Modulation of Lung Allergic Response by Renal Ischemia and Reperfusion Injury

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Key Words
Asthma • Renal ischemia and reperfusion • Th1/Th2 balance • Lung allergic inflammation • IRI

Abstract
The Th1/Th2 balance represents an important factor in the pathogenesis of renal ischemia-reperfusion injury (IRI). In addition, IRI causes a systemic inflammation that can affect other tissues, such as the lungs. To investigate the ability of renal IRI to modulate pulmonary function in a specific model of allergic inflammation, C57Bl/6 mice were immunized with ovalbumin/albumin on days 0 and 7 and challenged with an ovalbumin (OA) aerosol on days 14 and 21. After 24 h of the second antigen challenge, the animals were subjected to 45 minutes of ischemia. After 24 h of reperfusion, the bronchoalveolar lavage (BAL) fluid, blood and lung tissue were collected for analysis. Serum creatinine levels increased in both allergic and non-immunized animals subjected to IRI. However, BAL analysis showed a reduction in the total cells (46%) and neutrophils (58%) compared with control allergic animals not submitted to IRI. In addition, OA challenge induced the phosphorylation of ERK and Akt and the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in lung homogenates. After renal IRI, the phosphorylation of ERK and expression of COX-2 and iNOS were markedly reduced; however, there was no difference in the phosphorylation of Akt between sham and ischemic OA-challenged animals. Mucus production was also reduced in allergic mice after renal IRI. IL-4, IL-5 and IL-13 were markedly down-regulated in immunized/challenged mice subjected to IRI. These results suggest that renal IRI can modulate lung allergic inflammation, probably by altering the Th1/Th2 balance and, at least in part, by changing cellular signal transduction factors.

Introduction
Allergic asthma is a complex disease that simultaneously involves the participation of several cells, cytokines and inflammatory mediators. It is characterized by a specific pattern of airway inflammation, involving eosinophils and lymphocytes with a prevalence of the Th2 sub-population. Moreover, asthma induces structural changes in the airways (remodeling) that include an increased deposition of collagen in the bronchi, sub-
epithelial fibrosis, hypertrophy and hyperplasia of the flat airways muscle, angiogenesis and an increase in mucus secretion [1].

Several reports in the literature indicate that diseases with a Th1 pattern could modulate the inflammatory response in asthma [2-5]. A possible cause for this modulation is an increase in IL-10 production. The protective effect of IL-10 in asthma includes the induction of IgG [6], the modulation of the expression of IL-4 and IL-5 cytokines [7] and the inhibition of the production of histamine and other inflammatory mediators released by the mast cells [8]. These effects of an antigen challenge are the result of the activation of intracellular signaling cascades, including the mitogen-activated protein kinases (MAPK), which include the extracellular signal-regulated kinase (ERK) [9]. In addition, protein kinase B (Akt) is a downstream regulator of phosphatidylinositol 3'-kinase (PI3K) and is implicated in the PI3K-mediated regulation of extracellular signal-regulated kinase (ERK) [9]. In addition, protein kinase B (Akt) is a downstream regulator of phosphatidylinositol 3'-kinase (PI3K) and is implicated in the PI3K-mediated regulation of nuclear factor-κB (NF-κB), an important transcription factor for pro-inflammatory mediators [10]. The pathogenesis of renal ischemia-reperfusion injury (IRI) is complex and still not fully understood. However, the inflammatory response is thought to be an important pathogenic component, and other factors, including endothelium lesion, reactive oxygen and nitrogen species and the production of lipid mediators by the sub-letally damaged tubular cells, have also been implicated [11-13]. Experimental evidence has suggested that the Th1/Th2 balance represents an important factor in the pathogenesis of renal IRI. Corresponding evidence demonstrating that the Th1 profile is an important factor for the secretion of monocyte chemoattractant protein-1 (MCP-1) has also been presented in studies from our group [14].

Experimental models of respiratory stress syndrome in adults are characterized by the accumulation of neutrophils in the lung and an increase in microvascular permeability [15]. Acute respiratory distress syndrome and renal IRI frequently co-exist in patients in the intensive treatment units, and this combination is associated with higher mortality. Rabb et al. [16] and Kramer et al. [17] demonstrated that renal IRI can result in increased lung vascular permeability, similar to that observed in adults with acute respiratory distress syndrome. We recently demonstrated that mice subjected to IRI develop lung inflammation with increased expression levels of COX2, iNOS and prostaglandins E2. In addition, IRI induces the migration of inflammatory cells, such as neutrophils, lymphocytes and macrophages in the lung [18].

