Original Article

Role of asymmetric dimethylarginine in acute lung injury induced by cerebral ischemia/reperfusion injury in rats

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Abstract: Objective To determine the role of asymmetric dimethylarginine (ADMA) in acute lung injury induced by cerebral ischemia/reperfusion (I/R) injury in rats. Methods Adult male SD rats were randomly divided into 4 groups, namely the sham-operated group (S), cerebral I/R model group, ADMA + I/R group, and dimethylarginine dimethylaminohydrolase (DDAH)+I/R group. In the latter 3 groups, acute lung injury was induced by left middle cerebral artery occlusion for 120 min. After a 24-h reperfusion, the rats were sacrificed and the activities of nitric oxide synthase (NOS) and contents of nitric oxide (NO) were measured using reductase and colorimetric assay. The mRNA and protein expressions of protein kinase C (PKC) and myosin light chain kinase (MLCK) in the lung tissues were detected with RT-PCR and Western blotting, respectively. The contents of ADMA in the bronchoalveolar lavage fluid (BALF) and blood flowing into and out of the lungs were measured by ELISA. Results Cerebral I/R injury caused significantly elevated ADMA levels in the BALF and blood flowing into the lungs, and obviously lowered the NO concentration and NOS activity in the lung tissues (P<0.05). Following cerebral I/R injury, MLCK and PKC mRNA and protein expressions were significantly upregulated in the lung tissues (P<0.05). Exogenous DDAH obviously decreased the levels of ADMA in the BALF and blood flowing into the lungs, increased NO concentration and NOS activity, and down-regulated MLCK and PKC mRNA and protein expressions in lung tissues of rats with cerebral I/R injury (P<0.05). Conclusion ADMA contributes to the development of acute lung injury following cerebral I/R injury in rats by upregulating MLCK and PKC expression. ADMA may serve as a novel therapeutic biomarker and a potential therapeutic target for acute lung injury induced by cerebral I/R injury.

Key words: asymmetric dimethylarginine; dimethylarginine dimethylaminohydrolase; acute lung injury; cerebral ischemia/reperfusion injury

Introduction

Restoration of blood flow by thrombolysis or mechanical recanalization often remains the primary treatment option for ischemic stroke [1-2]. But in some cases, reperfusion may further exacerbate the brain injury caused by ischemia, resulting in a condition known as cerebral ischemia/reperfusion (I/R) injury [3]. The prognosis of ischemic stroke depends largely on the incidence of complications [4]. Pneumonia, an inflammatory lung injury caused usually by Gram-positive bacteria, is the most frequent severe complication and the most common cause of death in stroke patients [5-6]. Currently the mechanism of acute lung injury (ALI) induced by cerebral I/R injury is still not fully understood.

The plasma contents of asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthases (NOS) inhibitor and also a cardiovascular risk factor, are known to increase in conditions associated with endothelial dysfunction and vascular endothelial barrier function such as hypertension, diabetes, heart failure, atherosclerosis and pulmonary arterial hypertension. Recent studies have shown that elevated ADMA levels are closely associated with ischemic stroke and transient ischemia attacks [7-8]. Researchers found that ADMA contributes to the pathogenesis of acute lung injury [9-10], but so far no studies have been reported to examine its role in acute lung injury induced by cerebral I/R injury. In this study, we investigated the pathogenic role of ADMA in acute lung injury induced by cerebral I/R injury in rats.

Materials and Methods

Materials

ADMA and dimethylarginine dimethylaminohydrolase (DDAH) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The primers for protein kinase C
(PKC), myosin light chain kinase (MLCK), and β-actin were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (China). TRI Reagent (TR-118) was purchased from MRC (Cincinnati, OH, USA). Revert Aid First strand cDNA Synthesis kit, 2×PCR Master Mix and DNA ladder were products of Fermentas (Vilnius, Lithuania). Monoclonal antibodies of PKC, MLCK, and β-actin were purchased from Santa Cruz Biotechnology (USA). Quanti Chrom Nitric Oxide Assay Kit was supplied by BioAssay Systems (Cambridge, UK). PVDF-PLUS membranes were obtained from Osmonics, Leica company (Germany). Rat ADMA Direct ELISA Kit was purchased from Enzo Life Sciences, Inc (Switzerland).

