Mol Cell Biochem (2015) 400:153–162
DOI 10.1007/s11010-014-2271-4

PARP inhibitor, olaparib ameliorates acute lung and kidney injury upon intratracheal administration of LPS in mice

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Received: 25 August 2014 / Accepted: 29 October 2014 / Published online: 18 November 2014

Abstract We have previously shown that PARP-1 inhibition provides protection against lung inflammation in the context of asthma and acute lung injury. Olaparib is a potent new generation PARP inhibitor that has been approved for human testing. The present work was designed to evaluate its beneficial potential against LPS-induced acute lung injury and acute kidney injury upon intratracheal administration of the endotoxin in mice. Administration of olaparib at different doses, 30 min after LPS treatment showed that single intraperitoneal injection of the drug at 5 mg/kg b.wt. reduced the total number of inflammatory cells particularly neutrophils in the lungs. This was associated with reduced pulmonary edema as the total protein content in the bronchoalveolar fluid was found to be decreased substantially. Olaparib provided strong protection against LPS-mediated secondary kidney injury as reflected by restoration of serum levels of urea, creatinine, and uric acid toward normal. The drug restored the LPS-mediated redox imbalance toward normal in lung and kidney tissues as assessed by measuring malondialdehyde and GSH levels. Finally, RT-PCR data revealed that olaparib downregulates the LPS-induced expression of NF-κB-dependent genes namely TNF-α, IL-1β, and VCAM-1 in the lungs without altering the expression of total p65NF-κB. Overall, the data suggest that olaparib has a strong potential to protect against LPS-induced lung injury and associated dysfunctioning of kidney in mice. Given the fact that olaparib is approved by FDA for human testing, our findings can pave the way for testing of the drug on humans inflicted with acute lung injury.

Keywords PARP · Olaparib · LPS · Acute lung injury · Acute kidney injury

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Introduction

Acute lung injury (ALI) is a syndrome consisting of acute hypoxemic respiratory failure with bilateral pulmonary infiltrates that is associated with both pulmonary and nonpulmonary risk factors [1]. ALI and its severe form acute respiratory distress syndrome (ARDS) can result in acute respiratory failure with substantial morbidity and mortality. Even in patients who survive ALI, there is evidence that their long-term quality of life is adversely affected [2, 3]. ALI is characterized by a disruption of the endothelium and alveolar epithelial barriers involving increased micro vascular permeability, followed by an onset of dyspnea, severe hypoxemia, and pulmonary edema [4, 5] ALI can develop numerous devastating complications at later stages, including severe sepsis, severe trauma, and ischemia/reperfusion injury [6]. Sepsis due to nonpulmonary infections, aspiration of gastric contents, and major trauma with shock also commonly precipitates the injury. Less commonly, acute pancreatitis, transfusions, drug reactions, and fungal and parasitic lung infections are linked to ALI and ARDS. The coexistence of two or more of these risk factors can enhance the likelihood of developing ALI or ARDS [1].

Lipopolysaccharide (LPS) is a large molecule consisting of lipid and a polysaccharide composed of O-antigen,

found in the outer membrane of the gram-negative bacteria and is known to elicit strong immune response in animals [7]. Inflammatory stimuli from this endotoxin are well recognized for their ability to induce pulmonary inflammation. Experimental administration of LPS, both systemically and intratracheally, has been used to induce pulmonary inflammation in animal models of ALI [8–11]. The acute lung inflammation may have deleterious effects on remote organs such as kidney and may involve crosstalk between the lung and kidney [12]. Survival decreases drastically when acute kidney injury (AKI) and ALI occur together [13, 14]. The development of AKI in the setting of ALI carries an in-hospital mortality rate of 58 %, compared to 28 % in ALI patients without AKI [14].