Clearly, more studies are needed to elucidate the crosstalk between the kidney and lungs after an acute renal injury (ARI). The aim of the present study was to investigate the influence of renal IRI on lung dysfunction in a model of pulmonary allergic reactions. We evaluated the expression levels of the IL-1β, IL-2 and IL-12 Th1 cytokines and the IL-4, IL-5 and IL-13 Th2 cytokines, the phosphorylation of ERK and Akt, the expression levels of COX-2 and iNOS, inflammatory cell infiltration and mucus production.

Materials and Methods

Animals

Male C57Bl/6 mice weighing 20-25 g, 6-8 weeks old, from our own animal facilities were housed in a room with 12 h light-dark cycle with water and food ad libitum. Animal care and research protocols were in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Biomedical Sciences Institute/USP – Ethical Committee for Animal Research (CEEA).

Immunization protocol

Mice were sensitized on days 0 and 7 by intraperitoneal injection of a mixture containing 50 µg of ovalbumin (OA) and 1 mg of aluminium hydroxide [Al(OH)3] in saline (a total volume of 0.2 mL). At 14 and 21 days after first immunization the animal were challenged by exposure to an aerosol of OA (grade III, Sigma) generated by an ultrasonic nebulizer (ICEL US-800, SP, Brazil) delivering particles of 0.5-10 µm diameter at approximately 0.75 cc/min for 20 min. The concentration of OA in the nebulizer was 2.5% wt/vol. The control group consisted of animals immunized as described and challenged with saline solution, or non-immunized animals challenged with OA aerosol as above.

Experimental model of IRI

Surgery was performed 24 hours after the second OA challenge, as previously describe [19]. Mice were anesthetized with Ketamine-Xylazine (Agribrandos do Brazil, São Paulo, Brazil). A midline incision was made and both renal pedicles were cross-clamped. In the ischemia period, animals were kept well hydrated with saline and at a constant temperature (~37°C) through a heating pad device. Forty-five minutes later, microsurgery clamps were removed, abdomen was closed in two layers and animals were placed in single cages, warmed by indirect light until complete recovery from anesthesia. Animals were kept under adjustable conditions until sacrifice at 24 hours of reperfusion.

Analysis of renal function

Serum creatinine was used for evaluation of renal function after IRI. Blood samples were collected 24 hours after ischemic surgery from the abdominal inferior cava vein immediately after IRI. Blood samples were collected 24 hours after ischemic surgery from the abdominal inferior cava vein immediately...
before induced death. Serum creatinine levels were determined by absorbance readings at 520 nm in spectrophotometer using modified Jaffé technique.

**Bronchoalveolar lavage**

The animals were sacrificed by injection of ketamine/xylazine (50 µL of a 100 mg/mL solution, i.p.) 24 h after ischemia or sham surgery. A tracheal cannula was inserted via a midcervical incision and the airways were flushed twice with 1 mL of phosphate buffered saline (PBS, pH 7.4 at 4°C).

**Total and differential cell counts**

The bronchoalveolar lavage (BAL) fluid was centrifuged at 170 g for 10 min at 4°C, the supernatant was removed, and the cell pellet was resuspended in 0.5 mL of PBS. One volume of a solution containing 0.5% crystal violet dissolved in 30% acetic acid was added to nine volumes of the cell suspension. The total number of cells was determined by counting in a hemacytometer. Differential cell counts were performed after cytocentrifugation and staining with hematoxylin-eosin (Hema 3).

**Western blot**

Lung was collected after BAL fluid and homogenized in 1 mL of cold PBS by sonication in ice-cold lyses buffer containing 150 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 1 µg/mL leupeptin, followed by centrifugation at 10000 g for 20 minutes at 4°C. Protein content in the supernatant was determined using the BCA protein assay reagent kit (Pierce), according to the manufacturer’s protocol. Samples containing 20 µg protein were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membrane using the Biorrad Mini-Gel system and trans-blot® SD-semidy Transfer cells. For immunoblotting, the nitrocellulose membranes were incubated in TBS-T buffer (150 mM NaCl, 20 mM Tris, 1% Tween 20, pH 7.4) containing 5% non-fat milk dried milk, for 1 h. After that, the blots were washed with TBS-T and probed with antibodies (1:500 dilution) directed against phospho-ERK 1/2 MAPK (Thr183/Tyr185), or phospho-Akt (Ser473), or COX-2 or iNOS (1:1000) for 2 h at room temperature, then were washed three times with TBS-T, and incubated with 1:2000 dilution of peroxidase-conjugated monoclonal anti-rabbit IgG for 1 h at room temperature. Protein bands at 42/44 kDa (ERK), 60 kDa (Akt), 72 kDa (Akt), 60 kDa (Akt), 72 kDa (COX-2) or 130 kDa (iNOS) were identified by anti-mouse secondary antibody (1:2000). The band densities were determined by densitometric analysis using the AlphaEaseFC™ program (Alpha Innotech, San Leandro, CA, USA). Density values of bands were normalized to the total β-actin present in each lane and expressed in percentage of control.