Animas grouping and treatment

Adult male Sprague-Dawley rats weighing 200-220 g were obtained from the Animal Center of Kunming Medical University (Animal certificate: N00001450) and kept under standard laboratory conditions with 12-h light/dark cycles and free access to food and water. The experiments were conducted with approval by Kunming Medical University Administrative Panel on Laboratory Animal Care. The rats were divided into 4 groups (n=8), namely the sham group, I/R model group, ADMA+I/R group, and DDAH+I/R group. In the latter 3 groups with cerebral I/R injury, the rats were anesthetized by an intraperitoneal injection of chloraldurate (0.3 ml/100 g) and subjected to occlusion of the origin of the left middle cerebral artery following the procedures previously described [12]. Two hours after the occlusion, the rats were reanesthetized and the suture for artery occlusion was removed to allow reperfusion. The rats in the sham group received only sham surgery. The rats in ADMA+I/R and DDAH+I/R groups received an intravenous injection of 15 mg/ml of ADMA and DDAH, respectively, 2 h before occlusion of the left middle cerebral artery. Following a 24-h reperfusion, the rats were assessed for neurological deficits using the method previously described [12]. The rats were then anesthetized for blood sampling and sacrificed to collect the lung tissues and bronchoalveolar lavage fluid (BALF).

Collection of plasma samples

Following a 24-h reperfusion, the rats were anesthetized and carotid artery catheterization was performed to obtain 2 ml of blood flowing into and out of the lungs, extracted from the right and left ventricles, respectively. All the blood samples were transferred into pre-cooled (4 °C) test tubes containing 230 μl 10% EDTA-Na and 40 μl aprotinin. After centrifugation at 3 000 r/min for 10 min, the plasma was separated and stored at -70 °C until analysis.

Bronchoalveolar lavage

Bronchoalveolar lavage was performed immediately after the rats were killed. The left lung was ligated and the right lung was lavaged with 5 ml sterile saline through the tracheal cannula for 3 times. BALF were pooled and centrifuged at 5 000 r/min for 10 min at 4 °C. The supernatants were collected and stored at -70 °C until analysis.

Tissue samples

The rats in each group were perfused with cold saline after sacrifice. Lung tissue samples were harvested and placed on the mixture of dry ice and alcohol, and then stored at -73 °C until processed.

RT-PCR

The total RNA was extracted from the lung tissues using TRI Reagent according to the manufacturer’s protocol. The extracted total RNA (2 μg) from each sample was reverse-transcribed to complementary DNA (cDNA) using RevertAid First strand cDNA Synthesis kit. PCR amplification was performed with 35 thermal cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. The primers for PCR amplification of PKC (613 bp, MCLK (358 bp) and β-actin mRNA are listed below://

PKC Forward: 5’-AGGCAGCTTGTGAACGTGACTCC-3’,
Reverse: 5’-GAG GGA TGC CAACAGAGGCACC-3’,
MCLK Forward: 5’-TGGGTTGGGCTGACTTTCA-3’,
Reverse: 5’-CAG TGGGCCACTGCTGCA-3’,
β-actin Forward: 5’-CC ACCGCAAATGCTTCTAAAC-3’,
Reverse: 5’-GGG CG T TCCG CTC CAA CAT-3’

The PCR products were separated on a 2% agarose gel and stained by ethidium bromide. Semi-quantitative image analyses of the PCR products were performed with the GelDoc 2000 system and Quantity One software version 4.5 (Bio-Rad, Laboratories, Hercules, CA). The data were normalized to the transcription levels of β-actin gene and expressed as ratios of optical density.

Western blotting

The lung tissues were added into lysis buffer of sodium dodecyl sulfate (SDS) containing protease inhibitors, and the supernatants were stored at -70 °C after high-speed centrifugation. Equal amounts of protein extracts (25 μg) from the lung tissue samples were separated on 10% SDS-PAGE gels and transferred to PVDF-PLUS membranes (Osmonics, Leica company, Germany). Western blotting was performed with the antibodies against PKC (at a dilution of 1: 1 000), MLCK (1: 1 000) and DDAH (1: 2 000). After incubation with the respective secondary antibodies, the specific bands were visualized by autoradiography using enhanced chemiluminescence according to the manufacturer’s instructions (Lumigen, Amersham, Buckinghamshire).
Determination of NO concentration and NOS activity

The NO concentrations and NOS activity in the lung tissue extracts were determined using Quanti Chrom Nitric Oxide Assay Kit (BioAssay Systems, Cambridge, UK) according to the manufacturer’s instructions.

