as in the kidney disease cases[13-15]. Further, one metabolic enzyme namely lysine deacetylase was required for rapid cardio-protective metabolic adaptation[16]. The present study explored the altered metabolic pathway under ischemic preconditioning, as underlying metabolic mechanisms related to alleviated ischemia-reperfusion injury induced by remote ischemic preconditioning are still limited.

In contrast to the metabolic investigation of ischemic preconditioning, a line of studies revealing some metabolic mechanisms of I/R injury have been reported. There is evidence presenting that depletion of ATP after I/R injury is responsible for cell death in the kidney due to depletion

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of ATP protected renal function[17,18]. Of note, the accumulation of intracellular calcium could be triggered by the rapid loss of ATP, which finally activated the phospholipase A2 (PLA2) [19]. PLA2 metabolizes the membrane phospholipid to generate free fatty acids (FFAs) and a lysophospholipid. So, the above two by-products might have contributed towards I/R injury in kidney through TLR4-dependent signaling pathway[19-22]. Moreover, L-Carnitine, a vital component of activated fatty acids transport mechanism across the mitochondrial membrane[23], has been reported to improve energy metabolism and prohibit oxidative stress in various animal I/R models[24,25].

It is a well-known fact that gas chromatography/mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) are three most common analytical technologies in metabolomics investigation[26]. While each technology has its own unique advantages and disadvantages, GC-MS is specifically efficient for the analyses of volatile compounds[27,28]. Therefore, in the present study, GC-MS-based metabolomics have been used to exploit important metabolites and key pathways in the differentiation of I/R treatment from the treatment of remote ischemic preconditioning (RIP).

Materials and Methods

Animals

Adult female Sprague-Dawley rats weighing 200-220 g were kept in an environmentally controlled breeding room (temperature: 20 ± 2°C, humidity: 60 ± 5%, 12 h dark/light cycle). They were fed standard laboratory chow with water ad libitum. They were maintained in accordance with internationally accepted principles for laboratory animal use. All work was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care (Animal Welfare Assurance Number: 201508023).

Remote Ischemic Preconditioning (RIP) and Renal Ischemia-reperfusion

Animals were anesthetized with pentobarbital sodium (60 mg/kg intraperitoneally) and administered 1 ml of 0.85% NaCl (37°C) on the day of surgery. Body temperature was sustained at 36.0-37.5°C. Animals were divided into three groups. Rat kidneys were exposed by flank incisions. Animals in Group 1 (Control group) were subjected to sham surgery, and rats in Group 2 (I/R group) underwent bilateral renal ischemia by clamping both renal pedicles with nontraumatic microaneurysm clamps (Roboz Surgical Instrument Co., Inc., Gaithersburg, MD, USA). After 30 min, the clamps were removed, and reperfusion of the kidneys was visually confirmed. Rats in Group 3 (I/R+RIP group) received splenic artery occlusion (15 min) and reperfusion (15 min) before above-mentioned renal ischemia/reperfusion protocol.

Histology Analyses

Before paraffin embedding, hippocampal slices were fixed in 4% paraformaldehyde overnight at room temperature and then transferred to 70% ethanol. Organs were then embedded and freezed using liquid nitrogen-cooled isopentane. They were then sectioned at a thickness of 4 μm. For pathological analysis, paraffin sections were stained with hematoxylin and eosin (H&E). Scoring was performed by examining at least 40 consecutive fields at 40 X magnification.

Extraction of Metabolites of the Kidney

For the metabolomic investigation, the kidney tissues were homogenized and dissolved for 30 s in methanol at 4°C. To extract metabolites, a volume of 500 μL of methanol was used for each 100 mg of spleen tissues in the sample. The homogenates were centrifuged at 12,000 x g for 10 min at 4°C. The resulting supernatant (300 μL) was transferred to a GC sampling vial containing ribitol (10 μL, 0.1 mg/mL). Samples were concentrated before the subsequent derivatization.