**Histopathologic analysis**

Lungs were removed after (BAL) fluid collection, perfused via the right ventricle with 10 mL PBS to remove residual blood, immersed in 10% phosphate-buffered formalin for 24 h and then in 70% ethanol until embedding in paraffin. Tissues were sliced and 5 micron sections and stained with periodic acid–Schiff (PAS)/hematoxylin for evaluation of mucus-producing cells. The intensity of mucus production was evaluated as previously describe [20], in each preparation and scores from 0 to 3 were attributed: 0 when none of the bronchi show any sign of mucus; 1, 2 or 3 when 25%, 50% or more than 50% of the bronchi epithelium was covered by mucus. Values represent the sum of 10 bronchi scored randomly at x250 magnification.

**Statistical analysis**

Data are expressed as the means ± S.E.M. Statistical evaluation of the data was carried out by analysis of variance (ANOVA) and sequential analysis of differences among means was done by Tukey’s contrast analysis. A P value lower than 0.05 was considered to be significant. P<0.05, P<0.01 and P<0.001 were marked with one, two or three asterisks, respectively.

**Results**

**Renal dysfunction**

C57Bl/6 mice were subjected to 45 minutes of renal ischemia. After 24 h of reperfusion, blood was collected to evaluate renal function by measuring serum creatinine levels. The ischemic group showed increased serum creatinine compared to controls (1.14±0.14 vs. 0.44±0.02 mg/dl). Serum creatinine levels were also increased in the immunized ischemic group (1.0±0.11 vs. 0.34±0.01 mg/dl) compared to control ischemic group (1.0±0.11 vs. 0.34±0.01 mg/dl).
mg/dl) compared to the immunized sham group. However, serum creatinine levels in the immunized ischemic group were not different from those in the ischemic group. These results show that IRI causes severe renal dysfunction in both immunized and non-immunized ischemic animals (Fig. 1).

**Cells in BAL after IRI**

To analyze the systemic effects of renal IRI on the lungs, analyses of the BAL fluid were performed 24 hours after renal ischemic surgery in the immunized and non-immunized groups. In the non-immunized groups, the number of total cells was significantly higher in the ischemic animals than in the sham animals (32.1±2.8 vs. 17.5±2.5). The sham immunized group presented a higher number of total cells compared to both the sham non-immunized group (55±4 vs. 17.5±2.5) and the ischemic non-immunized group (55±4 vs. 32.1±2.8). When the immunized animals were subjected to ischemia, the increase in the number of cells was abolished. Differential cell counting showed that the number of neutrophils was increased after IRI (9.08±4.5 vs. 0.2±0.1) and in the sham immunized group (9.3±2.5 vs. 0.2±0.1). However, in the immunized ischemic group, the increase in the number of neutrophils was abolished. The number of eosinophils was increased in the sham immunized group (5.2±1.5 vs. 0.47±0.3) and was reduced after IRI (0.73±0.06 vs. 5.2±1.5) (Fig. 2).

**Mucus production**

In another series of experiments, the intensity of mucus production was evaluated in the bronchi (n=10) and scored from 0 to 3 as described in the Materials and Methods. In the sham and ischemic groups, none of the bronchi contained mucus (score 0), while the immunized group had an average score of 2.5. The immunized ischemic group showed significantly inhibited mucus production (2.5 vs. 0.9). Mucus plugs were not observed in any group (Fig. 3).
Expression of COX-2 and iNOS in pulmonary tissue

Two molecules linked to endothelial cell dysfunction, COX-2 and iNOS, were quantified in the lung using Western blot. Lung tissue was collected 24 h after the renal ischemic surgery. The density of bands in the Western blot demonstrated that both molecules were increased in the ischemic groups at 24 hours compared to sham-operated animals (COX-2: 1084±97.09 vs. 129±10; iNOS: 1172±126.2 vs. 529±59). Both molecules were increased in the immunized group (COX-2: 1867±69.4 vs. 1084±97.09; iNOS: 2428.5±185.7 vs. 1172±126.2) compared to the ischemic group, and in the immunized ischemic group, the levels of COX-2 and iNOS were significantly decreased (COX-2: 1867±69.4 vs. 1186.7±190.5; iNOS: 2428.5±185.7 vs. 985±132.3) (Fig. 4).
IRI modulates the OA-induced phosphorylation of signaling pathways