Measurement of ADMA levels

ADMA concentration in the plasma in/out of the lung and in the BALF was measured with a highly sensitive ELISA kit (Enzo Life Sciences, Inc, Switzerland). The intensity of the color reaction was inversely proportional to ADMA level in the sample, measured by reading the optical density at 450 nm with a microplate reader (QS-500, Leica Company, Germany) [13].

Statistical Analysis

All the data were analyzed using SPSS 11.0 and expressed as Mean±SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) and t test. P<0.05 was considered to denote a significant difference.

Results

Neurological score

The neurological deficit score was significantly higher in rats in I/R group (2.3±0.2) and ADMA+I/R group (2.4±0.3) than in the sham group (0) and DDAH+I/R group (1.5±0.3) (P<0.05). The deficit score in DDAH+I/R group was significantly lower than that in I/R and ADMA+I/R groups, but higher than that in the sham group (P<0.05). The results showed that cerebral I/R induced brain injury and neurological deficits were alleviated by DDAH pretreatment.

Expression of MLCK and PKC mRNA

The mRNA levels of MLCK and PKC in the lung tissues of rats were significantly higher in I/R group and ADMA+I/R group than in the sham group and DDAH+I/R group (P<0.05). MLCK and PKC mRNA levels in ADMA+I/R group were slightly higher than those in I/R group, but this difference was not statistically significant (Fig.1 and Tab.1). These results suggested that cerebral I/R injury caused up-regulated MLCK and PKC mRNA expression in the lung tissues of rats, and ADMA promoted their upregulation while DDAH counteracted the effect.

Expression of PKC and MLCK protein

The protein levels of MLCK and PKC in the lung tissues of rats were significantly higher in I/R group and ADMA+I/R group than in the sham group and DDAH+I/R group (P<0.05, Fig.2 and Tab.2). The protein expressions of MLCK and PKC in the lung tissues of rats were consistent with their mRNA expressions.

NO concentration and NOS activity

Compared with those in the sham group, NOS activity and NO content decreased significantly in the lung tissues in I/R group and ADMA+I/R group (P<0.05). The activity of NOS and NO content in the lung tissues in DDAH+I/R group increased significantly in comparison with those in I/R group and ADMA+I/R group (P<0.05). The activity of NOS in DDAH+I/R group were mildly lower and NO content mildly higher than those in the sham group (Fig.3 and 4). These
results showed that cerebral I/R injury decreased the activity of NOS and NO content in the lung tissues, and DDAH enhanced NOS activity and NO content.

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\text{Fig.3 Activities of NOS in the lung tissues (Means±SD, n=8). *P<0.05 vs sham and DDAH + I/R group; \*P<0.05 vs I/R and ADMA+I/R groups.}
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\text{Fig.4 Contents of NO in the lung tissues (Means±SD, n=8). *P<0.05 vs sham and DDAH + I/R groups; \*P<0.05 vs I/R and ADMA+I/R groups.}
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**ADMA levels in plasma and BALF**

Compared with the sham group and DDAH + I/R group, I/R group and ADMA + I/R group showed significantly increased levels of ADMA in the BALF and in the blood flowing into the lung (\(P<0.05\)); no significant difference was found between the sham group and DDAH+I/R group or between I/R group and ADMA + I/R group. The levels of ADMA in the blood flowing out of the lung were comparable among the 4 groups. The levels of ADMA in the BALF and blood (into and out of the lung) in ADMA + I/R group were slightly higher than those in I/R group, but this difference was not statistically significant (Tab.3). These results showed that cerebral I/R injury increased the levels of ADMA in BALF and in the blood flowing into (but not out of) the lung, and DDAH pretreatment could decrease the levels of ADMA in BALF and plasma.