It has been established that Poly(ADP-ribosyl)ation reactions, carried out by poly (ADP-ribose) polymerases (PARPs) enzymes play an important role in the pathogenesis of oxidative stress-mediated number of human diseases [15–18]. The PARPs are a family of around 18 members whose primary role is to help in the repair of DNA damage [19]. Activation of PARPs results in post-transcriptional modification of enzymes involved in DNA repair process [20, 21] by catalyzing the attachment of ADP-ribose units to acceptor proteins. Such modification of cellular proteins modulates their structure and function, which favors the DNA repair process [22, 23]. DNA damage through excessive generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) during inflammatory conditions can cause persistent activation of PARPs resulting in the depletion of their substrate NAD^+ and consequently leading to necrotic cell death and organ dysfunction [24, 25]. We and others have shown that PARP-1 also acts as a co-factor to enhance the nuclear factor (NF)-κB-dependent transcription of inflammatory cytokines [26–31]. Treatment with PARP inhibitors have been reported to improve the pathogenesis of septic acute lung inflammation [31, 32] and ventilator-induced lung injury [33]. Recently, PARP has been in the news as number of researchers have shown that PARP inhibition provides a treatment strategy against breast, ovarian, and prostate cancers [34–37]. Olaparib is one such potent PARP inhibitor that has been approved for clinical testing in such cancer patients and has acceptable level of toxicity [38, 39]. A recent study has suggested that the activation of PARP mediates renal inflammation secondary to LPS-induced acute lung inflammation [40]. Since olaparib is approved for testing on humans, our aim for the present work was to evaluate the anti-inflammatory potential of the compound in LPS-induced lung injury and associated kidney injury using mouse model of the disease for its potential application in humans inflicted with ALI/ARDS.

Materials and methods

Animals

Female LACA mice weighing 25–30 g were obtained from central animal house of Panjab University, Chandigarh. All the animals were housed in polypropylene cages and were fed with standard diet and were given water ad libitum. The animals were housed, cared, and used for experiments in accordance with the "Guidelines for the Care and Use of Experimental Animals" approved by University Ethics Committee.

Chemicals

All the chemicals used in the study were of analytical grade. LPS procured from E. coli, serotype O111:B4 (TLRGradeTM) was purchased from Enzo life sciences, Farmingdale, USA. Olaparib was purchased from Selleck chemicals, Houston, USA. All other chemicals needed for several biochemical assays including 2-thiobarbituric acid (TBA), Sulfosalicylic acid, and 5,5′-Dithiobis-2-nitrobenzoic acid (DTNB) were purchased from either Himedia Laboratories, Mumbai, India or Sisco Research Laboratories Pvt. Ltd, Mumbai, India.

Experimental design

The mice were divided randomly into four groups and were given treatment as below:

Control group Mice were given standard diets and no drug was given to them.

Olaparib group Mice were given a single intraperitoneal *(i.p.)* injection of olaparib at a dose of 5 mg/kg b.wt.

LPS group Mice were administered LPS intratracheally (*i.t.*) at dose of 50 μg per mouse as reported previously [41].

LPS+ olaparib group Mice were treated with LPS as explained above and olaparib was given as single injection (*i.p.*) at dose of either 1, 5, or 10 mg/kg b.wt. 30 min post LPS administration. The dose range of drug was chosen on the basis of our earlier study where we have shown that administration of olaparib at different doses 30 min post-allergen (ovalbumin) challenge conferred a robust reduction in lung inflammation using mouse model of asthma [42]. Based on the outcome of different doses of olaparib on LPS-induced lung inflammation, we selected 5 mg/kg b.wt. as final dose for subsequent experiments.

The mice were sacrificed either 6 or 18 h after the administration of LPS by cervical dislocation. The blood was drawn from the heart by using 1 ml syringe and was processed for the preparation of serum. The lungs were subjected to bronchoalveolar lavage (BAL) and fixed on microscopic slides as described [43]. Some groups of mice

were sacrificed for procuring whole lung and kidney tissues for preparation of tissue homogenate as explained earlier [44]. The lungs of the mice sacrificed 6 h after LPS treatment were processed for extraction of total RNA and reverse transcribed to cDNA.

Analysis of biochemical parameters

The total protein content was assayed by method of Lowry [45]. Assessment of serum urea, creatinine, uric acid, alkaline phosphatase, glutamate oxaloacetate transaminase (GOT), and glutamate pyruvate transaminase (GPT) was conducted by using commercially available kits from Transasia Bio-medical Limited, Solan. Malondialdehyde (MDA) as a marker of lipid peroxidation was assayed by method of Beuge and Aust [46]. The reduced glutathione (GSH) was measured according to Ellman [47].