Derivatization and GC-MS Analysis

80 μL of methoxamine/pyridine hydrochloride (20 mg/mL) was added to dried samples to induce oximation for 1.5 h at 37°C. It was then followed by addition of 80 μL of the derivatization reagent N-Methyl-N-(TrimethylSilyl)Trifluoroacetamide (MSTFA) Sigma-Aldrich (St. Louis, MO, USA) and was reacted with the samples for 0.5 h at 37°C. 1 μL aliquot of the derivative of the supernatant was added to a tube and analyzed using GC-MS Trace DSQ II Thermo Scientific (Waltham, MA, USA). The separation conditions of GC-MS consisted of an initial temperature of 70°C (5 min) with a uniform increase to 270°C at a speed of 2°C/min (5 min).

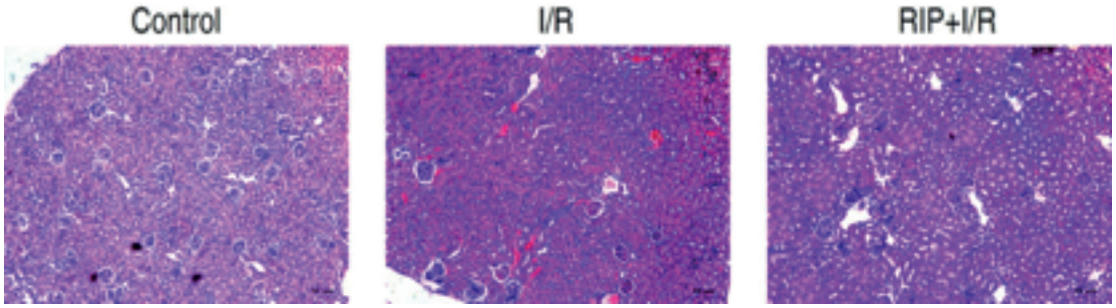

Figure 1. RIP relieves the renal I/R injury. Light microscopy photomicrographs depicting sections from kidney of rat with operation. 40 x.

Statistical Analysis

Metabolomic data were obtained using Thermo Foundation 1.0.1. The resulting data matrix was normalized using the sum abundance value, and then we centered the computed abundance of metabolites for each tissue sample on their median value and scaled by their inter-quartile range (IQR) to reduce between-sample variation[12,29]. We analyzed the differential metabolites using significant analyses of microarray (SAM)[30,31]. Independent component analysis (ICA) was selected as the pattern recognition method[32]. All data were expressed as means ± standard deviation (SD) and subjected to the analysis of variance (one-factor-ANOVA) followed by LSD (Least Significant Difference) as its Post Hoc Test to perform the treatment effects SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). The Student's t-test was applied to test for the statistically significant differences between individual groups. $p<0.05$ was considered statistically significant.

Results

Histology Analysis Shows the Helpful Effect of RIP on Renal I/R injury

The histology revealed that the kidney structure of control group was normal, while the tissue structure of I/R group was significantly disrupted. Notably, betterment in the structure was observed in RIP+I/R group (Figure 1).

Metabolomic Profiling of rat Kidney

To identify the pivotal metabolic pathway, key metabolites were found responsible for the protective effects of RIP on renal I/R injury. GC-MS was utilized to quantitatively measure levels of known metabolites in rat kidney from six individuals in each group. Typical total ion current chromatograms (TIC) are shown in Figure 2A. 68 metabolites with reliable signal were detected in each sample. The correlation coefficient of two technical repeats indicated the reliability of the detection technology (Figure 2B). The category showed that 50.00%, 14.71%, 33.82% and 1.47% of metabolites belonged to carbon sources, amino acids, lipids, and nucleotides, respectively (Figure 2C). The abundance of the metabolites sourced from the three groups was clustered as a heat map (Figure 2D). The metabolome variations among the three groups suggested an association between the metabolomics responses and degree of I/R injury.

