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Therapeutic potential of c-Raf in LPS-stimulated acute lung injury: Anti-inflammatory and protective effects in mice model

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Abstract

Background and objective: Acute lung injury (ALI) it considered as a very serious illness characterized by inflammatory lung swelling, leading to acute hypoxic respiratory failure. c-Raf G-protein has been shown to regulate inflammation we predicted that c-Raf could regulate neutrophil infiltration in LPS induced sepsis in mice model.

Methods: 18 albino mice (6 weeks age, weighing 26±2 grams) were randomly assigned to three groups: the control group (they were injected by normal saline intraperitoneally, n=6), the LPS (lipopolysaccharide) group (50 mg/kg LPS Were injected intraperitoneally, n=6), the GW5074 + LPS group (2 mg/kg of the selective c-Raf inhibitor GW5074 were given intraperitoneally 15 minutes before LPS injection, n=6). After 24 hours, the mice were sacrificed, then inflammatory cell counts, proinflammatory cytokines (TNF-α, IL-1β, MPO activity, CXCL-2, IL-6) were measured. The lungs were then isolated for molecular target analysis, histopathological, and immunohistochemical evaluations.

Results: Resemble to the LPS group, pretreatment of mice with c-Raf inhibitor significantly decreased MPO, CXCL-2, TNF-α, IL-1β and IL-6, activity. GW5074 weakened migration and infiltration of inflammatory cells, showing a marked reduce in MPO (6.0 ±0.2 vs. 2.2±2.0 *P* <0.05) as well as CXCL-2(132.8± 2.0 vs. 80.4±6.0 *P* <0.05) in the sham, LPS, and GW5074 + LPS. Additionally, IL-6 levels in treatment group significantly decrease compared with LPS group (123 ± 4.0 vs. 71.2 ± 6.0 *P* <0.05).

Conclusion: The conclusions of this research propose that c-Raf may have anti-inflammatory and protective effects against LPS-stimulated ALI in mice.

Keywords: c-Raf; Chemokine; Sepsis; LPS; ALI.

Introduction

Acute lung injury (ALI) is a known complexity of systemic inflammation and is related with increasing the level of mortality in critically ill patients. Intestinal aperture and the subsequent release of bacteria and their toxins into the abdominal socket trigger the regional production of pro-inflammatory agents, which then enter the bloodstream. (1)

These pro-inflammatory molecules induce innate immune cells like neutrophils and platelets, leading to a systemic inflammatory reply. While white blood cells play an important role in defending against to bacterial infections, their over accumulation in the pulmonary microcirculation can also impair gas exchange in the lungs. As an example, neutrophil decreasing has shown preservation against lung damage in many different sepsis models, suggesting that pulmonary white blood cell infiltration is important step in lung injury. (2,3) However, the detailed spatial mechanisms of white blood cell-endothelium interactions in the lungs very small vasculature during systemic inflammation are not well understood, largely because of the difficulty of studying these interactions in vivo. Recently, new model of lung microcirculation using intravital

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fluorescence microscopy has been created,[4] which may help explain the processes underlying the buildup of pulmonary leukocytes in abdominal sepsis.

In many organs, including colon, liver, pancreas, brain, and muscle, leukocyte recruitment is typically thought of as a multi-step process that starts with rolling contacts and progresses to firm adhesion and trans endothelial movement. β2-integrins, such as CD11a/CD18 (lymphocyte function-associated antigen-1) and CD11b/CD18 (macrophage-1 antigen), have been shown in numerous studies to mediate leukocyte adhesion.[5]

The recruitment of leukocytes in the lung is much more complicated than in other organs. Under normal circumstances, neutrophils, which are bigger than the diameter of pulmonary capillaries, must distort in order to move through the pulmonary microcirculation.[6] Neutrophils become more rigid when activated in systemic inflammation, which facilitates their mechanical sequestration in lung capillaries. The β2-integrin receptor ICAM-1, however, has strong evidence suggesting (intercellular adhesion molecule -1) promotes leukocyte migration to the lungs during systemic inflammation and is increased on pulmonary endothelial cells.[7] Furthermore, research has demonstrated that GW5074, a particular c-Raf inhibitor, inhibits it, indicating a role in the decrease of severe inflammation.[8]

Building on these discoveries, our research intends to examine how the c-Raf inhibitor controls leukocyte extravasation and chemokine and cytokine activity in the pulmonary system during systemic inflammation, particularly in relation to abdominal sepsis.