Extraction of RNA from lung tissue and RT-PCR analysis

Lung tissue was stored in RNA *later* for RNA extraction using Qiagen kit, according to the procedure given in the kit manual. The extracted total RNA was used for the generation of cDNA using reverse transcriptase III (Invitrogen) and analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) using primers specific for TNF-a, IL-1β, VCAM-1, *p65*NF-κB, and β-actin. The amplification program was as follows: 5 min at 95 °C, 30 s at 95 °C, 45 s at 60 °C, and 1 min at 72 °C. The cycle numbers were optimized for each primer pair. The PCR products were then incubated for 15 min at 72 °C. The resulting PCR products were subjected to electrophoresis in a 2 % agarose gel and stained with ethidium bromide. The sequence of primers used are given below: TNF-α: Forward- 5′-TAT GGC TCA GGG TCC AAC TC-3′, Reverse-5′-CTC CCT TTG CAG AAC TCA GG-3′; IL-1 β: Forward-5′-GAC CTT CCA GGA TGA GGA CA-3′, Reverse-5′AGG CCA CAG GTA TTT TGT CG-3′; VCAM-1: Forward-5′-ACA GAC AGT CCC CTC AAT GG-3′, Reverse-5′ACC TCC ACC TGG GTT CTC TT-3′; *p65*NF-κB: Forward- 5′-CTTGGCAACAGCACAGA CC-3′, Reverse- 5′-GAGAAGTCCATGTCCGCAAT-3′; β-actin: Forward- 5′-TACAGCTTCACCACCACAGC-3′, Reverse- 5′-TCTCCAGGG AGGAAGAGGAT-3′.

Statistical analysis

Results are depicted as mean ± SEM. Statistical analysis was performed by one way Anova test followed by Tukey's multiple comparison using graph-pad prism software (GraphPad Software, Inc. La Jolla, CA). $P < 0.05$ was considered as statistically significant.

Results

Olaparib administration markedly reduced the number of inflammatory cells in the lungs upon LPS treatment

The major components of the inflammatory and immunological reaction in the lungs are phagocytes, in particular, macrophages and polymorphonuclear neutrophils (PMNs). Macrophages are the normal resident cells of the normal lower respiratory tract whereas PMNs are mostly absent. But, PMNs are known to accumulate in the lung in case of injury, trauma, or infection. In order to evaluate the effect of olaparib on LPS-induced recruitment of inflammatory cells in lungs, the drug was given to mice at different doses (i.e., 1, 5, or 20 mg/kg b.wt.) upon LPS administration. Figure 1a depicts that the total number of cells in BAL fluid of LPS-treated mice was increased by 672 % as compared to the mice of control group and statistically found to be highly significant ($P < 0.001$). When olaparib was given at dose of 1 mg/kg, the number of inflammatory cells in BAL was observed to be similar to that seen in LPS-treated mice. However, when the dose of drug was increased to either 5 or 20 mg/kg, the number of total cells in BAL fluid was restored toward normal by 62.94 and 66.47 %, respectively in comparison to LPS-treated group ($P < 0.001$). We next quantified the number of neutrophils in the BAL fluid. The data (Fig. 1b) showed that LPS treatment indeed enhanced the number of neutrophils in BAL fluid sharply ($P < 0.01$ w.r.t. control) and olaparib administration at 1 mg/kg did not cause any major change in such increase. However, olaparib at 5 or 20 mg/kg reverted the neutrophil number in BAL toward normal significantly by 62.85 % ($P < 0.01$) and 69.7 % ($P < 0.01$), respectively when compared with LPS-treated group. Since olaparib at a dose of 5 mg/kg ameliorate the neutrophil number in BAL toward normal efficiently, we selected this dose for subsequent experiments.

The pathogenesis of ALI includes injury of endothelial and epithelial barriers leading to protein-rich edema and inflammation induced by cytokines and chemokines released from inflammatory cells, lung epithelial cells, or fibroblasts. Weakening of the endothelial barrier enhances the transendothelial diapedesis of leukocytes into lung tissues, further contributing to pulmonary dysfunction. Our results (Fig. 1c) indeed confirmed the earlier reports that LPS administration leads to pulmonary edema as total protein content in the BAL fluid was found to be elevated by 103.65 % when compared with control ($P < 0.001$). Olaparib administration at dose of 5 mg/kg after LPS treatment restored BAL protein content by 43.46 % toward normal quite efficiently ($P < 0.001$ w.r.t. LPS).