Differential Metabolomic Profiling Between I/R and I/R+RIP Samples

To further explore altered metabolome identification, the I/R+RIP group from the I/R group, a two-sided Wilcoxon rank-sum test coupled with a permutation test was utilized to ascertain differential metabolites. Forty-six (67.65%) and forty-three (63.24%) metabolites out of the 68 metabolites were differential at $p < 0.05$ in I/R and I/R+RIP group (Figure 3A), respectively. Further, z-score plot showed that it spanned from -5.29 to 67.96 in I/R group and from -5.62 to 53.22 in I/R+RIP group (Figure 3B). Higher varied abundances of metabolites were found in the I/R group than in the I/R+RIP group. Specifically, 17 metabolites down-regulated and 29 metabolites were up-regulated in the I/R group. On the other hand, 16 metabolites were decreased, and 27 metabolites showed an increase in the I/R+RIP group. Metabolic categories of these differential metabolites in abundance were further investigated. They showed analogous varying percentage in the two groups (Figure 3C). Figure 3D shows the number of up-regulated and down-regulated metabolites in these categories. Amino acid metabolism was likely to be more affected in the I/R+RIP than I/R

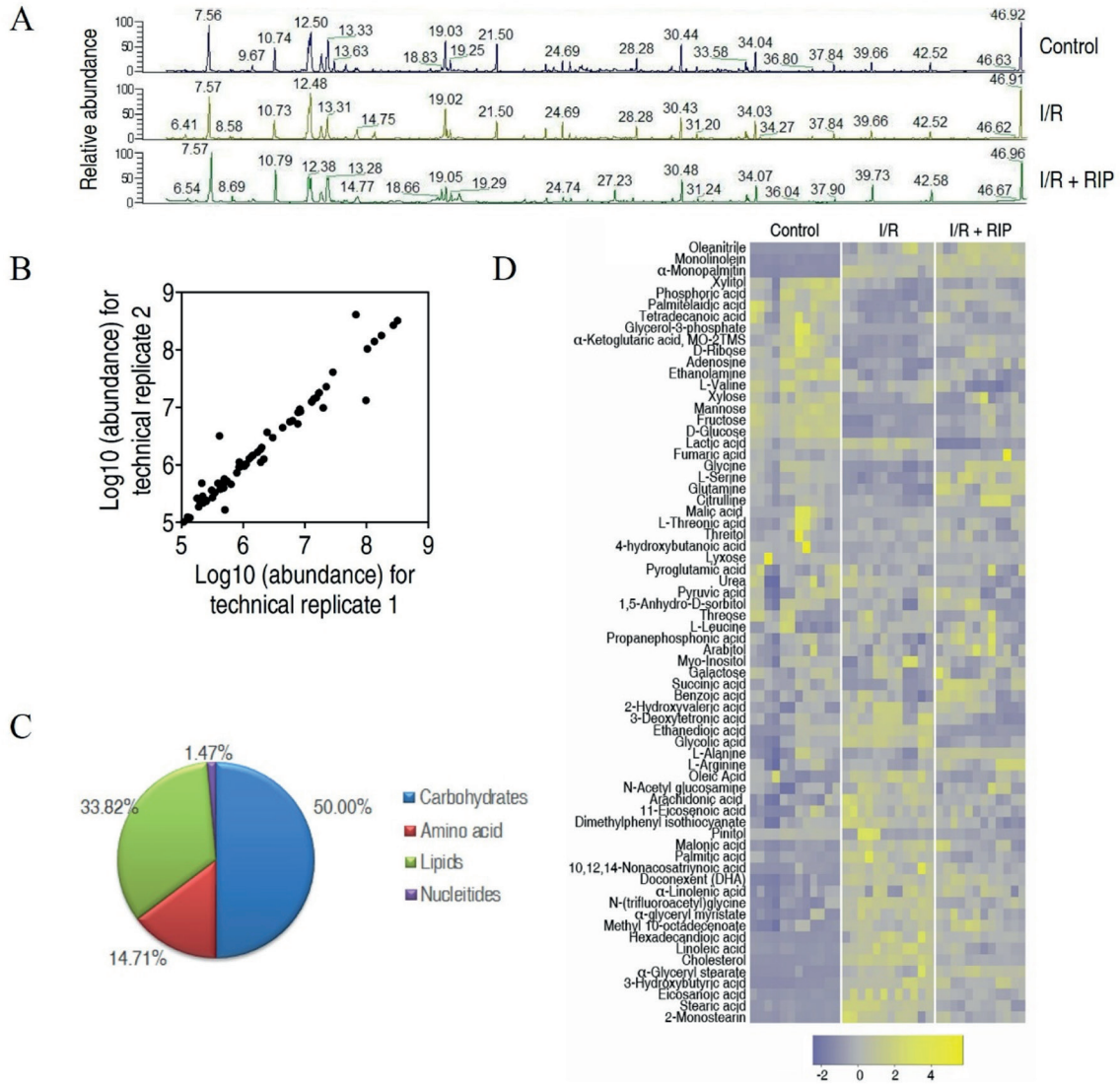

Figure 2. Metabolomic profiling of rat kidney. ***(A)*** Representative total ion current chromatograms from control, the I/R and I/R+RIP samples. ***(B)*** Reproducibility of metabolomic profile platform used in the discovery phase. The abundances of metabolite quantified in cell samples over two technical replicates are presented. Correlation coefficient between technical replicates varies between 0.995 and 0.999. This plot reveals the two replicates with the smallest correlation of 0.995. ***(C)*** Metabolic category of recognized metabolites. ***(D)*** Heat map exhibiting the 68 metabolites. Yellow and navy indicate increase and decrease of metabolites relative to the median metabolite level, respectively (see color scale).

groups. These results suggested that changes in metabolites were related to protective response induced by RIP.

Characterization of Pathways Involved in Helpful Response Induced by RIP