Methods

Male albino mice were (6–8 weeks age, 25–30 g) from the laboratory animal facility of Hawler Medical University (College of Pharmacy, Erbil). The ethic committee at Hawler Medical University/ College of Pharmacy gave their approval for the animal studies. The animals' living conditions included a 12-hour light/12-hour dark cycle, a fixed room temperature of 25° C, and 70% related humidity with unlimited access to food and water.

The LPS-induced septic shock model: 0.9% NaCl (control), LPS (20 mg/kg), pretreatment with GW5074(2 mg/kg) + LPS, LPS + GW5074 as a post treatment and GW5074 alone. In order to generate experimental sepsis, mice in the GW5074 (2 mg/kg) pretreatment sepsis groups received an intraperitoneal injection of GW5074 10 minutes before they receive an intraperitoneal injection of 20 mg/kg LPS.[9] Ten minutes prior to the LPS injection, mice in the LPS group were given identical volume of 0.9% NaCl (vehicle). Only the equivalent volume of 0.9% NaCl was given to the mice in the 0.9% NaCl group. 24 hours after the injection, all of the animals were scarified. We have used GW5074 as c-Raf inhibiter 2 mg -kg as previous study.[10]

Enzyme-Linked Immunosorbent Assay

Blood samples and peritoneal lavage fluid were collected and centrifuged at 3500 rpm for 15 minutes. The concentrations of TNF-α, IL-1β, and IL-6 were measured by using ELISA kits (Neo Bioscience Technology, Shenzhen, China).

MPO Activity

One milliliter of a mixture of PBS and aprotinin (4:1) (10,000 KIE/ml, Trasylol®, Bayer HealthCare AG, Leverkusen, Germany) was used to homogenize lung tissues for one minute. After centrifuging the homogenate for 10 minutes at 15,000 g, the supernatant was kept at -20°C. mentioned, the myeloperoxidase (MPO) assay was conducted by using the pellet.

After resuspending the pellets in 1 milliliter of 0.5% hexadecyltrimethylammonium bromide, then the sample was left to thaw for a day. The thawed sample was sonicated, sonicated for 90 seconds, and then incubated for two hours at 60°C in a water bath. Next, the supernatant's MPO activity was assessed by measuring the change in absorbance during the

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MPO-catalyzed redox reaction of H2O2 at 450 nm, using a reference filter at 540 nm, at 25°C, MPO enzyme activity was measured spectrophotometrically. MPO units per gram of tissue were used to express the results.

CXCL-2 levels

CXCL-2 levels were measured in the blood, and stocked supernatants from homogenized pancreatic tissues. CXCL-2 concentrations were measured by using double-antibody Quantikine enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Europe, Abingdon, UK).

Statistical Analysis

The data was shown as mean values ± standard error of the mean (SEM). Non-parametrical tests (Mann-Whitney) were used for statistical assessments. (n) represents the number of animals, and *P* <0.05 was considered statistically significant.

Results

c-Raf inhibitor controls proinflammatory cytokines

Plasma TNF-α levels were initially assessed as a marker of inflammation to investigate the involvement of c-Raf in acute lung injury using adult male mice. The sham, c-Raf alone, and treatment groups exhibited low concentrations of TNF-α in plasma, the LPS group displayed markedly elevated levels. Notably, pre-treatment with GW5074 resulted in a substantial decrease in plasma TNF-α compared to the LPS group, as evidenced by a statistically significant decrease (*P* = 0001) (Table 1).

Similarly, the IL-1β data exhibited a trend comparable to that of TNF-α. Specifically, the plasma concentrations of IL-1β in the LPS group were considerably high in compare to those in the Sham group (*P* = 0.001). Conversely, in the c-Raf pretreatment group, there was a high reduction in plasma IL-1β levels when contrasted with the LPS group (*P* = 0.001) (Table 2).