Twenty-four hours after OA challenge, the lungs were removed, as described in the Materials and Methods, and the phosphorylation of ERK and Akt were analyzed by Western blot. Fig. 5 shows that OA challenge was able to induce the phosphorylation of both molecules, ERK (2 fold) and Akt (3.3 fold), in the lung compared to the control group, which received saline aerosol. Interestingly, OA-challenged mice subjected to IRI showed an increase in ERK phosphorylation (1.5 fold) compared to sham OA-challenged mice. There was no difference in Akt phosphorylation between the two groups.

Expression of cytokines in the lungs after renal IRI

Real-Time PCR was used to quantify the expression of IL-1β, IL-2, IL-12, IL-4, IL-5 and IL-13 in pulmonary tissue after OA challenge and renal IRI. Lungs were collected 24 hours after reperfusion. Ischemic mice showed an increase in the expression of the Th1 cytokines IL-1β (18.59±0.5), IL-2 (6.12±1.25) and IL-12...
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(8.26±2.79) when normalized to the sham group but did not present an up-regulation in the expression of the Th2 cytokines IL-4, IL-5 and IL-13. However, the sham immunized group showed increased expression of IL-4 (2.84±0.08), IL-5 (23.26±1.99) and IL-13 (83.06±12.93). The immunized ischemic group showed decreased levels of the Th2 cytokines IL-4 (84%), IL-5 (68%) and IL-13 (72%) when compared to the sham immunized group (Fig. 6).

Discussion

The model we used to study allergic lung inflammation consisted of immunization with ovalbumin using aluminum hydroxide as an adjuvant, which was followed 14 days later by two ovalbumin aerosol challenges one week apart. This model presents many of the characteristic features of allergic asthma, such as eosinophil and lymphocyte infiltration, mucus production and airway hyperreactivity [21].

Genetic differences among mouse strains with regard to basal lung functions and airway responsiveness have been reported previously [22]. In fact, some papers have demonstrated that in BALB/c mice, the Th2 immune response develops a greater overall response when compared to C57BL/6 [23-25]. However, other authors have shown that C57BL/6 mice also exhibit marked pulmonary allergic inflammation compared to control animals [20, 21, 26-29].

Previous evidence from the literature has shown that distinct Th1-related pathologies could modulate the inflammatory response seen in asthma [2, 5]. Therefore, we wondered whether renal IRI could also modulate lung function in a model of allergic inflammation.

A number of studies have revealed that robust inflammatory responses mediated by both innate and adaptive immune systems in postischemic kidneys are a major factor in the pathogenesis of renal IRI. Inflammation starts during ischemia and accelerates upon reperfusion with endothelial activation, leukocyte recruitment, the up-regulation of chemokines and cytokines and the activation of the complement system. Postischemic kidneys are not merely the passive targets of immune-mediated injury but actively participate in the engagement of systemic immune factors, including the up-regulation of leukocyte adhesion molecules and toll-like receptors (TLRs) [30]. In addition to the local damage caused by renal IRI, distant organs can also be affected [31], including the lungs [17, 18]. Recently, we demonstrated that lung cellular infiltration and creatinine levels were increased simultaneously in renal IRI [18]. However, immunized and challenge mice after renal IRI showed reduced numbers of inflammatory cells in the BAL fluid compared to the immunized and challenged groups. Similar results were observed in relation to bronchial mucus production, where renal IRI reduced the amount of mucus in immunized and challenged mice.