Using an online tool, Metaboanalyst 3.0, seven and six pathways were developed in the I/R and I/R+RIP groups, respectively. Shared and differential enriched pathways between them are represented in Figure 4A. Among these pathways, two were uniquely related to the I/R injury, which were pentose, glucuronate interconversions, and nitrogen metabolism (Figure 4B). Metabolites enriched in the pentose and glucuronate inter-conversions were all decreased. Although all metabolites enriched in the biosynthesis of unsaturated fatty acids were augmented in I/R and I/R+RIP groups in contrast to the control group, abundance of most metabolites in I/R+RIP group were lesser than I/R group. In other words, RIP had the capability to reduce the abundance of these up-regulated metabolites enriched in the biosynthesis of unsaturated fatty acids in I/R-treated

kidney. These metabolites included eicosenoic acid, palmitic acid, stearic acid, eicosanoic acid, oleic acid, arachidonic acid and linoleic acid. Collectively, above data indicated that biosynthesis of unsaturated fatty acids, alanine, aspartate, glutamate metabolism, arginine and proline metabolism might be significantly related to the RIP-induced benefit during I/R injury.

ICA Analysis for the Identification of the Crucial Metabolites

Figure 5A, shows control group and I/R group were separated clearly on IC01, and IC02 depicting the differentiation between I/R and I/R+RIP groups. In Figure 5B, the loading of different independent components IC01 and IC02 were visualized in a heat map. Ranking of the varied metabolites displayed lactic acid, d-glucose, palmitic acid, stearic acid, oleic acid, glycolic acid, phosphoric acid, docosahexaenoic acid (DHA), monolinolein, 3-hydroxybutyric acid, 2-monostearin, linoleic acid, cholesterol, α-monopalmitin as the metabolites with the largest loading in IC01 and IC02. Out of these metabolites, palmitic acid, stearic acid, oleic acid, DHA, 3-hydroxybutyric acid and linoleic acid were significant metabolites detected in the above pathway enrichment analysis, while others were the new crucial metabolites