Table 1 TNF-α activity measured in plasma (pg./ml)

Parameters	Mean	SE	*P* value
Sham	28	11±3	<0.05
Vehicle + LPS	112*	6.4±6	
cRaf + LPS	46#	25±4	

c-Raf controls LPS-induced TNF-α. TNF-α levels in the plasma in sham normal saline (NS) animals and LPS exposed mice were pretreated with vehicle or the c-Raf inhibitor (2mg/kg). Samples were collected 24 h after sepsis induction. Data demonstrate means ± SEM and n = 6. **P* <0.05 vs. NS and #*P* <0.05 vs. Vehicle + LPS.

Table 2 IL-1β activity measured in plasma (pg./ml)

Parameters	Mean	SE	*P* value
Sham	22.5	±4	<0.05
Vehicle + LPS	123*	±8	
C-Raf + LPS	51.2#	6.5	

c-Raf controls LPS-induced proinflammatory cytokines. IL-1β levels in the plasma in sham normal saline (NS) animals and LPS exposed mice were pretreated with vehicle or c-Raf inhibitor (2mg/kg). Samples were collected 24 hr. after sepsis induction. Data were represented by means ± SEM and n = 6. **P* < 0.05 vs. NS and #*P* <0.05 vs. Vehicle + LPS.

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c-Raf inhibitor regulates neutrophil infiltration in lung

Levels of myeloperoxidase (MPO) in tissue were utilized as a marker of neutrophil infiltration. Our study revealed that lung MPO activity significantly rose by LPS injection (P = 0.003). Conversely, inhibition of c-Raf led to a 25% reduction in LPS-induced lung MPO levels (P = 0.001) (refer to Table 3). During the systemic inflammatory response in acute lung injury, induced neutrophils aggregate in the lung microvasculature. Notably, MPO activity in the lungs increased substantially in response to LPS challenge.

Furthermore, the application of LPS resulted in a significantly increase in chemokine CXCL-2 concentrations in the plasma, rising from an average of 30.8 ± 3 to 132 ± 2 pg/mg (P = 0.003). Subsequent administration of a c-Raf inhibitor led to a marked reduction in CXCL-2 levels within the inflamed lungs (P = 0.0024) (Table 4).

Within 24 hours of sepsis onset, IL-6 levels were considerably increased. The LPS group exhibited the highest serum IL-6 levels (mean ± SE: 123 ± 8) compared to the sham group (mean ± SE: 4.6 ± 4; P = 0.003). Conversely, plasma IL-6 levels were significantly reduced in LPS-treated mice (mean ± SE: 71.2 ± 6 vs. LPS 123 ± 8; P = 0.003), suggesting systemic c-Raf activation in this experimental model (Table 5).

Table 3 MPO activity measured in lung tissue (U/g/Tissue)

Parameters	Mean	SE	*P* value
Sham	1.3	±3	<0.05
Vehicle + LPS	6.0*	±2	
c-Raf = LPS	2.2#	±2	

c-Raf controls LPS-stimulated neutrophil aggregation. MPO levels in the lung in sham normal saline (NS) animals and LPS injected mice were pretreated with vehicle or the c-Raf inhibitor (2mg/kg). Samples were collected 24 h after sepsis induction. Data were represented by means ± SEM and n = 6. *P <0.05 vs. NS and #P <0.05 vs. Vehicle + LPS.

Table 4 CXCL-2 activity measured in plasma (pg/ml)

Parameters	Mean	SE	*P* value
Sham	30.8	± 3	<0.05
Vehicle + LPS	132.8*	±2	
c-Raf + LPS	80.4#	±6	

c-Raf controls LPS-induced chemokine production. CXCL-2 levels in the plasma in sham normal saline (NS) animals and LPS exposed mice were pretreated with vehicle or the c-Raf inhibitor (2mg/kg). Samples were collected 24 h after sepsis induction. Data were represented by means ± SEM and n = 6. *P <0.05 vs. NS and #P <0.05 vs. Vehicle + LPS.