**Discussion**

As a potent endogenous NOS inhibitor and a cardiovascular risk factor, ADMA is implicated in endothelial dysfunction and the pathogenesis of a number of cardiovascular diseases [14]. Recent studies have shown that ADMA contributes to the pathogenesis of acute lung injury [9][10]. Acute lung injury is associated with severe alterations in the lung structure and function and is characterized by hypoxemia, pulmonary edema, low lung compliance and widespread capillary leakage. ADMA can increase pulmonary endothelial permeability, and this effect is mediated by nitric oxide (NO) via protein kinase G (PKG) and independent of reactive oxygen species. ADMA metabolism critically determines pulmonary endothelial barrier function by modulating Rac1-mediated remodeling of the actin cytoskeleton and intercellular junctions [10]. But the role of ADMA in the pathogenesis of acute lung injury following cerebral I/R injury still remains to be clarified. In this study, we found that cerebral I/R injury caused a significant increase in the levels of ADMA in the BALF and blood flowing into the lung. ADMA is metabolized via hydrolytic degradation to L-citrulline and dimethylamine by the enzyme DDAH [10], known as the ADMA/DDAH pathway. We found that pretreatment with exogenous DDAH significantly decreased the levels of ADMA in BALF and in the blood flowing into the lung, suggesting that ADMA/DDAH pathway may contribute to the development of acute lung injury following cerebral I/R injury.

Evidence suggests that the lung structure and function are partially maintained by a balance between the competing arginine-metabolizing enzymes, namely arginase and NOS [11]. Excessive lung collagen deposition and reduced pulmonary function in acute lung injury are mediated through the arginase pathway [12][13]. Our results showed that NO concentration and NOS activity were decreased obviously in the lung tissues of rats with cerebral I/R injury, while pretreatment with exogenous DDAH restored the NO concentration and NOS activity to normal levels. This demonstrates that increased ADMA levels leads to reduced NO production and NOS activity in the lung tissues following cerebral I/R injury, and DDAH can restore NO concentration and NOS activity by degradation of ADMA.

Protein kinase C (PKC) is a serine/threonine kinase with 11 different PKC isotypes, which differ in substrate utilization and mechanisms of activation [14][15]. The classical group includes the \(\alpha\), \(\beta\), and \(\gamma\) isotypes. PKC-\(\alpha\) and PKC-\(\varepsilon\) are the most common isotypes in endothelium [15]. PKC-\(\alpha\) mediate tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\))-induced increases in permeability of pulmonary microvessel endothelial monolayers (PEM) [16]. MLCK is a serine/threonine-specific protein kinase that phosphorylates the regulatory light chain of myosin II [17], and functions as an important regulatory protein in the signal transduction pathways of vascular endothelial permeability [18-20]. In
order to determine whether PKC-α and MLCK participate in acute lung injury following cerebral I/R injury, we assayed the mRNA and protein expression of PKC-α and MLCK in the lung tissues of rats, and found that their expression were significantly upregulated at both the mRNA and protein levels in the lung tissues of rats in I/R group and ADMA + I/R group. This finding suggests that PKC-α and MLCK mediate the increase of pulmonary microvascular endothelial permeability, thus leading to acute pulmonary edema in rats following cerebral I/R injury.

In conclusion, our findings suggest that ADMA contributes to the development of acute lung injury following cerebral I/R injury in rats by up-regulating MLCK and PKC. ADMA may be a novel therapeutic biomarker and also a potential therapeutic target for acute lung injury induced by cerebral I/R injury.

References

非对称二甲基精氨酸在大鼠脑缺血再灌注损伤诱导的急性肺损伤发病过程中的作用

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摘要: 目的 研究非对称二甲基精氨酸(ADMA)在大鼠脑缺血再灌注(I/R)诱导的急性肺损伤发病过程中的作用。方法 成年雄性SD大鼠随机分为假手术组(S)、模型组(I/R)、ADMA处理组(ADMA+I/R)和DDAH处理组(DDAH+I/R)。通过大鼠脑缺血2 h后恢复血流灌注诱导急性肺损伤。在恢复血流灌注24 h后，采用比色法检测各组大鼠肺组织一氧化氮合酶(NOS)活性和NO含量。采用RT-PCR和Western blotting分别检测肺组织蛋白激酶(PKC)和肌球蛋白轻链激酶(MLCK)mRNA和蛋白表达水平; ELISA法检测支气管肺泡灌洗液及肺组织中的ADMA水平。结果 脑I/R损伤大鼠支气管肺泡灌洗液中ADMA水平明显升高,肺组织中NO含量和NOS活性明显降低(P<0.05),同时MLCK和PKC mRNA和蛋白表达明显上调(P<0.05)。结论 ADMA通过上调肺组织MLCK和PKC的表达参与了脑I/R损伤后急性肺损伤的发病过程。

关键词: 非对称二甲基精氨酸; 二甲基精氨酸-二甲胺水解酶; 急性肺损伤; 大鼠脑缺血再灌注

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