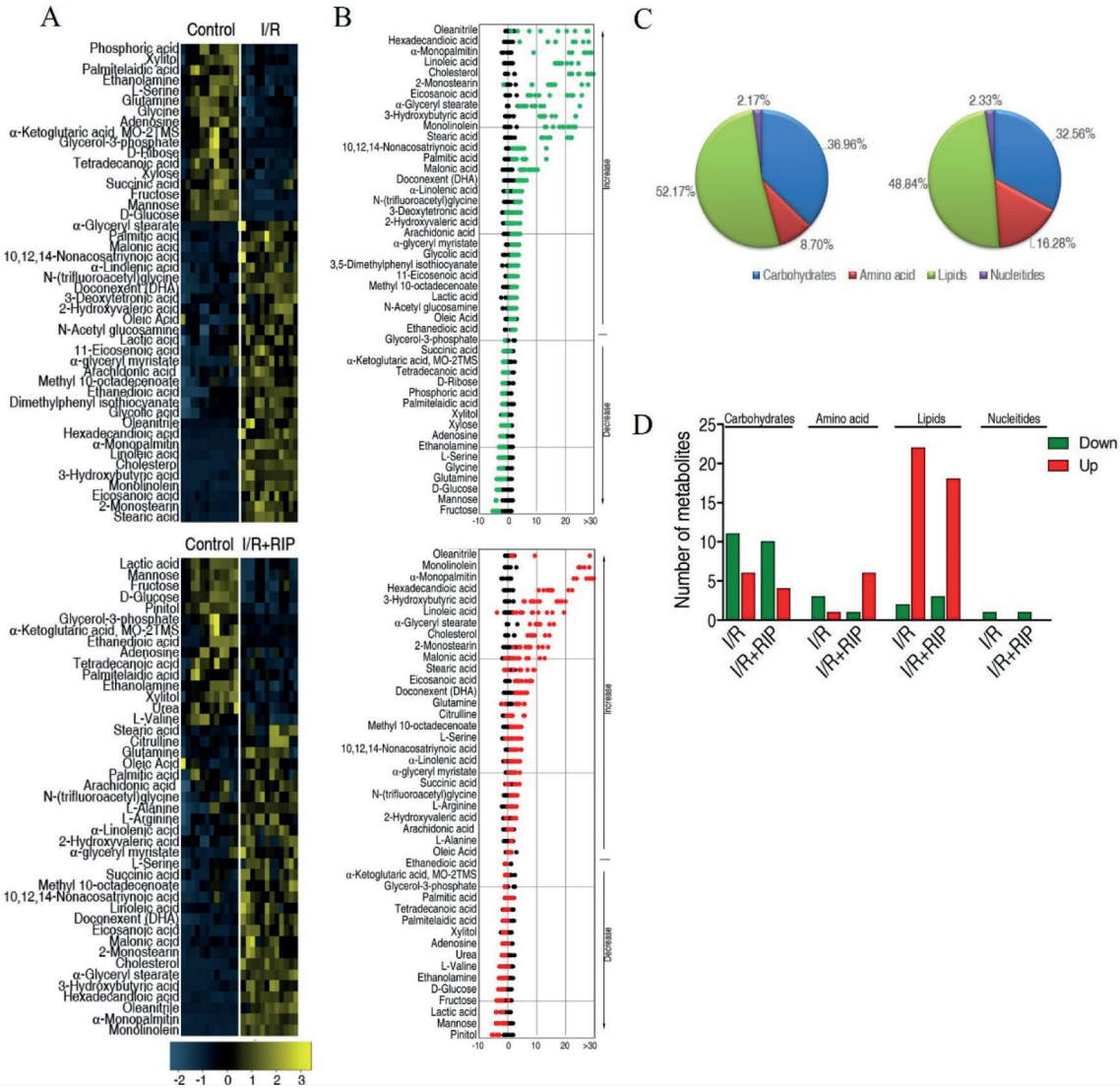

Figure 3. Varied metabolomes differentiating I/R+RIP from I/R in rat kidney. ***(A)*** Heat map revealing relative abundance of 46 and 43 significantly varied metabolites in the I/R and I/R+RIP as indicated, respectively. ***(B)*** Z-scores (standard deviation from average) corresponding to data in ***(A)***. Upper, the I/R group; lower, the I/R+RIP group. ***(C)*** Percentage of varied metabolites in four categories. ***(D)*** The number of metabolites increased and decreased in different categories.