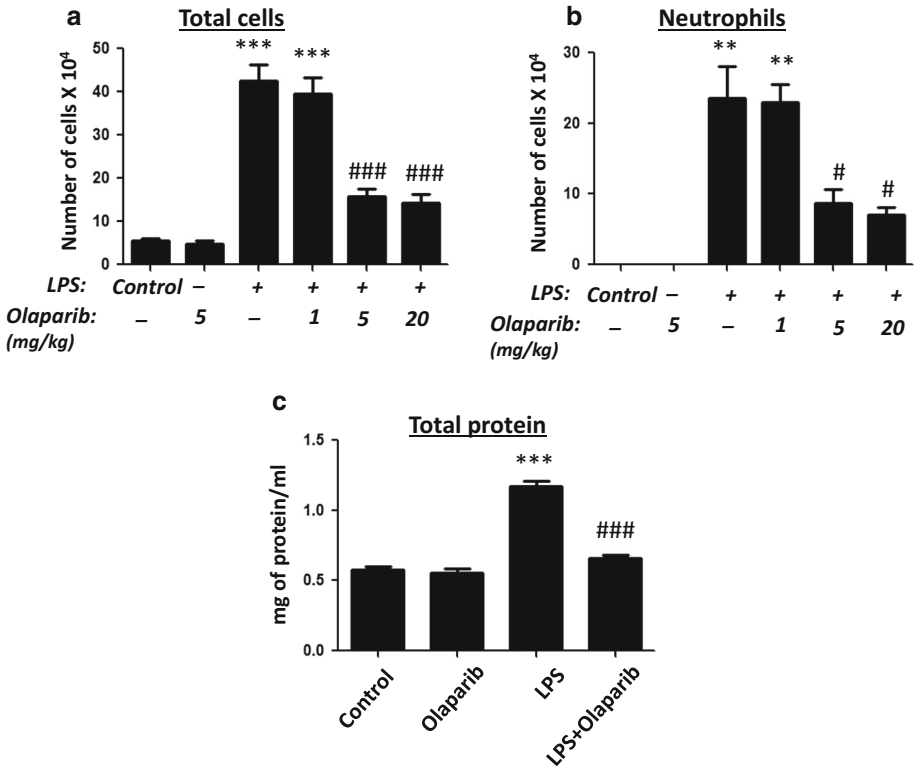

Fig. 1 Olaparib administration markedly reduced the number of inflammatory cells in the lungs upon LPS treatment. Mice were treated with LPS followed by different doses of olaparib as explained in materials and methods section and were subjected to BAL 18 h after LPS treatment. BAL cells thus obtained were differentially stained for counting total cells (**a**) and neutrophils (**b**). Supernatant of BAL was assessed for total protein content (**c**). Results are depicted as mean ± SEM. ***significant w.r.t. control, $P < 0.001$; **significant w.r.t. control, $P < 0.01$; ###significant w.r.t. LPS, $P < 0.001$; #significant w.r.t. LPS, $P < 0.05$