Many inflammatory mediators are released during IRI, including tumor necrosis factor and nitric oxide (NO), which are both possible candidates for mediating the lung dysfunction that results from intestinal IRI [32-34]. NO seems to play an important role in asthma, not only in the induction of the Th2 immune response but also in amplifying and perpetuating the Th2-mediated inflammatory response. It has been speculated that the large amount of NO generated in the asthmatic airways may result in the suppression of Th1 cells and a concomitant reduction of interferon-γ (IFN-γ), leading to the proliferation of Th2 cells [35]. In previously reported results, we showed that after an antigen challenge, there was transient expression of NOS2 in lung tissue, airway cells, and BAL fluid cells and that the infiltrating neutrophils in the airway produce NO [36]. We also observed that the administration of L-NAME prior to the antigen challenge significantly reduced eosinophil infiltration and mucus production [26]. Constitutive NOS is protective in IRI, whereas inducible NOS is responsible for lung dysfunction [34]. Turnage et al. [37, 38] demonstrated the deleterious role of inducible NOS in the lung after intestinal IRI. Matsuyama et al. [39] and Kosaka et al. [40] reported that COX-2 and iNOS are up-regulated and have deleterious effects on renal IRI, which is in agreement with the present results. In fact, our group previously demonstrated that COX-2 is important in renal damage after IRI by showing that the blockage of COX-2 ameliorates IRI [41]. As expected, immunized mice showed an up-regulation in COX-2 and iNOS expression, similar to our previous findings [18]. Interestingly, in the immunized ischemic group, the levels of COX-2 and iNOS were significantly decreased. These results could suggest that renal IRI has a protective effect on lung tissue, as renal IRI might attenuate the previously established allergic lung inflammation.

The MAPK signaling cascade plays a pivotal role in the activation of inflammatory cells. It has been shown that the activity of p42/44 MAPK, also known as extracellular signal-regulated kinase (ERK), in the lungs was significantly higher in asthmatic mice than in normal controls [9]. This pathway is involved in the activation of
several transcription factors that control gene activity and expression. The results presented herein showed that OA induced ERK phosphorylation in the lung compared to aerosol saline-treated mice. In addition, IRI/OA also increased the phosphorylation of this signaling molecule compared to sham-operated mice. We also analyzed PI3K, which is composed of two subunits, a 100 kDa (p110) catalytic subunit and an 85 kDa (p85) regulatory subunit [42]. A direct interaction between p21ras and p110 and the demonstration that p21 ras regulates PI3'-kinase activity [43] suggest that the activation of p21ras could initiate both the ERK and PI3K signaling pathways. The data presented herein show that IRI/OA increased the phosphorylation of ERK and Akt. This suggests that IRI could act directly on p21ras, which favors the hypothesis that the modulation of lung allergic responses by renal ischemia and reperfusion injury could occur, at least in part, by changing the activity of cellular signal transduction factors.

To confirm this hypothesis, we evaluated the expression levels of IL-1β, IL-2, IL-12, IL-4, IL-5 and IL-13 because these cytokines play a critical role in orchestrating this type of inflammatory response [44]. Some studies have demonstrated that the Th1 pattern in IRI is deleterious, while the Th2 pattern is protective [14, 45, 46]. IL-1β, a pro-inflammatory cytokine, is involved in endothelial activation, whereas IL-4 and IL-5 play an essential role in the Th2 immune response [18]. In the present study, IL-1β, IL-2 and IL-12 expression was increased only in ischemic mice, whereas IL-4 and IL-5 play an essential role in the Th2 immune response [18]. In the present study, IL-1β, IL-2 and IL-12 expression was increased only in ischemic mice, whereas IL-4 and IL-5 were increased in ischemic and challenged mice; however, in asthmatic mice subjected to renal IRI, the expression of Th2 cytokines was reduced to levels similar to that of ischemic mice. These results corroborate another study that demonstrated the modulation of IL-4 and IL-5 production in helminthes-infected patients with asthma [7]. It is well known that IL-4 and IL-5 stimulate IgE differentiation and the expression of high-affinity IgE receptors on mast cells and eosinophilia, all of which are involved in the pathogenesis of asthma [47]. IL-13 induces isotype switching of B cells towards immunoglobulinE synthesis, increases the expression of immunoglobulinE receptors on mast cells and causes an up-regulation of vascular cell adhesion molecule-1, which is implicated in the microvascular recruitment of eosinophils and basophils. On the other hand, IRI stimulates the synthesis of pro-inflammatory cytokines, including IL-1, IL-6, TNF-α and IFN-γ [48, 49], and the blockage of IL-1, IL-6, and keratinocyte-derived chemokine (KC; a mouse analog of human IL-8) mitigates renal injury in murine renal IRI models [50]. Marques et al. [14] showed that IL-4 acts as a protective factor in renal IRI. IL-4 KO mice showed reduced renal dysfunction compared to wild-type mice, and in models of liver and intestine ischemia and reperfusion injury, the induction of IL-13 was cytoprotective [51, 52]. Therefore, we could conclude that the reduced expression of IL-4, IL-5 and IL-13 promotes the down-regulation of the lung allergic response after renal IRI. In this model, renal IRI modulates the lung allergic response via the Th1/Th2 balance.

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