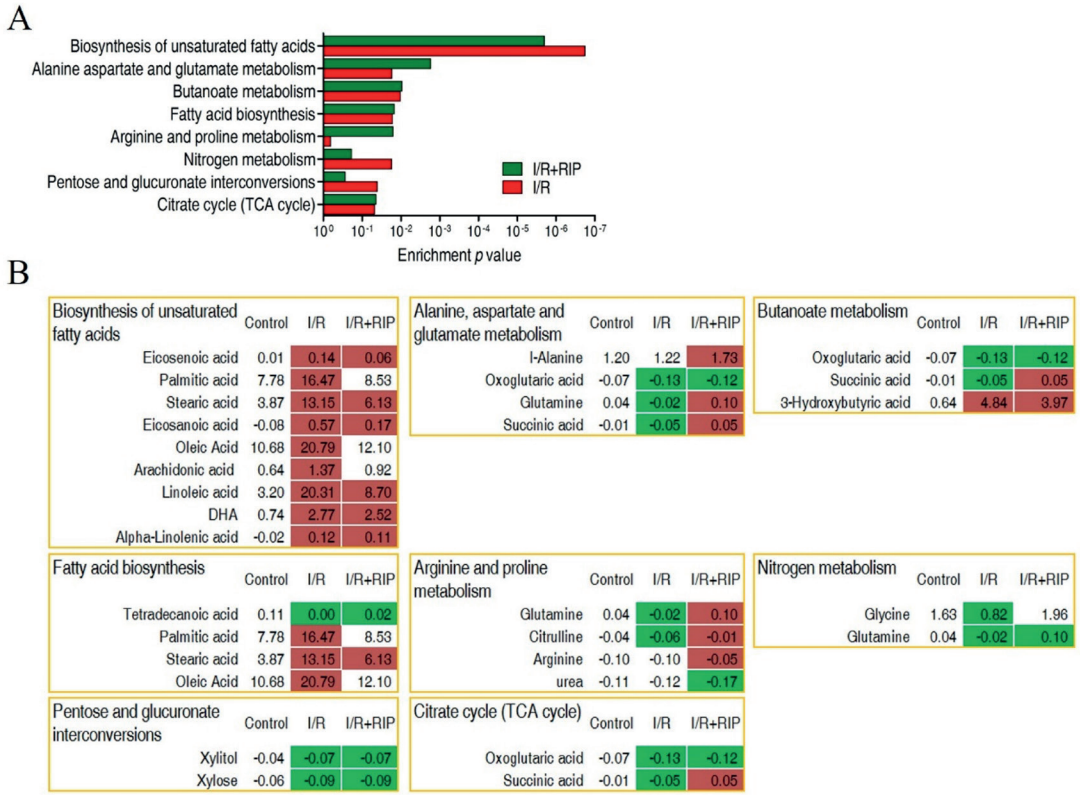

Figure 4. Pathway analysis and integrative analysis. ***(A)*** Pathway enrichment analysis of differential metabolites form I/R and I/R+RIP using an online tool, metaboanalyst 3.0 (http://www.metaboanalyst.ca/). Significantly enriched pathways are selected to plot. ***(B)*** Integrative analysis of metabolites in significantly enriched pathways. Up-regulation and down-regulation of metabolites are indicated as red and green, respectively. The number reveals the ratio of differential metabolites.

found in ICA analysis. Further, only d-glucose, lactic acid and cholesterol were differentiated significantly between control, I/R group, as well as between I/R and I/R+RIP group (Figure 5C).

Discussion

The underlying mechanisms of RIP have not been investigated in detail to date[33,34]. Further, some new beneficial molecules, including capsaicin-activated C sensory fibers, hypoxia-inducible factor 1α (HIF-1α), connexin 43, extracellular vesicles, microRNA-144, microRNA-1, and nitrite, modulated by RIP were identified[35]. Recent evidence[17-25] revealed that the dysfunctional metabolisms were responsible for I/R injury. However, information regarding protection offered by RIP against I/R injury through mounting metabolic strategy is still not known. Therefore, in the present study, we aimed to examine a metabolic response of RIP-pretreated I/R injury using GC/MS-based metabolomic. Our study not only suggested that metabolic response was likely linked to the degree of I/R injury, but also discovered some crucial pathways and key metabolites.