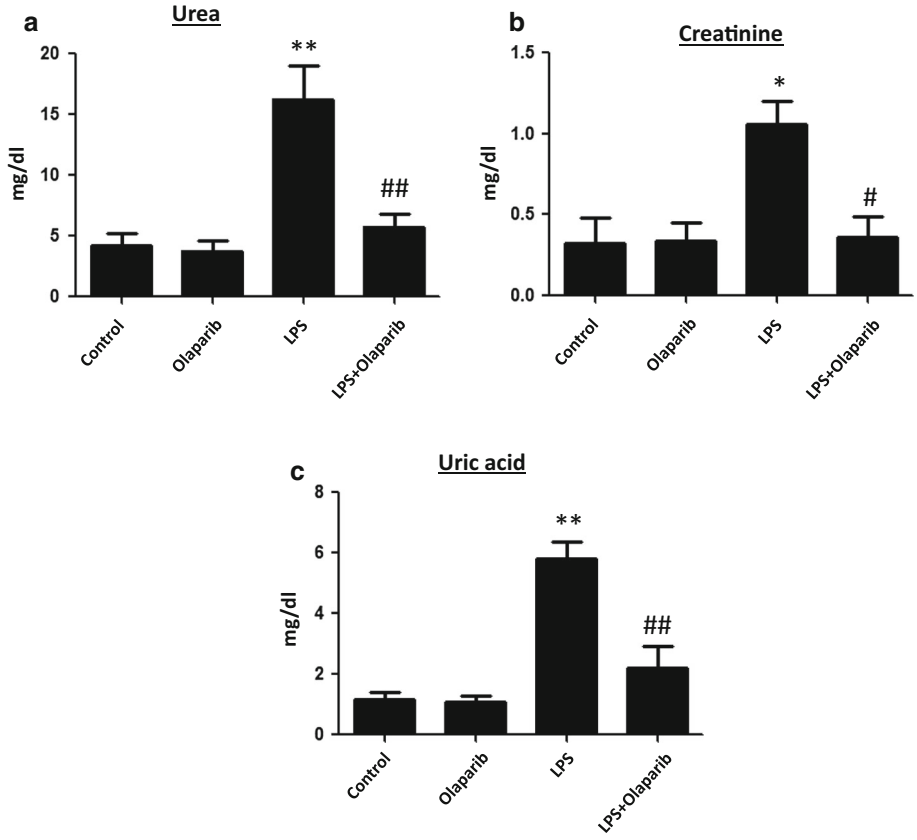

Fig. 2 Olaparib administration ameliorate kidney function upon intratracheal administration of LPS. Mice were treated with LPS and/or olaparib as explained earlier. Blood obtained 18 h after LPS treatment were processed for serum preparation and tested for urea (**a**), creatinine (**b**), and uric acid (**c**). Results are depicted as mean ± SEM. **significant w.r.t. control, $P < 0.01$; *significant w.r.t. control $P < 0.05$; ##significant w.r.t. LPS, $P < 0.01$; #significant w.r.t. LPS, $P < 0.05$

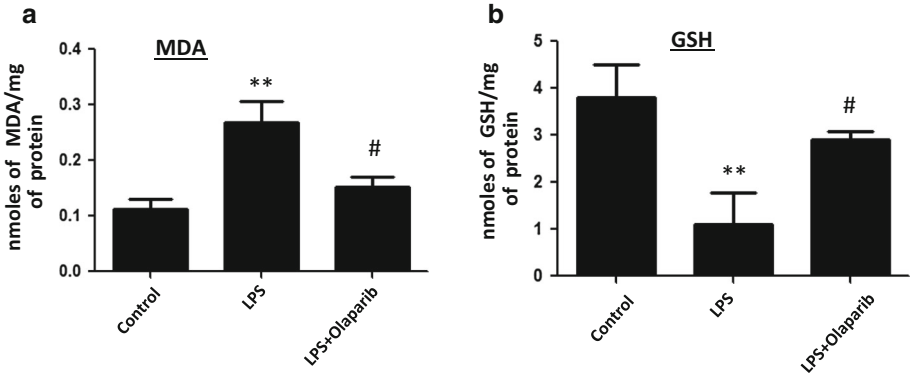

Fig. 3 Administration of olaparib restored the redox balance of lungs upon LPS treatment. Lungs were procured from different group of mice 18 h after LPS treatment and were processed for preparing total tissue homogenate. Tissue homogenate was then assessed for MDA (**a**) and GSH (**b**). Results are depicted as mean ± SEM. **significant w.r.t. control, $P < 0.01$; #significant w.r.t. LPS, $P < 0.05$

Olaparib administration ameliorate kidney function upon intratracheal administration of LPS

Figure 2a shows effect of olaparib on serum urea levels upon intratracheal administration of LPS. LPS treatment in the lungs increased the serum urea content by 288 % ($P < 0.01$ w.r.t. control) and olaparib administration reverted such increase by 64.61 % toward normal ($P < 0.01$ w.r.t. LPS). Figure 2b depicts the effect of olaparib administration on LPS-induced changes in serum content of creatinine. As shown in the figure, we noted that creatinine level increased by 230.0 % ($P < 0.05$ w.r.t. control) upon LPS treatment and olaparib administration suppressed such increase toward normal by 65.99 % ($P < 0.05$ w.r.t. LPS). Figure 3a shows effect of olaparib on uric acid levels in serum upon LPS treatment. LPS treatment in the lungs resulted in increase of uric acid level by 391.0 % ($P < 0.01$ w.r.t. control) while olaparib administration restored it toward normal by 61.87 % ($P < 0.01$ w.r.t. LPS).