Table 5 IL-6 activity measured in plasma (ng/ml)

Parameters	Mean	SE	*P* value
Sham	4.6	± 4	0.05
Vehicle + LPS	123*	± 8	
c-Raf = LPS	71.2#	± 6.5	

c-Raf controls LPS-induced IL-6. Il-6 levels in the plasma in sham normal saline (NS) animals and LPS exposed mice were pretreated with vehicle or the c-Raf inhibitor (2mg/kg). Samples were collected 24 h after sepsis inducement. Data were represented by means ± SEM and n = 6. *P <0.05 vs. NS and #P <0.05 vs. Vehicle + LPS.

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Discussion

It is unknown which signaling cascades govern the pro-inflammatory pathways in sepsis. Our study offers the first evidence that c-Raf plays significant part in controlling the pathophysiology of sepsis. These findings show that the surface overexpression of Mac-1 on neutrophils is mediated by c-Raf. In addition to reducing neutrophil infiltration in the lung, inhibition of c-Raf activity also significantly reduces proinflammatory cytokine and chemokine levels. Additionally, we recorded that c-Raf inhibition eliminated neutrophil buildup in the lung and reduced MPO levels, indicating that c-Raf regulates both systemic and local inflammation in ALI.

We discovered that the tissue that tissue damage in severe AP was considerably decreased by c-Raf inhibition using a particular c-Raf inhibitor (GW5074). For example, GW5074 therapy decreased the rise in sepsis plasma levels of TNF-α amylase by 25% and IL1B by 45%, suggesting that c-Raf activity regulates a large portion of the proinflammatory cytokines in ALI.

These results present the first hard proof in the literature that c-Raf signaling pathway blockage significantly reduces the risk of sepsis. It is important to notice that c-Raf is primarily employed in cancer therapy to target the RAF–MEK–ERK pathway in order to control lung cancer.[11] Research has shown that GW5074 decreases the expression of chemokines such as CXCL5, CXCL9, and CXCL10[12] in addition as proinflammatory cytokines. Therefore, the anti-inflammatory benefits of c-Raf in ALI may possibly be explained by our current findings. Next, we looked into the possibility that the synthesis of CXCL chemokines, such as CXCL-2, a potent neutrophil activator, could be linked to the indirect effects of c-Raf inhibitory actions.

This made evaluating CXCL-2 levels in the lungs particularly important. Our results showed that LPS significantly increased CXCL-2 levels in sepsis. However, in LPS-induced acute lung injury (ALI), c-Raf suppression notably reduced CXCL-2 production. Neutrophil infiltration is widely acknowledged as a critical component of ALI.[13-15] For example, it has frequently been noted that neutrophil depletion lessens lung tissue damage.[16-18] In the current study, we found that the LPS exposure markedly elevated the number of extravascular neutrophils in the lung as well as MPO activity.

The injection of GW5074 led to significant decline in MPO levels and extravascular neutrophil counts, indicating that c-Raf activity is a crucial modulator of neutrophil recruitment in the inflammatory lung. Given the crucial part neutrophils play in the pathophysiology of lung,[18] it is plausible that c-Raf's protective benefits in sepsis could be explained by its inhibitory influence on neutrophil responses.

One of the systemic effects of severe sepsis is neutrophil buildup in the lungs.[19] We found that there was a noticeable increase in lung MPO levels following the LPS challenge. Notably, GW5074 decreased pulmonary MPO activity, suggesting that c-Raf regulates systemic neutrophil infiltration and activation in sepsis as well.

IL-6, a key mediator of inflammatory processes in the lungs, is upregulated in both animal models and human patients with pulmonary diseases. Although IL-6 is significantly elevated in septic mice, our study reveals that c-Raf inhibition can significantly reduce these levels and ameliorate LPS-induced ALI. These findings are consistent with the proposed mechanism of c-Raf action in acute lung injury, which involves suppressing inflammatory responses by inhibiting chemokine, cytokine, and neutrophil recruitment to the lung.

Conclusion

The c-Raf signaling regulates lung tissue destruction in sepsis. According to our findings, c-Raf suppression reduces neutrophil infiltration in lung sepsis. indicating that in sepsis, c-Raf regulates

both systemic and local inflammation. Therefore, these findings not only identify a crucial signaling pathway in ALI, but they also suggest that c-Raf targeting may be a helpful strategy for reducing pathological inflammation in sepsis ALI.

Competing interests

The authors declare that they have no competing interests.

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