The results of current metabolic category showed that I/R+RIP group has a higher percentage of amino acids than I/R group. Subsequent pathway enrichment analysis further made it clear that alanine, aspartate, glutamate, arginine and proline metabolism might be involved in the beneficial effect of RIP. Four amino acids enriched in this metabolism were l-alanine, l-glutamine, citrulline and l-arginine. These enriched amino acids showed stronger abundance in I/R+RIP than I/R group, confirming that the high levels of these amino acids are beneficial for the alleviation of renal I/R injury. In general, l-alanine, a significant energy substrate for the cell, is beneficial for supporting gluconeogenesis and leukocyte metabolism through unknown mechanisms[36]. One paper studying metabolomic profiling of ischemic preconditioning also observed that l-alanine was

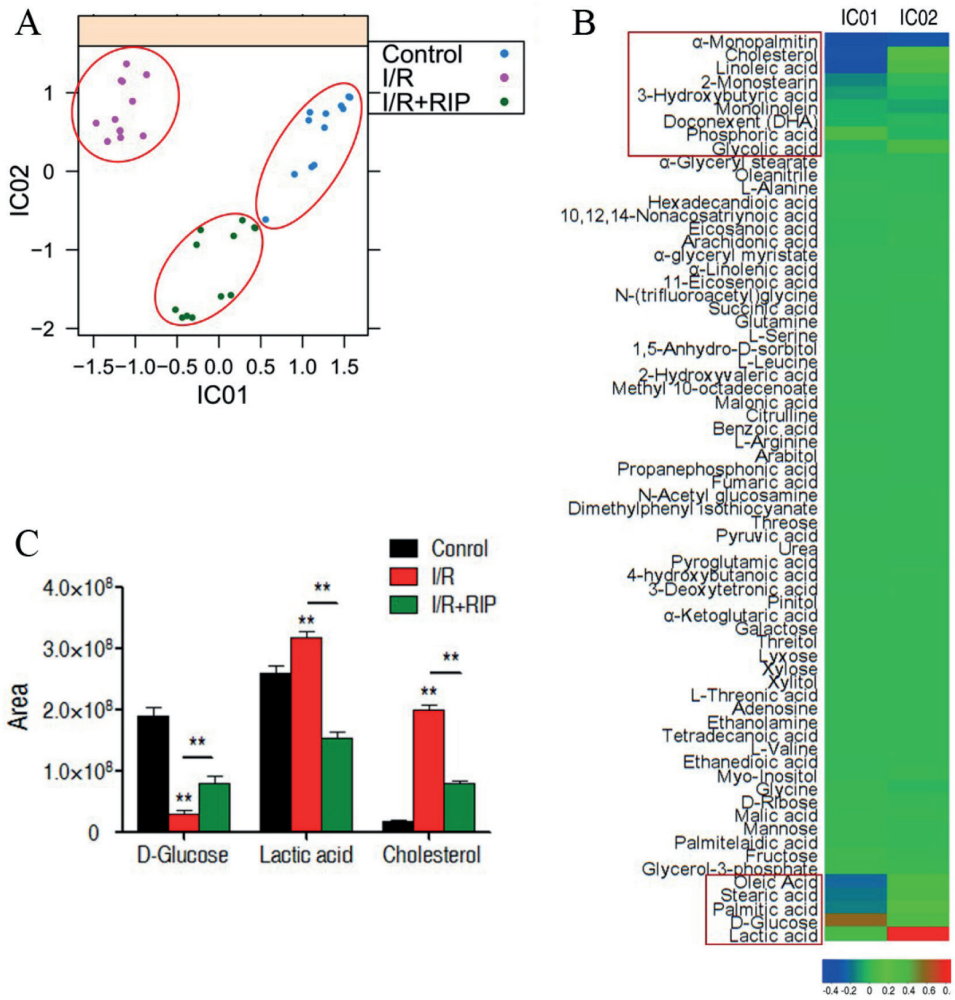

Figure 5. Investigation of vital metabolites that separate I/R+RIP from the I/R using independent component analysis (ICA). ***(A)*** ICA directly represents variation of metabolites among control, I/R and I/R+RIP. Each dot in the plot represents the replicate analysis of samples. ***(B)*** The weight distribution on IC01 and IC02 for the metabolites is shown. The weight (also called loadings) is proportional to the importance or significance of a metabolite for a corresponding independent component, in other words, the observed biological phenomenon. The interpretations of IC01 and IC02 correspond to (A). Red box indicates the metabolites which have largest the loadings. ***(C)*** Comparison of D-glucose, lactic acid and cholesterol among control, I/R and I/R+RIP. Error bars ± SD, $^{**}p < 0.01$.

up-regulated, leading to consistent protective nature of ischemic preconditioning[16]. L-glutamine is known to support optimal cytokine production and high concentration of glutamine in serum and was helpful in maintaining an effective immune function in patients undergoing surgery[36,37]. Moreover, glutamine-dependent anaplerosis produced succinic acid, a TCA cycle intermediate, which in turn stabilized the hypoxia-inducible factor-1α (HIF-1α)[38]. HIF-1 activated Il10 gene transcription which war required by RIP[39]. As the higher abundance of succinic acid was found in I/R+RIP group than that in control and I/R groups, indicating a possibility that modulation of succinic acid concentrations rendered strong host protection in renal I/R injury[40]. The boosted citrulline and declined urea found in current study revealed that NO might be the major metabolic director of l-arginine in RIP. Further, pharmacological and genetic inhibition of NO generation by endothelial NOS within the target organ has been noticed to abrogate the cardioprotection by RIP[41,42].