We also examined the effect of olaparib on liver function tests at a dose of 5 mg/kg b.wt. to determine if the drug causes any liver toxicity. Our data based on the analysis of alkaline phosphatase, GOT, and GPT in serum suggests that olaparib did not cause any untoward changes in liver function (data not shown).

Administration of olaparib restored the redox balance of lung and kidney tissue upon LPS treatment

Oxidative stress reflects an imbalance between the systemic manifestation of ROS and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. We assessed the levels of MDA (as a marker of lipid peroxidation) and GSH as index of oxidative stress levels in tissues. Figure 3a shows the effect of olaparib on MDA levels in lungs upon intratracheal administration of LPS. The MDA level in lung tissue was increased by 138.0 % ($P < 0.01$ w.r.t. control) upon LPS treatment. Administration of olaparib after LPS treatment reduced the level of MDA by 43.28 % ($P < 0.05$ w.r.t. LPS) toward normal. The increase in the MDA level was found to be associated with decline in GSH content by 70.63 % ($P < 0.01$ w.r.t. control) in lungs of LPS-treated mice (Fig. 3b). Administration of olaparib after LPS treatment however restored GSH content toward normal by 161.73 % ($P < 0.05$ w.r.t LPS).

Next we assessed the level of MDA and GSH in kidney tissue to ascertain if oxidative stress plays an important role in causing kidney toxicity upon i.t. administration of LPS. The data in Fig. 4a show that LPS treatment increased the MDA level by 101.12 % ($P < 0.01$ w.r.t. control) and administration of olaparib suppressed the MDA level toward normal significantly by 42.45 % ($P < 0.05$ w.r.t. LPS). Figure 4b shows the effect of olaparib on GSH level in kidney upon LPS treatment. GSH content was found to be decreased by 59.65 % ($P < 0.05$ w.r.t. control) in kidney tissue of LPS-administered mice while olaparib administration following LPS treatment restored its level toward normal by 149.18 % ($P < 0.05$ w.r.t. LPS).

Olaparib administration downregulates the expression of NF-κB-dependent genes in the lungs upon LPS treatment

Our data suggests that increase in lung inflammation and ensuing kidney injury upon intratracheal administration of LPS was associated with increase in oxidative stress in

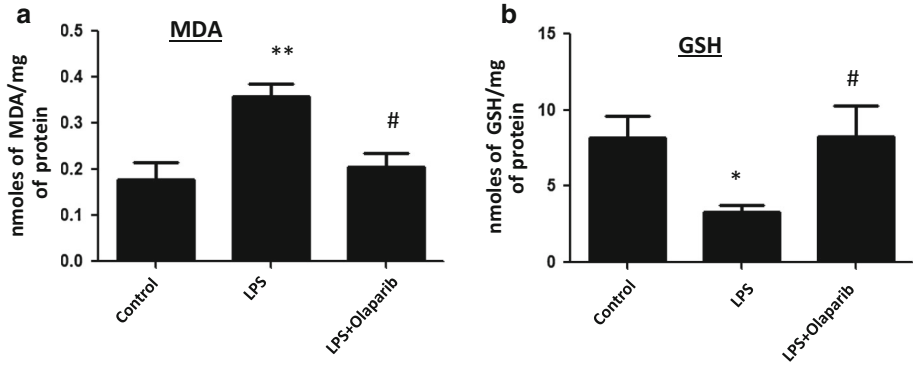

Fig. 4 Administration of olaparib restored the redox balance of kidney tissue upon LPS treatment. Kidneys were procured from different groups of mice after 18 h of LPS treatment and were processed for preparing total tissue homogenate. Tissue homogenate was then assessed for MDA (**a**) and GSH (**b**). Results are depicted as mean ± SEM. **significant w.r.t. control, $P < 0.01$; *significant w.r.t. control, $P < 0.05$; #significant w.r.t. LPS $P < 0.05$

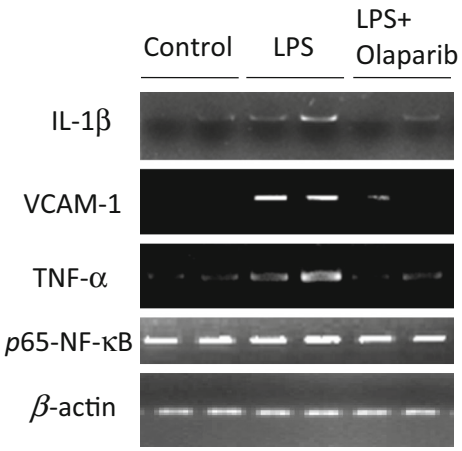

Fig. 5 Olaparib administration downregulates the expression of NF-κB-dependent genes in the lungs upon LPS treatment. Lung tissue obtained 6 h after LPS treatment was processed for total RNA extraction and mRNA was transcribed to cDNA. cDNA was then analyzed by RT-PCR for expression of TNF-α, IL-1β, p65NF-κB, and VCAM-1 using specific primers. β-actin was used as loading control

both the tissues. Consistent increase in oxidative stress is known to trigger pertinent cellular signaling pathways which activate the factors responsible for promoting inflammatory process in the tissues [48, 49]. One of such factors is transcription factor NF-κB [48, 49]. Expression of numerous NF-κB-dependent genes including several cytokines, chemokines, and adhesion molecules participate in the efficient recruitment of the inflammatory cells in the tissues [50, 51]. Accordingly we determined the effect of olaparib on the expression of factors which are known to participate in the inflammatory process in the lungs upon LPS treatment. Our data on RT-PCR (Fig. 5) certainly showed that olaparib administration after LPS treatment substantially downregulates the tissue expression of several NF-κB-dependent genes such as TNF-α, IL-1β, and VCAM-1. However, there was no major difference observed in the mRNA expression of total p65-NF-κB in lung tissues derived from different groups of mice.

Discussion

ALI in animals is characteristic of excessive neutrophil infiltration, release of pro-inflammatory, cytotoxic mediators, and loss of vascular barrier integrity [52]. Neutrophils can release damaging mediators, such as oxidants and elastase, leading to the injury of epithelial-vascular barrier [53–55]. It is a well-known fact that the intratracheal administration of LPS initiates a lung inflammatory response [33, 56–58] useful for preparing an animal model of ALI and ARDS [59, 60]. It is also known that LPS has the potential to induce symptoms of sepsis, even when administered intratracheally [59]. In our study, we found that LPS induced significant neutrophil infiltration in lungs which is in agreement with earlier reports [61–63]. Previously, we have shown that PARP inhibition blocks the lung inflammation in the context of ALI and asthma [29, 31, 32, 64–67]. Pharmacological inhibition of PARP has been investigated in various experimental conditions of ALI or other organ(s) injury mediated by systemic exposure of LPS [31, 32, 67–70]. Olaparib, which is a new generation PARP inhibitor has been reported to have anti-cancer potential [34–37] and approved by FDA for testing in humans. It is pertinent to mention that the compound has acceptable level of toxicity when tested on patients who have breast or ovarian cancer [38, 39]. Our data strongly suggest that the compound harbors strong anti-inflammatory potential against the LPS-induced ALI as well as associated kidney injury. A single injection of olaparib protected against LPS-induced infiltration of inflammatory