In combined current pathway enrichment analysis with ICA, the shared lipid-related metabolites were palmitic acid, stearic acid, oleic acid, and linoleic acid. It had been reported earlier that palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1) and linoleic acid (18:2) are capable of uti-

lizing TLR4 signaling to induce an inflammatory response[41], which eventually contributed to I/R injury in kidney[20]. Also, other lipid-related metabolites separately found in pathway enrichment analysis or ICA analysis were arachidonic acid or cholesterol, which also have a remarkable ability to induce the severe inflammation[43,44]. These metabolites were up-regulated by I/R treatment, but got down regulated by RIP. This confirmed that RIP possessed an anti-inflammatory action.

Furthermore, d-glucose and lactic acid were the two highest loadings in differentiating I/R+RIP from the I/R. D-glucose was decreased and lactic acid was increased by I/R. RIP resumed the abundance of d-glucose and declined the lactic acid abundance. In corporation with our report, a recent study also observed that ischemic preconditioning was slightly lower[16]. Tong et al[45] demonstrated that ischemic preconditioning increases glucose transport and is mediated by the p38 Mitogen-Activated Protein (MAP) kinase. Ji et al[46] had examined the role of glucose metabolism during RIP using genetic modulation *in vivo*. The above work proved that myocardial augmented glucose via co-activation of myocardial AMPK and Akt in the reperfused myocardium was essential to RIP-alleviated I/R injury. So, RIP might a relieved the renal I/R injury by elevating the d-glucose uptake and reducing the lactic acid production.

Conclusions

The present research used GC/MS-based metabolomics to characterize the variation of metabolomes in response to I/R and RIP before I/R. Metabolic category using differential metabolites showed the lower percentage of amino acid in I/R group than RIP+I/R group, revealing that amino acid metabolism might play an important role in RIP-treated rat kidney to combat I/R injury. Subsequent pathway enrichment analysis further revealed that alanine, aspartate glutamate arginine and proline metabolisms were involved in the protective effects of RIP during renal I/R injury. So, these findings elicited a viewpoint that underlying mechanisms of RIP are linked to the metabolic strategy and highlight the importance of metabolic strategy against I/R injury.

Conflict of interest

The authors declare no conflicts of interest.

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