cells in the lungs. Considering the fact that oxidative stress is known to play an important role in the pathogenesis of number of human inflammatory disorders including those inflicting lungs [71, 72], and PARP being reported to be key mediator in such a process [73–75], we analyzed the levels of MDA and GSH in the lungs as markers of oxidative stress. Indeed LPS treatment in mice was found to alter redox balance as MDA level was found to be increased and conversely GSH was found to be decreased upon LPS treatment. Excessive production of ROS has been known to cause DNA damage, which results in activation of PARP. Over activation of PARP consumes its substrate NAD^+ substantially and thus depletes ATP, which results in energy crisis leading to cell death and tissue damage [24, 25]. Tissue damage causes recruitment of greater number of inflammatory cells which further leads to enhanced production of ROS and eventually culminates in the vicious inflammatory cycle [73, 76]. Number of investigators have shown that PARP inhibition, in general, blocks the inflammation and helps restore the redox status of the cell by disrupting the persistence of this vicious cycle [73–75]. Our results, that PARP inhibition by olaparib lowered lipid peroxidation and increased GSH content toward normal suggests that the drug can normalize the tissue redox status. ROS species can directly stimulate the cell signaling pathways involving activation of several pro-inflammatory transcription factors such as MAP kinases and NF-κB [48, 49]. During the course of ROS-mediated activation of NF-κB, expression of various pro-inflammatory and adhesion molecules are known to be elevated that are responsible for the complications of various inflammatory disorders including ALI [50, 51] [77]. Several reports suggest that PARP inhibition modulates several signaling pathways including NF-κB activation for their anti-inflammatory effects [26, 28] [78]. TNF-α has been suggested as one of the important early mediators of ALI [79]. Our data utilizing RT-PCR demonstrated that olaparib suppresses the expression of lung TNF-α drastically in LPS-treated mice. It has been reported that TNF-α can further alter the cellular redox status by depleting GSH [80, 81]. Given the fact that TNF-α is a NF-κB-dependent gene and PARP is known to influence NF-κB activation, we next examined the effect of olaparib on expression of total p65NF-κB and other factors dependent on the activation of transcription factor such as IL-1β and VCAM-1. Our data revealed that olaparib did not alter the expression of total p65NF-κB but its activation, as it downregulates the expression of IL-1β and VCAM-1 in addition to TNF-α in lungs of LPS-treated mice. It is possible that reduction in the levels of TNF-α and IL-1β by olaparib in lungs of LPS-treated mice would hamper the mobilization of inflammatory cells. In addition, blunted expression VCAM-1 in lungs of LPS-treated mice by olaparib potentially adds to the defect in recruitment of inflammatory cells by hindering their transmigration across the lung endothelial barrier. Taken together, it appears that defective NF-κB-dependent transcription activation resulting from PARP-1 suppression by olaparib may partly ameliorate the LPS-induced ALI.

As mentioned earlier, induction of ALI by intratracheal administration of LPS is reported to cause symptoms of sepsis. It is known that sepsis not only mediates the ALI but also other organ failure/dysfunction including kidney [12, 82]. One such study has shown the occurrence of lung–kidney crosstalk by intratracheal instillation of LPS [40]. Our results have also shown that LPS-induced ALI is associated with marked and significant increase in kidney toxicity as reflected by increase in serum levels of urea, creatinine, and uric acid. On the other hand administration of olaparib after LPS treatment restored these parameters toward normal. It is possible that olaparib protect against kidney injury by partly restoring redox status in the tissue toward normal in addition to blunting the secondary sepsis like conditions by blocking production of pro-inflammatory factors at the level of lungs. Overall, our results suggest strong anti-inflammatory potential of olaparib against LPS-induced ALI as well as secondary kidney injury. Very recently Si et al. (2013) have also shown that 3-amino benzamide (3-AB), an old generation PARP inhibitor reduces the acute lung and kidney injury caused by intratracheal administration of LPS in rats [40]. Our results are in agreement with these observations. Olaparib has been reported to be a potent inhibitor of both PARP-1 and PARP-2 [83]. Further studies are needed to determine whether the drug shows its beneficial effects on LPS-induced inflammation in lungs/kidney by blocking both of these isoforms equally or selectively in our model. Nevertheless, we conclude that olaparib as PARP inhibitor possesses a strong anti-inflammatory potential against the LPS-induced acute lung as well as kidney injury in mice. Since, the drug is approved for human testing in cancer patients, our results can pave the way for testing it on humans presented with respiratory complication because of ALI/ARDS.

Acknowledgments The grant support (BT/RLF/Re-entry/36/2012) from Department of Biotechnology, Government of India in the form of Ramalingaswami Fellowship to Dr. Amarjit S. Naura is highly acknowledged. We also acknowledge the Junior Research Fellowship to Bijayani Sahu from University Grant Commission, New Delhi, India.

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